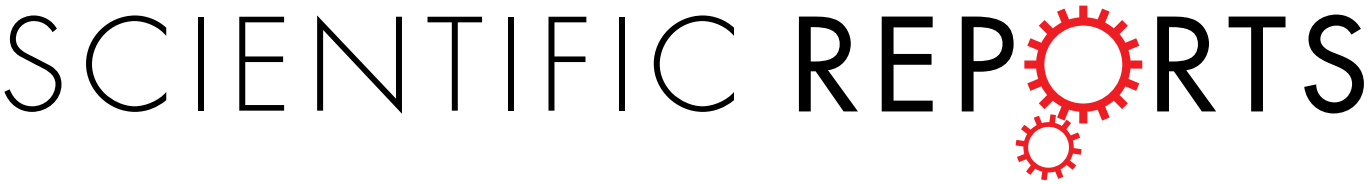

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Epidemiological characteristics of nasopharyngeal *Streptococcus pneumoniae* strains among children with pneumonia in Chongqing, China

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Streptococcus pneumoniae **(pneumococcus) is the most common respiratory pathogen worldwide. Nasopharyngeal carriage with *S. pneumoniae* is the major source of lower respiratory tract infection and horizontal spread among children. Investigating nasopharyngeal *S. pneumoniae* is crucial for clinicians to control pneumococcus disease. Here, we retrospectively analyzed clinical information of 5,960 hospitalized children, focusing on pneumonia children less than five years with positive nasopharyngeal pneumococcal cultures. Nasopharyngeal aspirates (NPAs) were collected between June 2009 and December 2016, which were outside the pneumococcal conjugate vaccine(PCV) period. NPAs were subjected to common bacterial culture and antibiotic susceptibility tests, and serotypes were identified by both multiplex PCR and DNA sequencing. Results clearly revealed that clinical manifestations of the children whose NPAs were *S. pneumoniae* culture positive were serious, especially in those less than twelve months old. Fifteen different serotypes of nasopharyngeal *S. pneumoniae* were detected, the most common ones being 19F (35.2%), 6A/B (23.8%), 19A (11.4%), 15B/C (9.3%) and 23F (7.8%). Eight serotypes, accounting for 85.5% of the isolates, corresponded to the PCV13 serotypes. Approximately one-third of all *S. pneumoniae* strains were susceptible to penicillin. Overall, we consider nasopharyngeal *S. pneumoniae* culture is beneficial in assessing the situations of pneumonia children. Moreover, PCV13 could be useful in preventing pneumococcal disease in Chongqing, China.**

Streptococcus pneumonia (pneumococcus) is a significant human pathogen that can cause pneumonia, otitis media, septicemia and meningitis, and constitutes an important cause of death among children under the age of five years[1]. Nasopharyngeal carriage with *S. pneumoniae* can be a reservoir of lower respiratory tract (LRT) infection, and is a major prerequisite towards the development of pneumococcal diseases[2–5]. The environment of the LRT is normally not sterile[6,7]; nasopharyngeal microbes can in fact be microaspirated in healthy individuals, and have a higher prevalence during seasons of respiratory diseases[8,9]. Ongoing surveillance of nasopharyngeal *S. pneumoniae* characteristics is, therefore, a significant source of epidemiological information. Nonetheless, data on nasopharyngeal *S. pneumoniae* strains in China have been limited, and here, we describe 7½ years (June 2009-December 2016) retrospective longitudinal study that characterized nasopharyngeal *S. pneumoniae* strains among a large cohort of pneumonia children. The study is based on *S. pneumoniae* bacterial culture, serotype distribution and antibiotic susceptibility. The characteristics of nasopharyngeal *S. pneumoniae* strains of pneumonia children are summarized here, which should be an important resource for clinicians as well as for local and national immunization programs.

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Results

Prevalence and clinical correlates of nasopharyngeal *S. pneumoniae*. Series of clinical data were compared between nasopharyngeal *S. pneumoniae* culture-positive and -negative cohorts of a range of ages, grouped as less than 12 months (m), 13–36 m and 37–59 m (Table 1). Among these age groups, *S. pneumoniae* culture-positive rates were 13.9%, 20.4% and 18.9%, respectively. The following differences were observed, especially in children that were less than 12 m old. First, there were several factors associated with pneumococcal carriage and disease. They were more likely to have siblings (Chi-square test, p values were 0.019, 0.016 and 0.561 in the three age groups, respectively), histories of more than 5 days of prehospital antibiotic usage (Chi-square test, $p = 0.002$, 0.026, 0.222), repeated respiratory tract infection(RTI) (Chi-square test, $p = 0.000$, 0.37, 0.576), and the history of repeated wheezing (Chi-square test, $p = 0.000$, 0.042, 0.002). Second, the clinical manifestations were more serious in the positive groups. For example, the lengths of hospital stay were significantly longer in the positive groups (Mann-Whitney U test, $p = 0.023$, 0.013, 0.106). The morbidities of persistent or chronic pneumonia were more prevalent in the positive groups (Chi-square test, $p = 0.000$, 0.136, 0.277). Symptoms, such as fever (Chi-square test, $p = 0.001$, 0.992, 0.632) and wheeze (Chi-square test, $p = 0.031$, 0.004, 0.530), were also more severe in the positive groups. Third, inflammatory responses, both in the blood and the lungs, were more pronounced in the *S. pneumoniae*-positive groups, such as the counts of leukocyte (Mann-Whitney U test, $p = 0.019$, 0.005, 0.936), neutrophil (Mann-Whitney U test, $p = 0.001$, 0.756, 0.212)and thrombocyte(Mann-Whitney U test, $p = 0.031$, 0.254, 0.922). Another inflammatory marker, C-reactive protein (CRP), was also higher in positive groups (Chi-square test, $p = 0.006$, 0.148, 0.931).

Distribution of nasopharyngeal *S. pneumoniae* serotypes. Using multiplex PCR and DNA sequencing, fifteen different serotypes were identified among 193 nasopharyngeal *S. pneumoniae* culture-positive pneumonia children whose demographic characteristics and clinical data were summarized in Table 2. The most common serotypes were 19 F(35.2%, 95% CI: 28.5–42.4), 6 A/B (23.8%, 95% CI: 18–30.5), 19 A(11.4%, 95% CI: 7.3–16.8), 15B/C(9.3%, 95% CI: 5.6–14.3), 23 F(7.8%, 95% CI: 4.4–12.5) and 14(5.2%, 95% CI: 2.5–9.3)(Fig. 1). The results clearly showed that 19 F and 6 A/B were the most common serotypes, accounting for more than half of all serotypes, whereas 19 A, 15B/C, 23 F and 14 were relatively less prevalent. The other serotypes were seldom detected in this study. All detected serotypes were validated by sequencing. The table of serotypes distribution (Supplementary Table 1) and the detected sequences are provided in the online Supplementary Information files. Currently, two PCVs are dominant: PCV10 and PCV13. In this study, six serotypes of PCV10 representing 73.6% (142/193, 95% CI: 67.4–79.8%) and eight serotypes of PCV13 representing 85.5% (165/193, 95% CI: 80.5–90.5%) were observed, respectively.

Antibiotic susceptibility of different *S. pneumoniae* serotypes. The antibiotic susceptibility tests were performed with nine classes of agents by the Kirby-Bauer disc diffusion method (Table 3). The outcomes were divided into susceptible, intermediate and resistant to specific antibiotics. The different antibiotics susceptibility of *S. pneumoniae* strains were summarized, which we generalize here. First, 63 (32.6%, 95% CI: 26.1–39.8) and 66 (34.2%, 95% CI: 27.5–41.4) of the 193 *S. pneumoniae* strains analyzed in the study were respectively susceptible and resistant to penicillin. Second, nearly all of the detected *S. pneumoniae* strains were susceptible to vancomycin(100%, 95% CI:98.1–100), linezolid(100%, 95% CI:98.1–100), levofloxacin(100%, 95% CI:98.1–100) and chloramphenicol(92.2%, 95% CI:87.5–95.6). Third, most of the *S. pneumoniae* strains were resistant to clindamycin (81.3%, 95% CI:75.1–86.6), tetracycline (88.6%, 95% CI:83.3–92.7), sulfamethoxazole(89.6%, 95% CI:84.5–93.6) and erythromycin(96.9%, 95% CI:93.4–98.8). 94.3% (182/193, 95% CI:90–97.1) of all strains in the NPAs were MDR strains in this study, and about 66.3% (128/193, 95% CI: 59.6–73%) of all strains were resistant to erythromycin, sulfamethoxazole, tetracycline and clindamycin simultaneously. No PDR *S. pneumoniae* strain has been detected so far. Lastly, antibiotic susceptibilities of different *S. pneumoniae* serotypes were summarized in Table 4. The penicillin resistance of 19F and 19A serotypes were similar, which might suggest that the 19 serogroup *S. pneumoniae* overall is more resistant to penicillin than other serogroups. Tables of antibiotic susceptibility presented as percentages of the total (%) and 95% CI were provided in the online Supplementary Information files (Supplementary Table 2 and Table 3)

Discussion

About 800,000 children die each year due to pneumococcal disease[2]. As potential pathogen, *S. pneumoniae* can colonize the nasopharynx at low density without causing symptoms in healthy children, and are less likely to be detected by culture methods[10,11]. This study documented that positive *S. pneumoniae* culture of nasopharyngeal aspirates is an important reference for clinicians. *S. pneumoniae* culture-positive children had several specific characteristics, such as more than one siblings, history of repeated wheezing or respiratory tract infection (RTI) and more common antibiotic usage. Several previous studies had shown a clear association between siblings and the isolation of nasopharyngeal *S. pneumoniae*[12,13]. This is likely because close contact can transmit nasopharyngeal *S. pneumoniae* between siblings in the same family. Children with repeated wheezing were more likely to have positive nasopharyngeal *S. pneumoniae* detection, which is in agreement with other references[14,15]. Nasopharyngeal *S. pneumoniae* species appeared to contribute to respiratory symptoms[16], and therefore, avoidance of exposure to *S. pneumoniae* pathogen or PCV inoculation should be beneficial for repeated RTI or wheezing in children[17]. As noted, the clinical manifestations of culture-positive children were obviously more serious than those of the negative ones, especially in younger children. The positive group not only had a longer recovery time, but also displayed higher levels of inflammatory markers than the negative group, which was consistent with previous reports[15,18]. These results supported *S. pneumoniae* carriage as a prerequisite for pneumococcal infection or diseases[5,19]. Nasopharyngeal colonization of *S. pneumoniae* resulted in increased numbers of mucosal

Variables	*S. pneumoniae* (+)	*S. pneumoniae* (−)	P value
0–12 m (n = 1337)	n = 186 (13.9%)	n = 1151 (86.1%)	
General Information			
Male[a]	73.1 (66.1–79.3)	70.2 (67.5–72.8)	0.418
Premature History (≤36 week) [a]	9.1 (5.4–14.2)	9.4 (7.8–11.2)	0.916
Siblings (n ≥ 1)[a]	42.5 (35.3–49.9)	33.6 (30.9–36.4)	***0.019***
Usage of Antibiotic (≥5 day)*[a]	39.8 (32.7–47.2)	28.4 (25.8–31.1)	***0.002***
History of Wheezing (≥3times)[a]	9.7 (5.8–14.9)	3.3 (2.4–4.5)	***0.000***
History of RTI (≥3 times)[a]	25.8 (19.7–32.7)	14.1 (12.1–16.2)	***0.000***
Condition			
Length of Stay (day)[b]	6 (6, 8)	6 (5, 8)	***0.023***
Persistent/Chronic*[a]	22 (16.3–28.7)	12.5 (10.7–14.6)	***0.000***
Severe*[a]	18.3 (13–24.6)	15.5 (13.4–17.7)	0.33
Symptoms			
Fever[a]	57.5 (50.1–64.7)	44.3 (41.4–47.2)	***0.001***
Wheeze[a]	60.2 (52.8–67.3)	51.7 (48.8–54.6)	***0.031***
Cough[a]	98.9 (96.2–99.9)	96.9 (95.7–97.8)	0.118
Laboratory Parameters			
Leukocyte ($\times 10^9$/L)[b]	12 (9, 15.5)	11 (8.3, 14)	***0.019***
Neutrophil (%)[b]	38.5 (29, 52)	32 (24, 47)	***0.001***
Thrombocyte ($\times 10^9$/L)[b]	450 (367, 558)	429 (331, 530)	***0.031***
CRP*[a]	14 (9.3–19.8)	7.8 (6.3–9.5)	***0.006***
Imaging Features			
Pleural Effusion[a]	2.2 (0.6–5.4)	0.7 (0.3–1.4)	0.073
Lobar Consolidation[a]	3.2 (1.2–6.9)	4.8 (3.6–6.2)	0.346
13–36 m (n = 780)	**n = 159 (20.4%)**	**n = 621 (79.6%)**	
General Information			
Male[a]	59.7 (51.7–67.4)	63.9 (60–67.7)	0.33
Premature History(≤36 week)[a]	10.1 (5.9–15.8)	8.5 (6.5–11)	0.545
Siblings(n ≥ 1)[a]	37.1 (29.6–45.1)	27.4 (23.9–31.1)	***0.016***
Usage of Antibiotic (≥5 day)*[a]	35.8 (28.4–43.8)	26.9 (23.4–30.6)	***0.026***
History of Wheezing (≥3times)[a]	14.5 (9.4–20.9)	9 (6.9–11.6)	***0.042***
History of RTI (≥3 times)[a]	37.1 (29.6–45.1)	33.3 (29.6–37.2)	0.37
Condition			
Length of Stay (day)[b]	6 (5, 8)	6 (5, 7)	***0.013***
Persistent/Chronic*[a]	12.6 (7.9–18.8)	8.7 (6.6–11.2)	0.136
Severe*[a]	9.4 (5.4–15.1)	12.4 (9.9–15.3)	0.301
Symptoms			
Fever[a]	74.8 (67.4–81.4)	74.9 (71.3–78.3)	0.992
Wheeze[a]	57.2 (49.2–65)	44.4 (40.5–48.5)	***0.004***
Cough[a]	97.5 (93.7–99.3)	96.9 (95.3–98.2)	1.000
Laboratory Parameters			
Leukocyte ($\times 10^9$/L)[b]	11 (8.3, 14.5)	9.8 (7.3, 13.2)	***0.005***
Neutrophil (%)[b]	47 (34, 59.3)	45 (33, 59)	0.756
Thrombocyte ($\times 10^9$/L)[b]	330 (249, 425.5)	317 (245, 407.8)	0.254
CRP*[a]	22 (15.8–29.3)	17.1 (14.2–20.3)	0.148
Imaging Features			
Pleural Effusion[a]	1.3 (0.2–4.5)	2.4 (1.4–4)	0.546
Lobar Consolidation[a]	8.2 (4.4–13.6)	8.5 (6.5–11)	0.885
37–59 m (n = 238)	**n = 45 (18.9%)**	**n = 193 (81.1%)**	
General Information			
Male[a]	62.2 (46.5–76.2)	49.2 (42–56.5)	0.116
Premature History (≤36 week)[a]	0	6.7 (3.6–11.2)	0.136
Siblings (n ≥ 1)[a]	22.2 (11.2–37.1)	26.4 (20.4–33.2)	0.561
Usage of Antibiotic (≥5 day)*[a]	37.8 (23.8–53.5)	28.5 (22.3–35.4)	0.222
History of Wheezing (≥3times)[a]	24.4 (12.9–39.5)	8.3 (4.8–13.1)	***0.002***
History of RTI (≥3 times)[a]	44.4 (29.6–60)	39.9 (32.9–47.2)	0.576

Continued

Variables	*S. pneumoniae* (+)	*S. pneumoniae* (−)	P value
Condition			
Length of Stay (day)[b]	6.5 (5, 8)	6 (4, 7)	0.106
Persistent/Chronic*[a]	15.6 (6.5–29.5)	9.3 (5.6–14.3)	0.277
Severe*[a]	15.6 (6.5–29.5)	7.3 (4–11.9)	0.085
Symptoms			
Fever[a]	80 (65.4–90.4)	76.7 (70.1–82.5)	0.632
Wheeze[a]	26.7 (14.6–41.9)	22.3 (16.6–28.8)	0.530
Cough[a]	100 (92.1–100)	97.9 (94.8–99.4)	1.000
Laboratory Parameters			
Leukocyte ($\times 10^9$/L)[b]	9.1 (7.1, 13)	9.1 (6.7, 13.1)	0.936
Neutrophil (%)[b]	59 (40.5, 67)	61 (44, 71)	0.212
Thrombocyte ($\times 10^9$/L) [b]	312.5 (213.8, 390.5)	299 (219.8, 390.8)	0.922
CRP*[a]	24.4 (12.9–39.5)	23.8 (18–30.5)	0.931
Imaging Features			
Pleural Effusion[a]	6.7 (1.4–18.3)	3.1 (1.2–6.6)	0.377
Lobar Consolidation[a]	26.7 (14.6–41.9)	9.3 (5.6–14.3)	***0.002***

Table 1. Comparison of clinical data between nasopharyngeal *S. pneumoniae* culture positive and negative groups among different ages. Series of clinical data were compared between nasopharyngeal *S. pneumoniae* culture positive and negative cohorts of a range of ages. The conditions of children less than 5 years old were of utmost concern. [a]The results were presented as percentages of the total (%) and 95% CI. [b]The results were reported as median with IQR. Usage of antibiotic*: days of antibiotic usage before NPAs collection. Persistent/Chronic*: morbidities of persistent/chronic pneumonia. Severe*: morbidities of severe pneumonia. CRP*: the number of children whose CRP values were higher than normal range (8 mg/L). P values < 0.05 were considered statistically significant in bold and italic. Normal ranges of inflammation markers: Leukocyte: 4–10 $\times$ 10^9/L. Neutrophil: 33–79%. Thrombocyte: 100–300 $\times$ 10^9/L.

and systemic inflammatory cells and higher concentrations of proinflammatory cytokines, which may impact on disease severity[20–22]. High bacterial load in the nasopharynx and local inflammatory reactions were indeed shown to be important in bacterial invasion of the LRT[23]. The above mentioned reasons may cause transmission of the nasopharyngeal *S. pneumoniae* into the LRT and aggravate the conditions of the children. Moreover, the younger children exhibited more serious clinical manifestations, probably due to their immature and weaker immunity. Taken together, these findings lead us to conclude that the children who tested positive for nasopharyngeal *S. pneumoniae* culture had certain risk factors and serious clinical manifestations, especially in children less than 12 m of age.

Various factors may promote or facilitate nasopharyngeal *S. pneumoniae* invasion of the LRT. Polysaccharide capsules, for example, may play a crucial role in the process[24,25], which is not only significant for *S. pneumoniae* classification, but is also a cardinal determinant of vaccine target. Currently, according to the biochemical structures of the polysaccharide capsule and immunological distinction, *S. pneumoniae* can be divided into 48 serogroups and 97 serotypes[26]. The different serotypes have diverse characteristics, such as activation of complement, invasive ability and influence on biofilm formation[27,28]. It is believed that serotype epidemiology is quite variable both geographically and temporally. Prior to 2000, a large number of epidemiological studies reported that 19F, 6B, 23F and 14 serotypes accounted for the most common pneumococcal serotypes detected in the nasopharynx or in invasive diseases in the United States and several other countries[29]. Following the widespread use of PCVs, the incidence of pneumococcal diseases dramatically declined, bringing significant benefit to the developing countries[30–32]. PCVs not only protected the vaccinated individuals against disease but also reduced the carriage of vaccine serotypes that could induce herd effects across whole populations[33,34]. As shown in our study, 19F and 6A/B were the most common serotypes detected in nasopharynx in Chongqing while 19A, 15B/C, 23F, 14 and 22F were also detected, consistent with studies in other Chinese cities[35–38]. However, in these studies, *S. pneumoniae* strains were isolated from patients with invasive pneumococcal disease. These serotypes were mainly the PCV serotypes, likely because the PCVs had not yet been introduced in the national compulsory immunization program in China. Compared with the serotypes of nasopharyngeal *S. pneumoniae* strains detected in other studies[39], there were some serotypes that were seldom detected or not detected in this study, such as serotypes 1, 3 and 5. Geographical division may well be a reason for this difference. Current United States guidelines on vaccine use recommend that children aged 2 to 59 m receive PCV13 as routine care[40]. Moreover, PCV13 covers serotypes of significantly higher invasive propensity, such as 1, 3, 5, 7F, and 19A[39,41], of which 19A has exhibited high prevalence in China, as we have also shown. Furthermore, PCV13 covered the major proportion of serotypes in this study. We thus suggest that PCV13 could indeed be an effective strategy for prevention of invasive pneumococcal disease in Chongqing, and even nationally in China.

The rising occurrence of antibiotic resistance enables *S. pneumoniae* to be an alarming threat to children's health. In fact, three major risk factors (antibiotic use, younger age and attending day-care facility) have been identified for nasopharyngeal-resistant *S. pneumoniae*[42]. Most importantly, association between carriage or

Variables	0–12 m (n = 103)	13–36 m (n = 72)	37–59 m (n = 18)
General Information			
Male[a]	74.8 (65.2–82.8)	62.5 (50.3–73.6)	55.6 (30.8–78.5)
Premature History (≤36 week)[a]	8.7 (4.1–15.9)	6.9 (2.3–15.5)	0
Siblings (n ≥ 1)[a]	39.8 (30.3–49.9)	41.7 (30.2–53.9)	27.8 (9.7–53.5)
Usage of Antibiotic (≥5 day)*[a]	38.8 (29.4–48.9)	47.2 (35.3–59.4)	38.9 (17.3–64.3)
History of Wheezing (≥3times)[a]	14.6 (8.4–22.9)	16.7 (8.9–27.3)	11.1 (1.4–34.7)
History of RTI (≥3 times)[a]	28.2 (19.7–37.9)	41.7 (30.2–53.9)	38.9 (17.3–64.3)
Condition			
Length of Stay (day)[b]	7 (6, 8)	6 (5, 8)	5.5 (5, 8)
Persistent/Chronic*[a]	28.2 (19.7–37.9)	13.9 (6.9–24.1)	11.1 (1.4–34.7)
Severe*[a]	15.5 (9.2–24)	8.3 (3.1–17.3)	11.1 (1.4–34.7)
Symptoms			
Fever[a]	67 (57–75.9)	70.8 (58.9–81)	61.1 (35.8–82.7)
Wheeze[a]	71.8 (62.1–80.3)	66.7 (54.6–77.3)	27.8 (9.7–53.5)
Cough[a]	100 (96.5–100)	100 (95–100)	100 (81.5–100)
Laboratory Parameters			
Leukocyte ($\times 10^9$/L)[b]	12 (9.4, 16.7)	10.9 (8.8, 13.8)	7.5 (5.7, 11.5)
Neutrophil (%)[b]	37.5 (29.3, 52)	43 (34, 54)	49 (38.5, 64.8)
Thrombocyte ($\times 10^9$/L)[b]	432 (359, 529)	361 (272, 458)	287.5 (214.3, 363.5)
CRP*[a]	14.6 (8.4–22.9)	15.3 (7.9–25.7)	22.2 (6.4–47.6)
Imaging Features			
Pleural Effusion[a]	1.9 (0.2–6.8)	2.8 (0.3–9.7)	5.6 (0.1–27.3)
Lobar Consolidation[a]	5.8 (2.2–12.3)	4.2 (0.9–11.7)	11.1 (1.4–34.7)

Table 2. The characteristics of 193 pneumonia children presenting with nasopharyngeal *S. pneumoniae* serotypes. [a]The results were presented as percentages of the total (%) and 95% CI. [b]The results were reported as median with IQR. Usage of antibiotic*: days of antibiotic usage before NPAs collection. Persistent/Chronic*: morbidities of persistent/chronic pneumonia. Severe*: morbidities of severe pneumonia. CRP*: the number of children whose CRP values were higher than normal range (8 mg/L). Normal ranges of inflammation markers: Leukocyte: 4–10 × 10^9/L. Neutrophil: 33–79%. Thrombocyte: 100–300 × 10^9/L.

infection with resistant *S. pneumoniae* and antibiotic use is now widely accepted. Many clinical studies have indeed linked the usage of antibiotics to community-wide antibiotic resistance[43,44]. It is now confirmed that antibiotic selection pressure enhances antibiotic resistance, and is linked to a reduction of susceptible bacterial strains, shift of the competitive balance, and dissemination of the existing resistant clone(s). The situation is particularly grave in China, where antibiotic usage is popular, as bacterial pathogens occur more frequently in developing countries. Reports have shown that a longer duration of carriage leads to higher incidence of resistance due to the greater risk of antibiotic exposure[45]. In the *S. pneumoniae* positive groups, over 30% of children received antibiotics for longer than 5 days before hospitalization, and thus, it is possible that the antibiotics contributed to the observed antibiotic resistance. As shown in our study, almost all *S. pneumoniae* strains were resistant to clindamycin, sulfamethoxazole, tetracycline and erythromycin, and the most common pattern was co-resistance to former four drugs. The results were consistent with previous reports[46,47]. Antibiotic-resistant *S. pneumoniae* could be detected if the antibiotic treatment is conducted within 4 weeks preceding the susceptibility test[12,48]. But these antibiotics have seldom been used for treatment in Chongqing for a long time. It was also reported that antibiotic resistance was due to the spread of strains belonging to a limited number of clones[49]. It was speculated that *S. pneumoniae* clones were stably resistant to former four antibiotics in Chongqing, which may be widely spread by over-use of antibiotics. And the detailed mechanisms of antibiotics resistance await further research. Conversely, *S. pneumoniae* strains have remained susceptible to other antibiotics, such as vancomycin, linezolid, levofloxacin and chloramphenicol, which lend hope to the treatment of resistant *S. pneumoniae*. Specifically, the former two antibiotics are better choices, while levofloxacin and chloramphenicol are cautiously used in children in the pediatric clinic. It was found that approximately one-third of all *S. pneumoniae* was susceptible to penicillin, which is also consistent with other studies[43]. Taken together, efforts to promote judicious antibiotic use in children appear to be the most appropriate measures to control the spread of antibiotic-resistant clones.

Lastly, we would like to point out potential limitations of our study. First, this study was conducted in a relatively isolated hospital population, and the mild pneumonia children that did not require hospitalization were, therefore, excluded. Second, this was a retrospective, single-center study, and thus, larger and continuous multicenter prospective studies are needed, which should provide crucial data to assess the national immunization program and the effects of vaccines and antibiotics on *S. pneumoniae* strains. Thirdly, serotyping NPAs were only possible for about 50% of all samples, as NPA samples with a DNA concentration lower than 20 ng/ul did not detected serotypes further. Finally, we have described the characteristics of the nasopharyngeal carriage *S. pneumoniae*, which should be valuable in monitoring *S. pneumoniae* epidemiology. Nonetheless, it may be more appropriate to collect *S. pneumoniae* from the lower respiratory tract to comprehensively monitor invasive *S. pneumoniae* characteristics in the future.

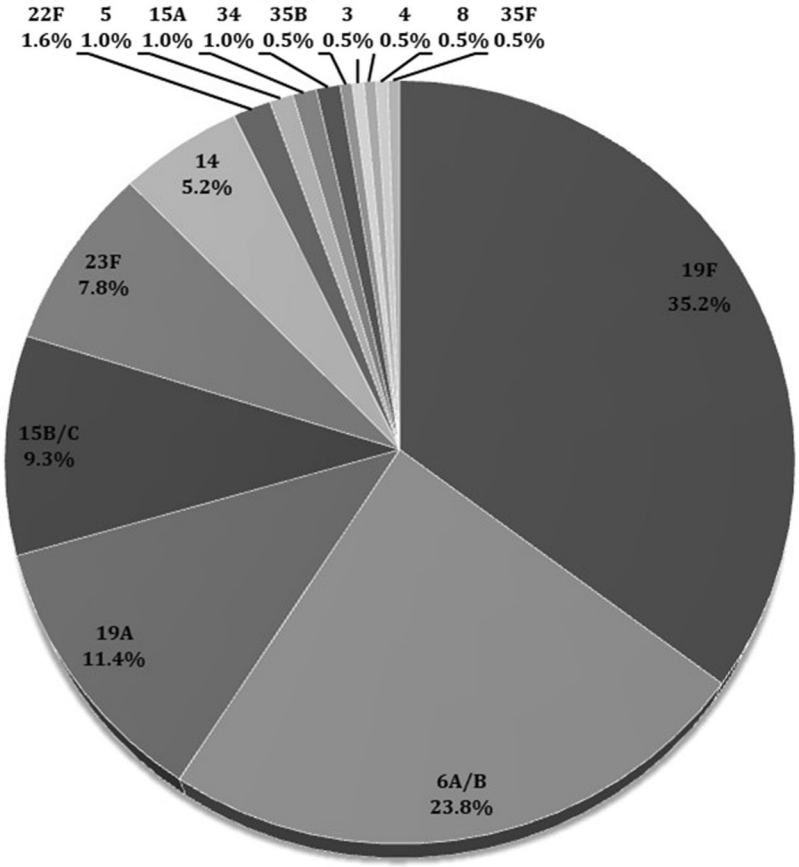

Figure 1. Distribution of nasopharyngeal *S. pneumoniae* serotypes among pneumonia children in Chongqing. Using multiplex PCR and DNA sequencing, fifteen different serotypes were identified among 193 nasopharyngeal *S. pneumoniae* culture positive pneumonia children. The most common serotypes were 19F(68/193, 35.2%), 6A/B(46/193, 23.8%), 19A(22/193, 11.4%), 15B/C(18/193, 9.3%), 23F(15/193, 7.8%) and 14(10/193, 5.2%), other serotypes were seldom detected. The table of serotypes distribution and the detected sequences have been provided in the online Supplementary Information file. Eight serotypes of PCV13 representing 85.5% were observed.

	Total (n = 193)		
	Susceptible	Intermediate	Resistant
Vancomycin	193 (100)	0 (0)	0 (0)
Linezolid	193 (100)	0 (0)	0 (0)
Levofloxacin	193 (100)	0 (0)	0 (0)
Chloramphenicol	178 (92.2)	0 (0)	15 (7.8)
Penicillin	63 (32.6)	64 (33.2)	66 (34.2)
Clindamycin	16 (8.3)	20 (10.4)	157 (81.3)
Tetracycline	7 (3.6)	15 (7.8)	171 (88.6)
Sulfamethoxazole	12 (6.2)	8 (4.1)	173 (89.6)
Erythromycin	6 (3.1)	0 (0)	187 (96.9)

Table 3. Antibiotic susceptibility of nasopharyngeal *S. pneumoniae* strains [n(%)]. The antibiotic susceptibility tests were performed with nine classes of agents by the Kirby-Bauer disc diffusion method. The guidelines for classifying isolates as susceptible, intermediate or resistant were according to Clinical and Laboratory Standards Institute(CLSI).

Methods

Ethics statement. The study was approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University (Permit number 2015–77). It was conducted in compliance with principles of the declaration of Helsinki. Informed consent was obtained from each parent or guardian on behalf of the children participants, prior to enrollment.

	19F (n = 68)			6A/B (n = 46)			19A (n = 22)			15B/C (n = 18)			23F (n = 15)		
	Sus-	Inter-	Res-	Sus-	Inter-	Res-	Sus-	Inter-	Res-	Sus-	Inter-	Res-	Sus-	Inter-	Res-
Vanco-	68 (100)	0 (0)	0 (0)	46 (100)	0 (0)	0 (0)	22 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
Linezo-	68 (100)	0 (0)	0 (0)	46 (100)	0 (0)	0 (0)	22 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
Levoflo-	68 (100)	0 (0)	0 (0)	46 (100)	0 (0)	0 (0)	22 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
Chlora-	64 (94.1)	0 (0)	4 (5.9)	38 (82.6)	0 (0)	8 (17.4)	21 (95.5)	0 (0)	1 (4.5)	18 (100)	0 (0)	0 (0)	15 (100)	0 (0)	0 (0)
Penici-	10 (14.7)	30 (44.1)	28 (41.2)	**20 (43.5)***	13 (28.3)	13 (28.3)	3 (13.6)	12 (54.5)	7 (31.8)	7 (38.9)[a]	3 (16.7)	8 (44.4)	6 (40)[b]	4 (26.7)	5 (33.3)
Clinda-	2 (2.9)	2 (2.9)	64 (94.1)	2 (4.3)	12 (26.1)	**32 (69.6)***	1 (4.5)	1 (4.5)	20 (90.9)	0 (0)	3 (16.7)	15 (83.3)	1 (6.7)	2 (13.3)	12 (80)
Sulfam-	2 (2.9)	1 (1.5)	65 (95.6)	2 (4.3)	1 (2.2)	43 (93.5)	2 (9.1)	0 (0)	20 (90.9)	0 (0)	1 (5.6)	17 (94.4)	0 (0)	0 (0)	15 (100)
Tetracy-	0 (0)	10 (14.7)	58 (85.3)	2 (4.3)	2 (4.3)	42 (91.3)	0 (0)	1 (4.5)	21 (95.5)	0 (0)	0 (0)	18 (100)	0 (0)	1 (6.7)	14 (93.3)
Erythr-	1 (1.5)	0 (0)	67 (98.5)	4 (8.7)	0 (0)	42 (91.3)	0 (0)	0 (0)	22 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)	15 (100)

Table 4. Antibiotic susceptibility of different *S. pneumoniae* serotypes [n(%)]. The differences of susceptible and resistant rates were compared among different serotypes.19 F serotype group was used as reference group. Compared among multiply groups, p values were adjusted by Bonferroni method. So p values less than 0.0125 (0.05/4 = 0.0125) in bold and marked with * were statistically significant. [a]p = 0.041; [b]p = 0.035. Abbreviations: Sus-:susceptible, Inter-: intermediate, Res-:resistant. Vanco-:vancomycin, Linezo-:linezolid, Levoflo-:levofloxacin, Chlora-:chloramphenicol, Penici-:penicillin, Clinda-:clindamycin, Sulfam-:sulfamethoxazole, Tetracy-: tetracycline, Erythr-: erythromycin.

Research subjects and sample collection. During the period from June 2009 to December 2016, a total of 9923 children were hospitalized at the Department of Respiration in Children's Hospital of Chongqing Medical University. A total of 5960 cases in this period were randomly selected and analyzed (minimum: 50 cases/month, 600 cases/year). In the primary diagnosis, children with no pneumonia were excluded. Pneumonia was diagnosed according to WHO clinical criteria[50], lung auscultation with moist rales or evidence of patchy alveolar opacities on chest radiographs. Cases with immune dysfunction/immunodeficiency or heart disease were excluded sequentially. Cases that were positive for other nasopharyngeal bacteria by culture or which *S. pneumoniae* was co-detected with other bacteria were also excluded, so that the focus was mainly on nasopharyngeal *S. pneumoniae* strains. Overall, 2583 cases were eligible, consisting of positive nasopharyngeal *S. pneumoniae* culture in 417 cases and no bacteria in 2166 cases. The conditions of children less than 5 years old were of utmost concern. They were further divided into three age groups: 0–12 m, 13–36 m, and 37–59 m. The demographic and clinical information of the children were collected after admission. NPAs and venous blood were collected within 24 h by trained clinical personnel in accordance with standard protocols. Venous bloods were used for detection and quantification of inflammation markers, such as the leukocytes, neutrophil, thrombocyte and CRP. The normal ranges of these markers are listed in Table 1. Clinical criteria for diagnosis of severe pneumonia was defined by WHO on the basis of cough, tachypnea, difficult breathing, and general danger signs (central cyanosis, inability to breastfeed or drink, severe chest indrawing, head nodding, reduced level of consciousness and convulsions)[51]. Persistent or chronic pneumonia were defined as the course of pneumonia for 1–3 months or more than3 months, respectively. Chest radiographs were reviewed by specialists. NPA samples with a DNA concentration greater than 20 ng/ul were considered eligible, so only 193 NPA samples (193/390, 49.5%) from children under 5 years of age were further tested for S. pneumoniae serotypes. The screening, eligibility and enrollment of children with pneumonia are summarized in Fig. 2.

NPA preparation. NPAs were collected into two tubes: one was immediately used for common bacteria culture and antibiotic susceptibility test by standard microbiological methods in the clinical bacteriology laboratory; the other one was sent to the respiratory laboratory for future analysis. The specimens were kept at 4 °C for a maximum of 4 h, and preserved at −80 °C until further use. DNA in the NPAs were extracted using a QIAamp DNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The concentrations of extracted DNA were then determined, and those exceeding 20 ng/ul were considered qualified. The DNA was preserved at −80 °C for subsequent tests.

Bacterial culture and antibiotic susceptibility test. NPA specimens were inoculated on blood plates and chocolate plates within 2 hours of collection, and the plates were cultured at 35 °C for 24–48 hours in a 5–10% CO_2 environment. *S. pneumoniae* was identified by colony morphology, gram staining, catalase test, optochin test, and biliary lysis test. Antibiotic sensitivity tests were performed using the Kirby-Bauer disc diffusion method to determine the sensitivity of all strains to vancomycin, linezolid, levofloxacin, chloramphenicol, penicillin, clindamycin, sulfamethoxazole, tetracycline, and erythromycin. Antibiotic susceptibility was determined according to the Clinical and Laboratory Standards Association (CLSI) guidelines of the year. *S. pneumoniae* ATCC49619 was included as the control strain. Multi-drug resistance (MDR) *S. pneumoniae* was defined as resistant to more than 3 classes of antibiotics, while pan-drug resistance (PDR) was defined as resistant to all antibiotics, including glycopeptides and linezolid.

Multiplex PCR and sequencing. *S. pneumoniae* capsular serotypes were determined both by multiplex PCR and DNA sequencing. Twenty eight oligonucleotide primers, described previously[52], were divided into 7

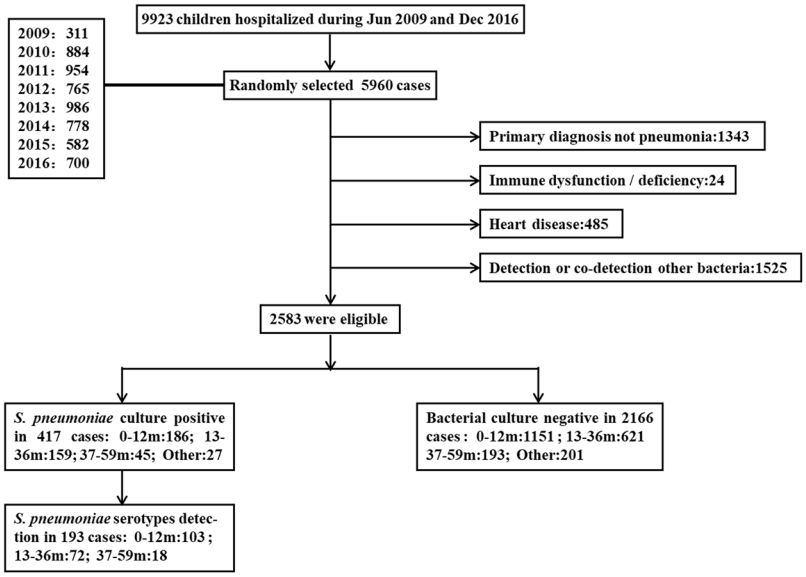

Figure 2. Flow chart of screening, eligibility and enrollment of children with pneumonia for comparison of nasopharyngeal *S. pneumoniae* culture. During the period from June 2009 and December 2016, a total of 9923 children with respiratory tract infection were hospitalized at the Department of Respiration in Children's Hospital of Medical University of Chongqing, of which 5960 cases were randomly selected(minimum: 50 cases/month, 600 cases/ year). The numbers of NPA samples each year were also listed. 1343 cases with no pneumonia primary diagnosis, 24 cases with immune dysfunction/deficiency, 485 cases with heart disease and 1525 cases of bacterial common culture with detection or co-detection other bacteria were excluded sequentially. Lastly, 2583 cases were eligible, among which 417 cases were *S. pneumoniae* culture positive. The conditions of children less than 5 years old were of utmost concern, among which *S. pneumoniae* culture-positive rate was 16.6% (390/2355). They were further divided into three age groups: 0–12 m, 13–36 m and 37–59 m. NPAs with *S. pneumoniae* culture positive were not all detected serotypes. DNA concentrations of NPA samples more than 20 ng/μl were qualified. Finally, 193 NPAs were detected for *S. pneumoniae* serotypes further. The numbers of different age group children were shown. The primary diagnose not pneumonia contained: Upper respiratory airway infection: 124, Bronchitis:191, Bronchiolitis:514, Asthma:362, Other:152. Immune dysfunction/deficiency contained: primary immune deficiency or secondary immune deficiency/dysfunction:24. Heart disease contained: Atrial septal defect: 246, Interventricular septal defect:69, Patent ductus arteriosus:43, others:127. Detection or co-detection with other bacteria contained: Haemophilusparainfluenzae:359, Cardamorasia:174, Haemophilusinfluenzae:156, Escherichia coli:149, Staphylococcus aureus:129, Klebsiella pneumoniae:158, Others:191, co- detections:209.

groups and used to detect *S. pneumoniae* serotypes (Supplementary Table 4), which included not only all common serotypes detected in China but also the PCV 13 serotypes. Multiplex PCR were performed in 25 μl volumes, each reaction mixture containing the following: 1 × PCR buffer (20 mM Tris-HCl, pH 8.0, 100mMKCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween 20, 0.5% NonidetP-40), 6.25 μM of each deoxy nucleoside triphosphate, 62.5 μM of $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. All samples were analyzed using a commercial detection kit (TaKaRaEx Taq, RR01AM, Dalian, China and Applied Biosystems, Japan). The PCR parameters were: 95 °C for 5 min, followed by 35 amplification cycles of 95 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis in 2% NuSieve agarose gels. Specific target primers were then used for further amplification, and the products were sent for sequencing to the Beijing Genomics Institute (BGI).

Statistical analysis. Continuous variables that do not satisfied the normal distribution were expressed as median with inter-quartile range(IQR);Categorical variables were reported as numbers (n), percentages of the total (%) and 95% confidence intervals (95% CI). Comparison between two groups, the Mann-Whitney U test and Chi-square test were used. Fisher's exact tests were appropriately performed. Comparison among more than three groups, p values were adjusted by Bonferroni correction for multiple comparisons. All tests were two-sided considered statistically significant. SPSS (version 21.0) was used for all analyses.

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Author Contributions

E.M.L., X.H.X. and L.R. conceived and designed this study. Y.G., Y. Z. and H.L. assisted to collect the samples and clinical information. Y.Y.Y. performed the experiments, analyzed the data and wrote the manuscript. Y.D., J.L.and Z.X.L. reviewed and revised the manuscript. All authors approved the final manuscript and agree to be accountable for all of the work.

Additional Information

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