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Phenotypic and molecular characterization of *Streptococcus pneumoniae* in pre-conjugate vaccine era: A Chinese hospital-based retrospective study

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ABSTRACT

Background: *Streptococcus pneumoniae* (*S. pneumoniae*) is an important pathogen in causing global morbidity and mortality among children. This study aimed to determine phenotypic and molecular characteristics of *S. pneumoniae* causing infections in children under five years in China.
Methods: A hospital-based retrospective study was conducted. All 537 *S. pneumoniae* isolates were tested for antimicrobial susceptibility by E-test method, molecular characteristics including resistance genes, virulence genes and serotypes by multiplex polymerase chain reaction (PCR) method, and sequence types (STs) by sequencing seven housekeeping genes. Minimum spanning tree and correspondence analysis were used to reveal the potential relationship between serotypes and STs.
Results: Most of *S. pneumoniae* isolates were resistant to erythromycin (93.9%) and tetracycline (86.4%), with the predominant resistance genes being *erm*(B) (92.6%) and *tet*(M) (95.5%). The prevalent serotypes were 19F, 6B, 19A, 23F and 14, the coverage rate of PCV13 was high in 85.8%, and the predominant STs were ST271, ST320, ST3173, ST81 and ST876. A significant correlation existed between STs and serotypes, with ST271/19F and ST320/19A as the most prevalent clones. Notably, ST271/19F and ST320/19A isolates were associated with resistance to specific antibiotics and carrying of *mef*(A/E), *rlrA* and *sipA* genes.
Conclusions: Our findings suggest the introduction of PCV13 vaccine to Chinese children, and underscore the value of monitoring multiple characteristics to detect new epidemiologic trends and provide implications for the formulation of multivalent pneumococcal vaccines.

1. Introduction

Streptococcus pneumoniae (pneumococcus, *S. pneumoniae*) is a leading cause of bacterial pneumonia, sepsis, and meningitis in children [1,2]. Recent estimates of children died from pneumococcal infections range from 0.7 to 1 million every year worldwide, mainly from developing countries [3]. Global burden of disease caused by *S. pneumoniae* in children indicated that the ten countries with the highest numbers and proportions of pneumococcal cases were all in Asia and Africa, with China as the second highest one [4]. Additionally, a recent systematic review revealed that *S. pneumoniae*-associated disease remains to be one of the leading causes of childhood deaths in China [5]. Antimicrobial resistance in *S. pneumoniae* remains a serious concern worldwide, particularly in Asia. Increasing data showed an extremely high prevalence of macrolide resistance and an increasing prevalence of multidrug resistance in many Asian countries, particularly in China [6–8]. Macrolide resistance is commonly conferred by ribosomal modification encoded by *erm* genes or through efflux pump mediated by *mef* genes [9,10]. Given the high prevalence of resistance and its clinical impact, continuous surveillance of pneumococcal epidemiology is strongly warranted.

Capsular polysaccharide is the main virulence determinant of *S. pneumoniae*, which so far can divide the serotypes into more than 90 [11]. Effective vaccine introduction, such as the 7-valent pneumococcal conjugate vaccine (PCV7: 4, 6B, 9V, 14, 18C, 19F, and 23F), provides a powerful strategy for the prevention of *S. pneumoniae* infection [8,12,13]. However, the epidemiology of *S. pneumoniae* has been changing in many countries after the introduction of PCV7. One of the most prominent changes is the emergence of non-vaccine serotypes (such as serotype 19A) worldwide [14]. To establish appropriate treatment strategies and new formulations of vaccine candidates, the epidemiology of pneumococcal

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diseases needs to be understood. *S. pneumoniae* possesses several virulence properties, such as autolysin A (*lytA*), pneumococcal surface adhesin A (*psaA*), pneumococcal surface protein A (*pspA*), pneumolysin (*ply*), and pilus (PI-1 and PI-2) [15,16]. Notably, the prevalence of PI-2 increased from 3.6% of the invasive pneumococcal isolates in 1999 to 21% in 2006 in Georgia, and the gene targets for virulence properties (e.g., *lytA*, *ply*, *psaA*, and *pspA*) appeared to be widely conserved among Malaysian human pneumococci, indicating that these targets may be worthwhile future vaccine candidates [17,18].

Since recent studies mainly focus on the serotypes and antibiotic susceptibility of *S. pneumoniae* [19,20], the potential relationship between STs, serotypes and molecular characteristics is still unclear. Therefore, we undertook a hospital-based retrospective study of *S. pneumoniae* causing infections in children younger than 5 years in Liuzhou, China. The objective of this study is to describe the antimicrobial susceptibility, serotypes, sequence types (STs) and other molecular characteristics of *S. pneumoniae*. Furthermore, we build on previous literature to explore the potential relationship between molecular characteristics in addition to differentiating *S. pneumoniae* clones based on multiple phenotypic and molecular characteristics.

2. Materials and methods

2.1. Study participants and bacterial identification

A hospital-based retrospective study was conducted between January 2015 and January 2017 in Liuzhou, China. The target population was children younger than 5 years and with *S. pneumoniae* infection recruited from the two hospitals. One hospital was the sole tertiary children's hospital in Liuzhou, with around 10,000–20,000 admissions of children annually. The other was a general tertiary hospital, with around 6000 admissions of children annually. Most of children patients in Liuzhou are treated in these two hospitals. The eligibility criteria for study participants included: (1) being children younger than 5 years; (2) not vaccinating against *S. pneumoniae*; (3) having clinical infectious manifestations (such as cough, respiratory secretions, abnormal auscultation, dyspnea, or fever higher than 38 °C without localising symptoms; and/or Chest X-ray infiltrates); and (4) positive for *S. pneumoniae* isolated from clinical sampling sites (specimens including blood, cerebrospinal fluid, pleural fluid, sputum, ascites, bronchoalveolar lavage fluid, sinus aspirates, and ear secretions). Specimens from each child upon enrollment were collected by the physicians or nurses as per routine medical practice. Patients with febrile symptoms over 38 °C were done blood culture within 24 h after admission routinely. We used standardized data forms for collection of demographic or clinical information (sex and age), any antibiotic use before admission (yes vs. no), and primary discharge diagnosis (e.g., pneumonia, otitis media, bacteremia, upper respiratory infections, sinusitis, pleural inflammation and hyperpyrexia).

The specimens were cultured on agar plates supplemented with 5% defibrinated sheep's blood and incubated overnight at 35 °C in 5% CO_2 atmosphere. *S. pneumoniae* was identified and confirmed by typical colony morphology, Gram staining, alpha-hemolysis, Optochin (Oxoid, Basingstoke, UK) susceptibility and bile solubility. Bacterial DNA was extracted using Biospin Bacteria Genomic DNA Extraction Kit (Bioer Ltd, Hangzhou, China) according to the manufacturer's instructions.

2.2. Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of antimicrobial agents (including penicillin G, erythromycin, clindamycin, tetracycline, chloramphenicol, cefotaxime, azithromycin, trimethoprim-sulfamethoxazole [SXT], levofloxacin, linezolid, and vancomycin) were determined using E-test method (Oxoid, Basingstoke, UK). Quality control was conducted using *S. pneumoniae* ATCC 49,619. The interpretations of MIC breakpoints and test results were made according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2016) [21].

2.3. Resistance and virulence genes

The polymerase chain reaction (PCR) assays were used to detect the macrolide resistance genes [*erm*(A), *erm*(B) *and mef*(A/E)], tetracycline resistance genes [*tet*(M), *tet*(O), *tet*(L) and *tet*(K)], virulence genes (autolysin A [*lytA*], pneumolysin [*ply*], pneumococcal surface protein A [*pspA*], pneumococcal surface adhesin A [*psaA*], and pilus genes (*rlrA* for PI-1 and *sipA* for PI-2) [18,22].

2.4. Serotyping

The serotypes of all pneumococcal isolates were identified by a multiplex PCR method using previously described primers and reaction conditions [23,24]. The scheme of multiplex PCR was six reactions to identify 18 serotypes (including serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 15A, 15B/C, 18C, 19A, 19F, 23A, 23F, 34, and 35B). A primer pair targeting *cpsA* found in all known pneumococcal serotypes was used as the positive control.

2.5. Multilocus sequence typing

Multilocus sequence typing (MLST) of the seven housekeeping genes (*aroE, gdh, gki, recP, spi, xpt, ddl*) was conducted using previously described primers and protocols [25]. STs and allelic profiles were confirmed by querying the pneumococcal MLST database (https://pubmlst.org/spneumoniae/).

2.6. Statistical analysis

All data were entered in duplicate into the EpiData version 3.0 database (The EpiData Association, Odense Denmark). Categorical variables were compared by Pearson chi-squared (χ^2) test or Fisher exact test when appropriate. The relationship between serotypes and STs of *S. pneumoniae* isolates was illustrated by minimum spanning tree (PHYLOVIZ software version 2.0; http://www.phyloviz.net) and was tested by correspondence analysis, which is a useful statistical method for studying the internal relations between variables. A two-sided p-value <.05 was considered as being of statistical significance. All statistical analyses were performed using Stata version 14.0 (StataCorp LP, College Station, Texas, USA).

2.7. Ethics statement

The study was approved by the Ethics Committee of Liuzhou Maternity and Child Healthcare Hospital, and it was performed in accordance with the approved guidelines. Written informed consent was obtained from parents or legal guardians on behalf of children involved in the study before enrollment.

3. Results

3.1. Demographic and clinical characteristics of study participants

A total of 537 children with *S. pneumoniae* infection were included. The demographic and clinical characteristics were provided in Table 1. The ages of study participants ranged from 0 to

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