Molecular epidemiology of pneumococcal isolates from children in China

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ABSTRACT

الأهداف: التحقيق في علم الأوبئة الجزيئية لعزل المكورات الرئوية في تشونغتشينغ، الصين.

الطريقة: في هذه الدراسة المقطعية كانت 51 سلالة غزوية عقدية رئوية (*S. pneumoniae*) من أطفال يعانون من مرض المكورات الرئوية الغازيه (IPD). و32 سلالة ناقلة من أطفال أصحاء في الفترة الممتدة من يناير 2010م إلى ديسمبر 2013م في مستشفى الأطفال التابع لجامعة تشونغتشينغ الطبية، تشونغتشينغ، الصين. واُستخدمت تقنية كتابة التسلسل متعدد المواضع لتحديد أنواع التسلسل (STs) وحدد تفاعل البوليميراز المتسلسل الأنماط المصلية المحفظية وحدد التركيز المثبط الأدنى مقاومة وحساسية الأدوية.

النتائج: حُدد في هذه الدراسة 11 نمط مصلي بين 83 من *S. pneumoniae* التي اختبرت سريرياً وعُزلت. كانت الأنماط المصلية السائدة 19A بنسبة (20.4%)، 6A/B بنسبة (20.4%)، 19F بنسبة (15.7%)، 14 بنسبة (14.5%)، و23F بنسبة (10.8%). كان النمط المصلي 19F أكثر السلالات الناقلة شيوعاً والنمط المصلي 19A أكثر السلالات الغزوية شيوعاً، ST983 أكثر نوع تسلسلي سائد للسلالات الناقلة وST320 أكثر نوع تسلسلي للسلالات الغازية. بالنسبة لتحليل الجينات كان psaA بنسبة (99.5%) وpiaA بنسبة (98.6%) موجودتنان ومحفوظتان في جميع المكورات الرئوية التي اُختبرت. الجينان cps2A وpcsB كانا أكثر انتشاراً بالسلالات الغازية المعزولة من سلالات الناقلة. كانت معدلات مقاومة مضادات الميكروبات من المكورات الرئوية الغازية المعزولة بالنسبة إلى الاريثروميسين، البنسلين، الميروبينيم، السيفوتاكسيم، والكليندامايسين أعلى من السلالات الناقلة المعزولة من الأطفال.

الخاتمة: ظلت 14، 19F، 6A/B، 19A، و23F أكثر الأنماط المصلية انتشاراً تبعاً لما أظهرته الأدلة الوبائية ويمكن أن يوجهها PCV13. تختلف الأنماط الجينية ومقاومة العقاقير من حيث السلالات الناقلة والغازية وقد يكون كلاً من PsaA وPiaA لقاحان بروتينيان مفيدان.

Objectives: To investigate the molecular epidemiology of pneumococcal isolates in Chongqing, China.

Methods: In this cross-sectional study, 51 invasive *Streptococcus pneumoniae* (*S. pneumoniae*) strains were from children with invasive pneumococcal disease (IPD) and 32 carriage strains from healthy children from January 2010 to December 2013 at the Children's Hospital of Chongqing Medical University, Chongqing, China. Multilocus sequence typing was used to identify the sequence types (STs). Capsular serotypes were determined by multiplex polymerase chain reaction. Drug susceptibility and resistance was determined by minimum inhibitory concentrations.

Results: In this study, 11 serotypes were identified among the 83 *S. pneumoniae* clinical isolates tested. Prevalent serotypes were 19A (20.4%), 6A/B (20.4%), 19F (15.7%), 14 (14.5%), and 23F (10.8%). Serotype 19F was the most frequent carriage strain, and serotype 19A was the most frequent invasive strain. The ST983 was the most prevalent ST for carriage strains, and ST320 was the most prevalent ST for invasive strains. For gene analysis, psaA (99.5%) and piaA (98.6%) were present and much conserved in all pneumococci tested. The cps2A and pcsB genes were more frequent in invasive isolates than carriage strains. Antimicrobial resistance rates of invasive pneumococcal isolates to erythromycin, penicillin, meropenem, cefotaxime, and clindamycin were higher than the carriage isolates from children.

Conclusion: Our epidemiological evidence shows that 19A, 6A/B, 19F, 14, and 23F remain the most prevalent serotypes, which can be targeted by PCV13. Genotypes and drug resistance varied between carriage and invasive strains. The PsaA and PiaA may be good protein vaccine candidates.

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Streptococcus pneumoniae (S. pneumoniae) is an important bacterial pathogen to cause invasive pneumococcal diseases (IPDs) in children, including bacteremia and meningitis. It has been estimated that more than 1.6 million children die from *S. pneumoniae* infections worldwide every year.[1] The increasing resistance to different antibiotics of *S. pneumoniae* in recent years is a new challenge for public health management worldwide.[2] This situation seems much more worrying in many Asian countries,[3] especially in China.[4] Heptavalent pneumococcal conjugate vaccine (PCV)7, composed of capsular polysaccharide antigens from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F has been demonstrated to have high effectiveness in controlling IPD in the developed areas.[5,6] It has been reported, however, that only 60% of the pneumococcal isolates could be covered by PCV7 in Asia.[7] The PCV7 has not been commonly used due to its high price in China. However, there are reports that non-vaccine type strains have a high prevalence in population with IPDs in China.[8,9] The PCV13 has recently been introduced in China; however, due to the lack of epidemic data, its coverage rate is still unknown in most regions of China. The *S. pneumoniae* commonly colonizes the respiratory tract asymptomatically, which may cause IPD when the balance between pneumococci and host immunity is broken. Transmission of *S. pneumoniae* occurs through the respiratory droplets, and is more commonly associated with healthy individuals who carry the organism in the upper respiratory tract.[10] Although previous studies suggested that the phenotype, genotype, serotype, and virulence factors were related with the pathogenesis,[11,12] it has not been investigated regarding the difference at the DNA sequence level of virulence factor between the invasive and carriage pneumococcal strains. Serotypes of carriage isolates may vary from geographic regions, vaccine policies, and over time.[13] The prevalence of the nasopharyngeal carriage of *S. pneumoniae* is related to invasive pneumococcal diseases.[14] There are more than 90 pneumococcal serotypes, but few serotypes account for most of the invasive diseases. The surveillance regarding the serotype distribution of nasopharyngeal carrier isolates in healthy children is necessary for a better management of IPDs, and understanding the information regarding the coverage of novel vaccines. So far, only a few studies have been performed to evaluate the nasopharyngeal carriage pattern of *S. pneumoniae* among children in mainland China. This study attempts to present the updated molecular epidemiology evidence and pathogenic features of the pneumococcal strains isolated from young children in Chongqing, China between 2010 and 2013. The phenotype, genotype, and serotype of invasive pneumococcal strains were compared with those of nasopharyngeal carriage strains.

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Methods.

Bacterial strains. In this cross-sectional study, all invasive *S. pneumoniae* strains were obtained from children less than 11 years old diagnosed with IPD by laboratory testing and professional clinicians based on typical clinical manifestation and imaging examination at the Children's Hospital of Chongqing Medical University, Chongqing, China from January 2010 to December 2013. Healthy children under 6 years old (n=789) who attended the community health center for national immunization (pneumococcal vaccines were not included in the national immunization program), or at kindergarten were also enrolled for the isolation of nasopharyngeal carriage strains of *S. pneumoniae*. Children with respiratory tract infections, chronic diseases, congenital cranio-facial anomalies, or those who had received antibiotics within 2 weeks prior to study entry were excluded. Nasopharyngeal specimens were collected by a trained technician. Nasopharyngeal swab specimens were collected with a cotton-tipped wooden swab, and immediately plated onto sheep blood agar and incubated at 37°C with 5% CO_2. Based on the typical colony morphology of *S. pneumoniae*, which include umbilical fossa, pale, translucent, grass green lysis, isolates were collected after 20-24 hours of incubation, and were further confirmed using the Optochin sensitivity test, bile solubility test, and automatic bacterial identified system phoenix100 (Becton, Dickinson Co., USA). All participants were truly informed (from their guardians where necessary) prior to their participation in the study; the above protocol was approved by the Clinical Research Ethics Committee of the affiliated Children's Hospital of Chongqing Medical University, and informed consent was obtained from all participants. This study was conducted according to the principles of Helsinki Declaration.

Serotyping. The serotype of each pneumococcal isolate was determined by multiplex polymerase chain reaction (PCR) amplification using a previously published method,[15] and the quellung reaction[16] with the sera of different reactivity from the Statens Serum Institut (Copenhagen, Denmark). The genomic DNA was extracted from bacteria using a bacterial DNA extraction kit (DP302-02; TIAGEN, Beijing, China). The amplification was performed in a 25 μl reaction volume with 30 thermal cycles, and other conditions were listed in **Table 1**.

Multilocus sequence typing. The sequence types (STs) were determined by multi-locus sequence typing (MLST). Internal fragments (approximately 550-600 base pair [bp]) of the aroE, gdh, gki, recP, spi, xpt, and ddl genes were amplified by PCR using previously described methods.[17] The sequences of each of the 7 loci were obtained by comparing with those of all known alleles at these loci and with the STs in the pneumococcal MLST website database (http://spneumoniae.mlst.net). The STs were analyzed for genetic relatedness using the eBURST v3 program (http://eburst.mlst.net).

Detection of virulence genes. Eleven known virulence factors including pneumolysin (Ply), autolysin (lytA), neuraminidase (nanA), polyhistidine triad protein D (PhtD), caseinolytic protease (clpP), capsular polysaccharide (cps2A), bacteriocin (antimicrobial peptide or protein), pneumococcal surface adhesin A (psaA), ion transporters (piaA), protein required for cell wall separation of group B streptococcus (pcsB), and serine/threonine protein kinase (stkP) were sequenced. The primers were designed according to the *S. pneumoniae* genomes available on the United States National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The primer sequences were listed in **Table 1**. The 11 virulence factors were amplified by PCR and sequenced to reveal any mutations, which were carried out by HuaDa Genomics Company (Beijing, China).

Antimicrobial susceptibility testing. Antimicrobial susceptibility patterns were determined by automatic bacteria identification/susceptibility system Phoenix100 (BD, USA), and used to assess the antibiotic susceptibility of all 12 isolated antibiotics

Table 1 - Primers and amplification conditions used to amplify the 11 virulence genes.

Virulence genes	Primer, 5' - 3'	Length, bp	TM, °C	Extending time
Ply	up 5'ATGGCAAATAAAGCAGTAAATGACT 3' dn 5'CTAGTCATTTTCTACCTTATCCTCT 3'	1415	55	1 min 30 s
LytA	up 5'ATGGAAATTAATGTGAGTAAATTAA 3' dn 5'TTATTTTACTGTAATCAAGCCATCT 3'	956	59	1 min
NanA	up 5'ATGATTGTAGGAGCAGTGGTATTTG 3' dn 5'TTATTGTTCTCTCTTTTTCCCTAGC 3'	2993	55	3 min
PhtD	up 5'TGGGTCTTAAAACTCTGA 3' dn 5'CTCCATAAAATATGCTCC 3'	2704	53	3 min
clpP	up 5'AACTCAAAAGGAGAAATG 3' dn 5'TCTTGGAATGATAGGTAAT 3'	791	54	1 min
Cps2A	up 5'TTCGCGGGAAGTCTACTAAG 3' dn 5'GGGACCGTCATCTACATCAA 3'	1606	65	2 min
Bacteriocin	up 5'AAGAGTAAGTTCGTTGTT 3' dn 5'TCTACAGTCTTTCCCATT 3'	577	58	1 min
PsaA	up 5'TAATGTTGCGGCAGGTTCT 3' dn 5'CGGAAATGTGGGCATAGAA 3'	1136	66	1 min 30 s
PiaA	up 5'GACTTGAAATATGTTTAAGGAGT 3' dn 5'TGTATTACCCATTCCAAAA 3'	1185	54	1 min 30 s
PcsB	up 5'ACGGTAAAACCTGAAAAGAG 3' dn 5'AAATGTAACAAAGGCGTAAT 3'	1305	60	1 min 30 s
StkP	up 5'TTCCATGCTTTCCTCCTT 3' dn 5'ACATTACGGTTGCCCTTG 3'	2142	61	2 min 30 s

Ply - pneumolysin, LytA - autolysin, NanA - neuraminidase, PhtD - polyhistidine triad protein D, ClpP - caseinolytic protease, Cps2A - capsular polysaccharide, Bacteriocin - antimicrobial peptide or protein, PsaA - pneumococcal surface adhesin A, PiaA - ion transporters, PcsB - protein required for cell wall separation of group B streptococcus, StkP - serine/threonine protein kinase, bp - base pair, TM - mean temperature, s - seconds

according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI).[18] The CLSI 2013 criteria_ENREF_39[19] for minimum inhibitory concentrations (MICs) were applied to classify susceptible, intermediate, and resistant isolates. The separate interpretive breakpoints for non-meningeal, meningeal were used to define cefotaxime and Cefepime resistance: MIC 2 μg/ml (meningeal) and ≥4 μg/ml (non-meningeal). For parenteral penicillin resistance: MIC ≥0.12 μg/ml (meningeal), and ≥8 μg/ml (non-meningeal). As to the exact MIC of vancomycin, it would only be given when the MIC value is greater than 1 μg/ml, otherwise, the MIC of vancomycin would be given as <1 μg/ml by analytical platform. The *S. pneumoniae* ATCC 49619 was used as a quality control strain during susceptibility testing.

Statistical analysis. Data were collected and classified. The detection rate of virulence factor and nonsensitive percentage of antibiotic was calculated. Numeration data were described by positive example and rate, and then analyzed using the X^2 test or Fisher's exact test (2-tailed). All analyses were performed using the Statistical Package for Social Sciences software for Windows version 10 (SPSS Inc., Chicago, IL, USA). $P<0.05$ were considered statistically significant.

Results. ***General properties of pneumococcal isolates.*** A total of 83 isolates including 51 invasive strains and 32 carriage strains were obtained from children in Chongqing. Among the 51 isolates of invasive *S. pneumoniae*, 32 strains (62.8%) were isolated from blood, 9 strains (17.7%) from cerebrospinal fluid, 7 strains (17.7%) from pleural effusion, 2 strains (13.7%) from pus, and one strain (2%) from bone marrow. Among the 51 strains, 30 strains (58.8%) were from males, and 21 (41.2%) strains were from females. Among the 789 healthy children attending the study, 44 subjects had a vaccination history of PCV, and 745 subjects do not have vaccination history of pneumococcal vaccines. In addition, 32 carriage strains that were mentioned above were isolated from the nasopharyngeal cavity of healthy children. The carriage rate of *S. pneumoniae* was 4.1%. Among the 32 *S. pneumoniae* carriages, 18 isolates were from infants under 2 years old, whereas 14 were from preschoolers 2 years old and above. Fifteen isolates were from boys, whereas 17 isolates were from girls.

Serotypes of the pneumococcal isolates. Serotype distribution was diverse and widely divergent among the carriage and invasive isolates (**Tables 2-5**). The 83 isolates were composed of 11 STs. Prevalent serotypes were 19A (20.4%), 6A/B (20.4%), 19F (15.7%), 14 (14.5%), and 23F (10.8%). Nine STs were identified in 51 invasive isolates. Most serotypes were 19A (25.5%), followed by serotype 14 (19.6%), 6A/6B (19.6%), 23F (13.7%), 19F (6.0%) and 1 (5.9%). In addition, there is only one isolate for serotypes 33/33B, 17F, and 11/11A (2%). Seven serotypes were identified in 32 of the carriage isolates. Most serotypes were 19F (25%), followed by 6A/B (22%), 19A (13%), 15 (9%), 6C (6%), 14 (6%), and 23F (6%). Four carriage isolates were non-typeable. Serotypes 14, 6A/6B, 23F, 19F and 19A were detected in both strain types. However, several serogroups among the invasive strains have never been identified in carriage strains, such as serogroups 1, 33/33B, 17F, and 11/11A. The serogroups 15 and 6C found in the carriage strains were rarely isolated as invasive groups.

The MLST. Eighty-three isolates were characterized using MLST, and 18 known STs were identified, 5 of which have not been previously recorded in the online pneumococcal database (http://spneumoniae.mlst.net). A strong association between the ST and serotype of isolates was observed (**Tables 2-5**). Isolates of closely related STs (clone complexes) almost invariably had the same serotype. In invasive isolates, ST320 and ST876 were the most frequent STs (serotypes 19A and 14) (23.5%), followed by ST876 (19.6%), ST2296 (7.8%), ST90 (7.8%), ST81 (7.8%), and ST271 (7.8%), T242 (5.9%), and ST3173 (5.9%), ST2754 (2%), ST902 (2%), ST4112 (2%), ST3263 (2%), and new STs account for 5.8%. Nine STs were identified in 32 of the carriage isolates. The ST983 (serotypes 19F [28.6%]) was the primary ST for carriage strain, followed by ST2912, ST7759, ST3397, ST7752, ST7392, ST876, ST81 and ST3173 (for all), and new STs account for 14.3%. Diversity comparison of STs showed that ST7752, ST7392, ST983, ST2912, ST7759 and ST3397 were not presented in invasive isolates, and ST2296, ST90, ST271, ST242, ST2754, ST902, ST4112 and ST3263 were rarely observed in carriage isolates. In addition, eBURST analyses using the stringent 6/7 identical loci definition grouped the 18 STs into 2 clonal complexes, and 16 singletons expressing a high level of genetic diversity among the isolates.

Detection of virulence genes. Virulence factors are important candidate targets for the design of next-generation protein vaccines. The conservation of the protein antigens in epidemic strains is taken as an important index to decide whether this antigen is conserved enough to protect broad protection against pneumococcal infections. Eleven virulence factors were

Table 2 - Results of sequence types (STs) and serotypes of 51 strains of invasive *Streptococcus pneumoniae (S. pneumoniae)*.

Sample	aroE, (%)	gdh	gki	recP	spi, (%)	xpt, (%)	ddl, (%)	ST	Serotype
D39	7	5	1	1	10	7	15	595	2
I1	4 (99)	16	19	15	6 (99)	20	1	320	19A
I2	10 (99)	31	4	34	6 (99)	4	94	2296	1
I3	15 (99)	29	4	21	30	1	14	242	23F
I4	15 (99)	29	4	21	30	1	14	242	23F
I5	10 (99)	31	4	34	6 (99)	4	94 (97)	2296	1
I6	8 (99)	13	14	4	6 (99)	4	14	876	14
I7	5 (99)	6	1	2	6 (99)	3	4	90	6/6B/A
I8	10 (99)	31	4	34	6	4 (99)	94	2296	1
I9	10 (99)	31	4	34	6	4	94	2296	1
I10	4 (99)	16	19	15	6 (99)	20	1	320	19A
I11	4 (99)	4	2	4	4	1 (98)	1	81	23F
I12	4 (99)	4	2	4	4	1 (98)	1	81	23F
I13	2 (99)	88	70	16	6 (99)	19 (98)	18	2754	33/33B
I14	4 (99)	16	19	15	6 (99)	20 (98)	1	320	19A
I15	2 (99)	13	2	1	6 (99)	121 (98)	121	902	6/6B/A
I16	5 (99)	6	1	2	6 (99)	3 (98)	4	90	6/6B/A
I17	4	16	19	15	6	20	1	320	19A
I18	8 (99)	13	14	4	6 (98)	4 (98)	14	876	14
I19	4	16	19	15	6 (98)	20 (98)	26	271	19F
I20	4 (99)	16	19	15	6 (99)	20	1	320	19A
I21	4 (99)	16	19	15	6 (99)	20	1	320	19A
I22	4 (99)	16	19	15	6	20	26	271	19F
I23	5	6	1	2	6 (99)	20	1	New	6/6B/A
I24	5 (99)	6	1	2	6 (99)	20 (99)	1	New	6/6B/A
I25	8 (99)	13	14	4	6	4 (98)	14	876	14

I - invasive *S. pneumoniae*. New - novel and unidentified ST.
aroE, Gdh, gki, recP, spi, xpt, and ddl were the 7 housekeeping genes of *S. pneumoniae*

Table 3 - Results of sequence types (STs) and serotypes of 51 strains of invasive *Streptococcus pneumoniae (S. pneumoniae)* (continued).

Sample	aroE, (%)	gdh	gki	recP	spi, (%)	xpt, (%)	ddl, (%)	ST	Serotype
I26	8 (99)	13	14	4	6 (99)	4 (98)	14	876	14
I27	4 (99)	16	19	15	6	20 (98)	1	320	19A
I28	4 (99)	4	2	4	4	1 (98)	1	81	23F
I29	4 (99)	16	19	15	6 (99)	20 (98)	1	320	19A
I30	137 (99)	5	9	16	6 (99)	1	18	4112	17F
I31	4 (99)	16	19	15	6 (99)	20 (98)	1	320	19A
I32	15 (99)	16	19	68 (99%)	6	20 (98)	26	New	19F
I33	7 (99)	30	8	6	6 (99)	6 (99)	1	3173	6A/6B
I34	4 (99)	16	19	15	6 (99)	20	1	320	19A
I35	2 (99)	8	70	16	6 (99)	19 (98)	31	3263	6B
I36	4 (99)	16	19	15	6 (98)	20 (98)	1	320	19A
I37	15	29	4	21	30	1	14	242	23F
I38	8 (99)	13	14	4	6 (98)	4 (97)	14	876	14
I39	8 (99)	13	14	4	6 (99)	4 (99)	14	876	14
I40	5 (99)	6	1	2	6 (99)	3	4 (98)	90	6B
I41	5 (99)	8	4	16	6 (98)	1	31	99	11/11A
I42	8 (99)	13	14	4	6	4 (97)	14	876	14
I43	7 (99)	30	8	6	6 (99)	6 (99)	1	3173	6B/6A
I44	7 (99)	30	8	6	6 (99)	6	1	3173	6B/6A
I45	8 (99)	13	14	4	6	4 (97)	14 (99)	876	14
I46	8 (99)	13	14	4	6	4 (99)	14	876	14
I47	8 (99)	13	14	4	6	4 (99)	14	876	14
I48	4	16	19	15	6	20	26 (99)	271	19F
I49	4	16	19	15	6	20 (99)	26 (99)	271	19F
I50	4	16	19	15	6	20	1	320	19A
I51	4	4	2	4	4	1	1	81	23F

I - invasive *S. pneumoniae*. New - novel and unidentified ST.
aroE, Gdh, gki, recP, spi, xpt, and ddl were the 7 housekeeping genes of *S. pneumoniae*

Table 4 - Results of sequence types (STs) and serotypes of 32 strains of carriage *Streptococcus pneumoniae (S. pneumoniae)*.

Sample	aroE, (%)	Gdh, (%)	gki	recP	spi, (%)	xpt, (%)	ddl, (%)	ST	Serotype
C1	15 (99)	16	19	15	3	104 (99)	63	983	19F
C2	15 (99)	16	19	15	3	104 (99)	63	983	19F
C3	15 (99)	16	19	15	3	104 (99)	63	983	19F
C4	15 (99)	16	19	15	3	104 (99)	63	983	19F
C5	242 (95)	263 (97)	44 (96)	54 (96)	265 (95)	46 (96)	131 (99)	New	NT
C6	2 (99)	8	9	38	17 (99)	1 (98)	9	2912	6C
C7	10 (99)	13	4	16	17	365 (99)	31	7759	NT
C8	8	13	14	4	6	4	14	876	14
C9	7	30	8	6	6	6	1	3173	6A/B
C10	4	4	2	4	4	1	1	81	23F
C11	7	187	122	87	17	1	290	3397	15
C12	2	8	83	16	6	19	18	7752	NT
C13	5	8	9	38	17	1	9	7392	6B
C14	7	321 (99)	4	1	6	12	14	New	NT
C15	15 (99)	16	19	15	3	104 (99)	63	983	19F

C - carriage strain, NEW - novel and unidentified ST.
aroE, Gdh, gki, recP, spi, xpt, and ddl were 7 housekeeping genes of *S. pneumoniae*

Table 5 - Results of sequence types (STs) and serotypes of 32 strains of carriage *Streptococcus pneumoniae (S. pneumoniae)* (continued).

Sample	aroE, (%)	Gdh	gki	recP	spi, (%)	xpt, (%)	ddl	ST	Serotype
C16	4 (99)	16	19	15	6 (99)	20	1	320	19A
C17	7	187	122	87	17	1	290	3397	15
C18	5	8	9	38	17	1	9	7392	6B
C19	7	30	8	6	6	6	1	3173	6A/B
C20	4 (99)	16	19	15	6 (99)	20	1	320	19A
C21	15 (99)	16	19	15	3	104 (99)	63	983	19F
C22	15 (99)	16	19	15	3	104 (99)	63	983	19F
C23	15 (99)	16	19	15	3	104 (99)	63	983	19F
C24	4 (99)	16	19	15	6 (99)	20	1	320	19A
C25	4 (99)	16	19	15	6 (99)	20	1	320	19A
C26	7	30	8	6	6	6	1	3173	6A/B
C27	7	187	122	87	17	1	290	3397	15
C28	7	30	8	6	6	6	1	3173	6A/B
C29	5	8	9	38	17	1	9	7392	6B
C30	2 (99)	8	9	38	17 (99)	1 (98)	9	2912	6C
C31	8	13	14	4	6	4	14	876	14
C32	4	4	2	4	4	1	1	81	23F

C - carriage strain, NEW - novel and unidentified ST.
aroE, Gdh, gki, recP, spi, xpt, and ddl were 7 housekeeping genes of *S. pneumoniae*

amplified by PCR and sequenced. Results showed the prevalence rates of cps2A (90.2%), bacteriocin (82.4%), and phtD (94.1%). The other 8 virulence factors could be amplified from all the invasive isolates. For the carriage strains, the prevalence rate for the virulence factors of pcsB was 57.1%, cps2A - 57.1%, clpP - 78.6%, bacteriocin - 71.4%, phtD - 78.6%, nanA - 78.6%, stkP - 85.7%, ply - 85.7%, lytA - 85.7%, psaA - 100%, and piaA - 100%. The prevalence of cps2A was significantly different between invasive (90.2%) and carriage (57.1%) isolates (p=0.029). Similarly, the prevalence of pcsB was significantly different between invasive (100%) and carriage (57.1%) isolates (p=0.008) (**Figure 1**). The conservation from DNA sequence level of the virulence factors, including psaA was 99.5%, piaA - 98.6%, clpP - 98.5%, ply - 97.8%, stkP - 96.7%, lytA - 96.3%, pcsB - 88.7%, phtD - 87.5%, cps2A - 83.7%, nanA - 83%, and bacteriocin was 66.3% in the invasive isolates. In general, a strong association between the ST and the DNA sequence of virulence factors was observed in this collection of isolates (**Table 6**). The DNA sequence of virulence factors was almost invariable in the same STs. In our study, ST271 and ST320 were mainly detected in invasive strains, while were rarely

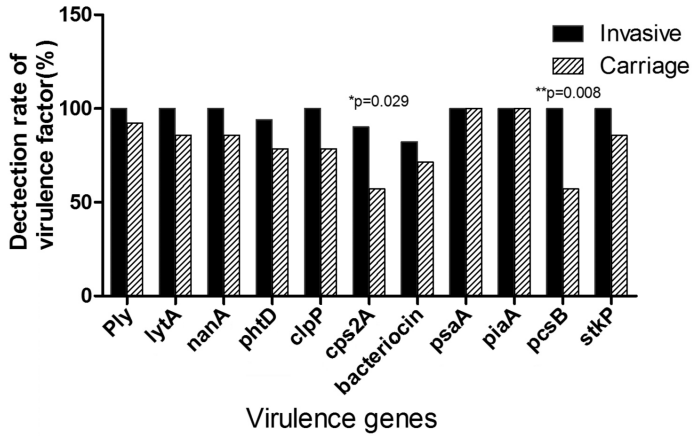

Figure 1 - Individual prevalence of the 11 virulence genes in invasive and carriage pneumococcal strains.

Table 6 - The association of sequence types (STs) and virulence factors.

Virulence factors	ST320	ST876	ST81	ST271	ST90	ST2296	ST983	ST3397
Ply	Y	Y	Y	Y	Y	Y	Y	Y
Cps2A	M	Y	Y	Y	Y*	Y	Y	Y
NanA	M	Y	Y	Y	Y	Y	Y	Y
LytA	Y	Y	Y	Y	Y	Y	Y	Y
PspA	M	Y	Y	M	Y	M	Y	Y
PcsB	Y	Y	Y	M	Y	Y	Y	Y
StkP	M	Y	Y	M	Y	Y	Y	Y
Bacteriocin	Y	Y	Y	Y	Y	Y*	Y	Y
PiaA	Y	Y	Y	Y	M	Y	Y	Y
ClpP	Y	Y	Y	Y	Y	Y	Y	Y
PsaA	Y	Y	Y	M	Y	Y	Y	Y

Ply - pneumolysin, Cps2A - capsular polysaccharide, NanA - neuraminidase, LytA - autolysin, PsaA - pneumococcal surface adhesin A, PcsB - protein required for cell wall separation of group B streptococcus, StkP - serine/threonine protein kinase, Bacteriocin - antimicrobial peptide or protein, PiaA - ion transporters, ClpP - caseinolytic protease, PsaA - pneumococcal surface adhesin A.
Y - nucleotide sequences of virulence factors were homologous in the same ST, M - nucleotide sequences of virulence factors have the base mutation, Y* - virulence factors were not amplified in some ST

Table 7 - Susceptibility testing of *Streptococcus pneumoniae* strains isolated from children younger than 6 years old.

Antimicrobial agent	Invasive (n = 51)		Carriage (n = 32)	
	S	NS	S	NS
	%			
Vancomycin	100	0	100	0
Linezolid	100	0	100	0
Levofloxacin	100	0	100	0
Moxifloxacin	100	0	100	0
Erythromycin	0	100.0	28.6	71.4
Penicillin	41.2	58.8	92.9	7.2
Meropenem	21.6	78.4	57.1	42.9
Cefotaxime	64.7	35.3	100	0
Clindamycin	5.9	94.1	29.6	71.4
Amoxicillin	69.1	31.0	92.9	7.1
Chloramphenicol	92.2	7.8	57.1	42.9
Trimethoprim/sulfa methoxazole	37.3	62.8	35.7	64.3
Tetracycline	15.7	84.3	0	100

S - susceptible, NS - non-susceptible (resistance and intermediate)

observed in the carriage *S. pneumoniae*. The virulence factor were conservation in most STs. Interestingly, the base sequence of virulence factor in ST271 and ST320 have more mutations than other STs.

Drug resistance test. The antibiotic resistance patterns of invasive and carriage isolates are summarized in **Table** 7. Drug resistance data showed that antimicrobial resistance of the invasive pneumococcal strains was generally higher than carriage strains. All 83 isolates were completely sensitive to vancomycin, linezolid, levofloxacin, and moxifloxacin at the current applied breakpoints. Penicillin's nonsusceptibility was displayed by 58.8% of the invasive isolates. Moreover, the nonsusceptibility of the invasive isolates to trimethoprim/sulfamethoxazole was 62.8%, tetracycline - 84.3%, clindamycin - 94.1%, and erythromycin was

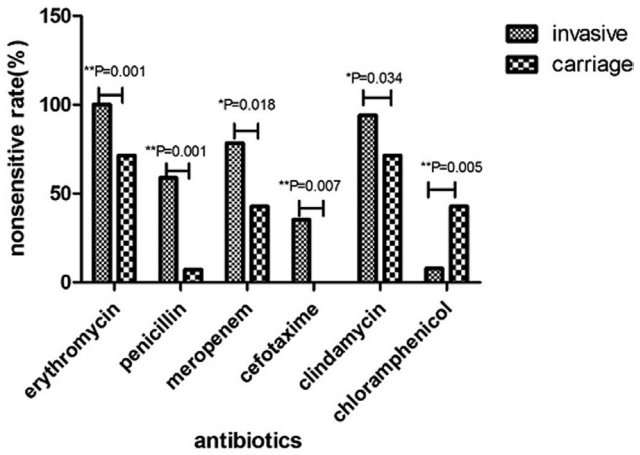

Figure 2 - Antimicrobial resistance between carriage and invasive pneumococcal isolates.

100% (**Table** 7). The nonsusceptibility rates of invasive strains to erythromycin (p=0.001), penicillin (p=0.001), meropenem (p=0.018), cefotaxime (p=0.007), and clindamycin (p=0.034) were significantly higher than the carriage strains. Only non-susceptibility to chloramphenicol (p=0.005) was significantly lower in invasive strains than carriage strains (**Figure 2**).

Discussion. The capsule is the main virulence determinant of *S. pneumoniae*. Only a few capsular types tend to be associated with invasive diseases, which may partly attribute to the differential ability of the capsular type variants in resisting phagocytosis.[20,21] Serotypes of invasive isolates may vary from region to region and over time.[22-24] Our study indicates that serotype 19A is the most prevalent invasive strain among children in Chongqing. This observation is similar to that of a study reported before in mainland China.[9] In contrast, serotype 14 was the most common invasive isolates reported among children in India.[25] The PCV7 coverage rate against invasive strains (62.7%) reported in this study is also similar to those of some Asian countries, such as Denmark and Singapore <65%).[26-28] However, this figure is lower than those observed in the United States (87%),[29] Canada, Europe (78-94%),[30] and Spain (72.3%).[31] Some researchers explained that the low percentage of PCV7-related serotypes may be due to the herd immunity effect, as reported in the United States,[32] and United Kingdom.[33] However, unlike the situation of high vaccine inoculation rate of PCV7 in the United States and United Kingdom, the prevalence of pneumococcal strains reflects the real circulating pneumococcal strains in Chongqing since the vaccine inoculation rate is only 4.4% in this region, which is quite the same as that in Singapore.[26] The PCV13 extends the serotype coverage, and serotypes 19F, 14, 6A, 6B, 23F, 18C, 9V, 4, 1, 5, 7F, 3, and 19A were included. The present finding showed that this vaccine could cover 94.1% (48/51) of the invasive isolates, and hence indicates the potential value of PCV13 in protecting pneumococcal infections in China, specifically in Chongqing city.

The MLST analysis reveals a significant diversity among all strains in terms of their STs. Our data show that ST320 is the most prevalent clone among the invasive isolates, whereas ST983 was the most prevalent clone among the carriage isolates in Chongqing, China. A strong association between the serotype and ST among carriage and invasive populations was observed.[34] Although previous studies[17] have shown that the pneumococcal serotype has a dominant role in determining its invasiveness, the genotype can affect the invasiveness of *S. pneumoniae*. Serotype 19F was identified to have 2 STs, ST271 and ST983 in the present study. The ST271 was only found in invasive serotype 19F, whereas ST983 was only found in the carriage strains. This phenomenon was also observed for serotype 6 A/B. Besides, 2 STs in our study including ST 90 and ST 2912 were associated with multiple serotypes indicating a history of serotype switching, which has been noted in West Africa.[35] The gene spectrums of 14.5% isolates were matched with those in pneumococcal molecular epidemiology network (PMEN) database. These isolates could be defined as the corresponding PMEN cloning plants or its mutant strains. In Chongqing, the most prevalent PMEN cloning was Spain23F-1.

Pneumococcal isolates with lower susceptibility to antibiotics and non-susceptible strains are increasing. This increase in the prevalence of drug-resistant pneumococci may result from the frequent and unnecessary use of antimicrobial drugs. Our study shows that drug resistance was higher in invasive strains than carriage strains, except for chloramphenicol, which carriage strains have a higher resistant rate than the invasive strains (p=0.005). The increase in chloramphenicol resistance may reflect the abusive use of this drug in communities. The incidence reported in this study is consistent with that previously reported in China.[8] The nonsusceptibility rate to penicillin is higher in China than in the United States and several other Asian countries.[36-38] Unlike the figure reported in the Western countries, macrolide nonsusceptibility of invasive strains is high in Chongqing.[39] The major reasons for this high level of macrolide resistance may be the widespread use of macrolides in clinical practice and clone spread of macrolide-resistant strains in China cities. Thus, the empirical use of macrolides alone for the treatment of pneumococcal infections can be an inappropriate option in China. The surveillance of the antibiotic resistance of carriage strains is useful to guide the empirical treatment against IPD.

Although PCVs are effective against IPDs, they are not widely accepted in undeveloped countries due to their high cost and limited serotype coverage. An alternative strategy is to design a protein-based pneumococcal vaccine and some protein candidates were protective against pneumococcal infections, either alone or in combinations.[40-42] The PsaA and PiaA have been demonstrated to decrease the number of bacteria in the lungs of mice challenged with *S. pneumoniae*, and increase the survival rate in a mouse pneumococcal lethal intranasal challenge model.[43] In our study from the aspect of antigen conservation, it shows that PsaA and PiaA are likely to be important protein vaccine candidates, and are worthy of further investigation. Notably, our study shows that cps2A and pcsB were less apt to be detected in carriage strains than in invasive strains, which suggests the positive effect of cps2A and pcsB of the invasive strains to combat with the host. The base sequence of virulence factors in ST271 and ST320, which were apt to be determined in invasive isolates have more mutations than in other STs. We hypothesize that these genetic mutations might strengthen the virulence of strains to cause invasive pneumococcal disease. In addition, the genetic mutations of invasive *S. pneumoniae* might be a result of "the survival of the fittest". Accordingly, those mutations might involve in the genes of virulence factor.

Nasopharyngeal colonization with *S. pneumoniae* is asymptomatic in most infants with carriage rates that vary from 3.8-90%.[13,44,45] Our study shows a very low nasopharyngeal carrier rate (4.1%) for pneumococci in Chongqing children. This low carrier rate observed in our study may be due to the hot climate in Chongqing, sample collection time or specimen processing methods.

This study has several limitations. First, the number of carriage strains was limited. Secondly, potential primer divergence implies that a PCR negative result does not necessarily indicate the absence of proteins. Thirdly, this study was performed in Chongqing, which may not represent the whole situation of China. Fourthly, some serotypes and STs have not been identified so far, and further studies are needed to identify them in the future.

In conclusion, pneumococcal infection remains an important burden in children population. Our study shows prevalent serotypes among the 83 *S. pneumoniae* clinical isolates were 19A, 6A/B, 19F, 14, and 23F. Of them, serotype 19A was the most prevalent invasive isolates, and serotype 19F was the most prevalent carriage isolates. The potential coverage for PCV13 against invasive strains is 94.1%. Drug resistance varied among different serotypes and between invasive and carriage strains. The Cps2A and PcsB may be partly responsible for the increased virulence of invasive strains with a higher attack rate. The PsaA and PiaA are highly conserved among the pneumococcal clinical isolates, which shall be important candidates to be considered in protein based vaccines.

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Ethical Consent

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.