

Molecular characteristics of penicillin-binding protein 2b, 2x and 1a sequences in *Streptococcus pneumoniae* isolates causing invasive diseases among children in Northeast China

X. Zhou¹ · J. Liu¹ · Z. Zhang¹ · Y. Liu¹ · Y. Wang¹ · Y. Liu¹

Received: 30 November 2015 / Accepted: 11 January 2016 / Published online: 14 March 2016
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Abstract *Streptococcus pneumoniae* is one of the common pathogens causing severe invasive infections in children. This study aimed to investigate the serotype distribution and variations of penicillin-binding proteins (PBPs) 2b, 2x and 1a in *S. pneumoniae* isolates causing invasive diseases in Northeast China. A total of 256 strains were isolated from children with invasive pneumococcal disease (IPD) from January 2000 to October 2014. All strains were serotyped and determined for antibiotic resistance. The amplicons of penicillin-binding domains in *pbp1a*, *pbp2b* and *pbp2x* genes were sequenced for variation identification. The most prevalent serotypes of isolates in IPD children were 19A, 14, 19F, 23F and 6B. 19A and 19F were the most frequent serotypes of penicillin-resistant *S. pneumoniae* (PRSP), which present with high resistance to amoxicillin, cefotaxime, ceftriaxone and meropenem. The numbers of amino acid substitutions of penicillin-non-susceptible *S. pneumoniae* (PNSP) isolates were higher than those of penicillin-sensitive *S. pneumoniae* isolates in all the PBP genes ($p < 0.01$). The patterns of amino acid mutation in PBP2b, PBP2x and PBP1a were unique and different from those of other countries. All of the serotype 19A and 19F PRSP isolates carried 25 amino acid mutations, including Ala618 → Gly between positions 560 and 675 in PBP2b and Thr338 → Ala substitutions in PBP2x. The amino acid alterations in PBP2b, PBP2x and PBP1a from *S. pneumoniae* were closely associated with resistance to β -lactam antibiotics. This

study provides new data for further monitoring of genetic changes related to the emergence and spread of resistance to β -lactam antibiotics in China.

Introduction

Streptococcus pneumoniae is one of the most common pathogens causing severe invasive infections (including sepsis, empyema and meningitis) in infants. Both morbidity and mortality of invasive pneumococcal disease (IPD) are high worldwide, especially in developing countries. The World Health Organization (WHO) estimated that there were ~8.8 million deaths among children aged <5 years worldwide in 2008, and 476,000 (333,000–529,000) were caused by pneumococcal infections [1]. More recently, a review showed that there were 12,815 cases/100,000/year of all-cause pneumonia and 14 meningitis cases/100,000/year among children aged 1–59 months, with 526 deaths/100,000/year in China between 1980 and 2008 [2].

The β -lactam antibiotics (BLAs) have played a leading role in the treatment of *S. pneumoniae* infection for a long time. However, in recent years, penicillin-resistant *S. pneumoniae* (PRSP) and penicillin-intermediate *S. pneumoniae* (PISP), which are collectively referred to as penicillin-non-susceptible *S. pneumoniae* (PNSP), were reported to be detected around the world continually [3, 4]. The detection rates of PNSP isolated from patients in mainland China with invasive and non-invasive disease were 27 % and 17 %, respectively, which were significantly higher than those of other Asian areas [5].

BLAs are involved in the synthesis of peptidoglycans by binding to the active site of penicillin-binding protein (PBPs), thus disrupting the formation of normal cell walls and inducing cell death by bacteriolysis [6, 7]. *Streptococcus*

X. Zhou and J. Liu contributed equally to this work.

✉ Y. Liu
liuy@sj-hospital.org

¹ Present address: Department of Clinical Laboratory, The Affiliated Shengjing Hospital, China Medical University, No. 36, Sanhao Street, Heping District, Shenyang 110004, China

pneumoniae expresses six kinds of PBPs (PBP1a, PBP1b, PBP2a, PBP2b, PBP2x and PBP3). The first three proteins belong to Class A, which have activities of both transglycosylation and transpeptidylation; Class B proteins (PBP2b and PBP2x) only have transpeptidylation function; and PBP3 has D, D-carboxypeptidase activity [8, 9]. The active catalysis structure of each PBP is formed by three conserved amino acid motifs, SXXK, SXN and KT/SG [10]. Resistance to penicillin and other BLAs in *S. pneumoniae* is mainly due to alterations within or flanking these motifs, which results in alteration of PBPs and lowers the affinity of PBPs to penicillin [10–12].

Although the variants of *pbp1a*, *pbp2b* and *pbp2x* genes of *S. pneumoniae* have been studied extensively worldwide, few data are available on *S. pneumoniae* strains from IPD in mainland China. It may be that the improper use of antibiotics results in the low detection rates of *S. pneumoniae*. Vaccination is important in preventing *S. pneumoniae* infection due to the rapid spread of bacteria that are resistant to commonly used antibiotics. For the effective control of pneumococcal disease, the composition of the vaccine must closely match the prevalent serotypes in the native area. In view of the regional disparity of serotypes of *S. pneumoniae*, the coverage rate of pneumococcal conjugated vaccines (PCVs) and the distribution of PNSP, it is necessary to investigate the serotype distribution and resistance pattern of *S. pneumoniae* isolates from IPD patients in Northeast China. Thus, this study aimed to determine the amino acid variations encoded by *pbp1a*, *pbp2b* and *pbp2x* genes from clinical isolates of IPD patients in Northeast China. It also aimed to analyse the effect of amino acid modification on resistance to penicillin and other BLAs, and explore the associations between amino acid modification and serotypes.

Materials and methods

Strains

A total of 256 strains were collected from patients with IPD admitted to Shengjing Hospital of China Medical University from January 2000 to August 2014. All the strains were isolated from blood (135, 52.7 %) and other sterile body fluid samples, including pleural fluid (57, 22.3 %), cerebral spinal fluid (CSF) (55, 21.5 %), joint cavity fluid (5, 2.2 %) and abdominal fluid (4, 1.8 %). Most of the strains were isolated from infants aged ≤ 2 years (165, 64.5 %), whilst others were isolated from children aged 2–5 years (52, 20.3 %) and children aged 5–14 years (39, 15.2 %). All isolates were placed in a skimmed milk preservation medium and stored at -80 °C until further analysis. The study was approved by the Shengjing Hospital Ethics Committee.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of penicillin (PEN) were measured using Etest strips (AB Biodisk, Solna, Sweden). The MICs of amoxicillin (AMC), cefotaxime (CTX), ceftriaxone (CRO) and meropenem (MEM) were determined by the VITEK 2 compact system (bioMérieux, Marcy l'Etoile, France) using AST-GP68 cards. Breakpoints were based on the 2013 revised criteria of the Clinical and Laboratory Standards Institute (CLSI) [13]. *Streptococcus pneumoniae* ATCC 49619 was used as a quality control strain.

Serotyping

All isolates were serotyped by a Quellung test using type-specific antisera (Statens Serum Institut, Copenhagen, Denmark). Serotyping was performed by phase-contrast microscopy, as previously described [14]. Only one isolate was accepted when two isolates isolated from one patient had the same serotype.

Analysis of *pbp1a*, *pbp2b* and *pbp2x* genes by PCR and sequencing

Bacterial genomic DNA was extracted using the TIANamp Bacteria DNA Kit (TIANGEN Biotech, Beijing, China). The extracted DNA was stored at -20 °C until use. To amplify *pbp1a* (1299 bp), *pbp2b* (1504 bp) and *pbp2x* (1148 bp) genes by polymerase chain reaction (PCR), we used three sets of primers, as described previously [15–17]. PCR was run in a 50- μ l reaction mixture containing ~ 40 ng DNA template, 0.2 μ M each primer, 2.5 mM each dNTP, 1.5 U Taq DNA polymerase and 1 \times PCR buffer (2.0 mM Mg^{2+} plus). An initial denaturation step of 94 °C for 5 min was followed by 35 amplification cycles: 94 °C for 40 s, 60 °C for 40 s and 72 °C for 50 s. A final extension was performed at 72 °C for 7 min. The purified PCR products were sequenced from both directions using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled by DNA Baser software, then the nucleotide and amino acid sequences were compared with those of the reference strain (GenBank accession no. X90527 for *pbp1a*, X16022 for *pbp2b* and X16367 for *pbp2x*) by BioEdit software and the Basic Local Alignment Search Tool (BLAST) online.

Statistical analysis

The antibiotic susceptibility analyses were made using WHONET 5.6 software (WHO, Geneva, Switzerland). The number of amino acid alterations are presented as the mean \pm standard deviation (SD). Statistical analyses were performed with SPSS version 16.0. The resistance rates were compared

using Fisher's exact test or Pearson's χ^2 test. One-way analysis of variance (ANOVA) was used to analyse the differences in the mean number of mutations in isolates with different degrees of sensitivity to PEN. A p -value < 0.05 was considered statistically significant.

Results

Serotype distribution and vaccine coverage

Among the total 256 IPD isolates, serotypes 19A (92, 35.9 %), 14 (44, 17.2 %), 19F (41, 16.0 %), 6B (23, 9.0 %) and 23F (21, 8.2 %) were the most common, which accounted for 86.3 % of all the isolates. The other 13 serotypes only accounted for 13.7 % (35/256) of the isolates. The most frequent serotypes were 14 (20 %), 19F (18.2 %), 19A (16.4 %), 6B (14.8 %) and 23F (10.9 %) in strains isolated from CSF (Fig. 1). All serotypes covered by PCV7, PCV10 and PCV13 were 53.9 %, 56.3 % and 93.8 %, respectively.

Antimicrobial susceptibility testing

According to the recommendations of the CLSI in 2013, the breakpoints of parenteral PEN were an MIC ≤ 0.06 $\mu\text{g/ml}$ for susceptibility and MIC ≥ 0.12 $\mu\text{g/ml}$ for resistance for meningitis isolates [13]. For non-meningitis isolates, the breakpoints of parenteral PEN were ≤ 2 , 4 and ≥ 8 $\mu\text{g/ml}$ [13]. Of the 256 *S. pneumoniae* isolates, 55 were meningitis isolates, including penicillin-susceptible *S. pneumoniae* (PSSP) isolates (11, 20.0 %) and PRSP isolates (44, 80.0 %). More than 60 % of PRSP isolates were susceptible to AMC, while the strains susceptible to CTX, CRO and MEM were relatively few. Among 201 non-meningitis isolates, 67 (33.3 %), 69 (34.3 %) and 65 (32.3 %) were susceptible, intermediate susceptible and resistant to PEN, respectively. For 69 PISP isolates, only the rate of susceptibility to AMC was >50 %. The rate of susceptibility to CTX was <10 %, and that to AMC,

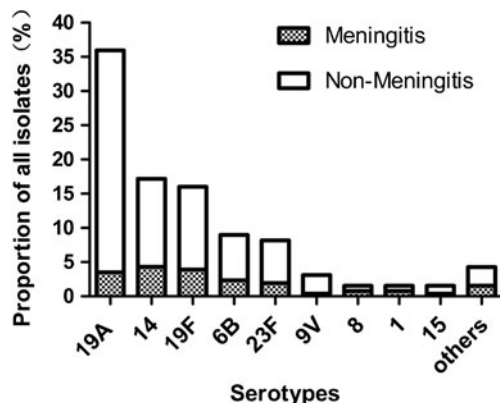


Fig. 1 Serotype distributions among children with invasive pneumococcal diseases (IPDs)

CRO and MEM was <5 % for PRSP isolates. PNSP isolates accounted for 69.5 % (178/256) of all the *S. pneumoniae* isolates, which comprised 44 isolates from CSF and 134 from other samples. All 78 PSSP isolates (including meningitis and non-meningitis isolates) were susceptible to AMC, CTX, CRO and MEM (Table 1).

Association between serotypes and susceptibility to PEN

Among the 256 isolates, the five common serotypes exhibited a significantly higher level of resistance to penicillin than did other serotypes ($p < 0.05$), especially serotypes 19A, 19F and 14 (Table 2). Serotype 19A had the most PNSP (91.3 %) overall, followed by 19F (85.4 %) and 14 (76.2 %). PNSP of serotype 14 from non-meningitis isolates were all PISP without PRSP. Serotypes 19A (84, 47.2 %), 19F (35, 19.7 %), 14 (32, 17.9 %), 23F (14, 7.9 %) and 6B (8, 4.5 %) were the most common serotypes recovered among 178 PNSP isolates. These five serotypes accounted for 97.2 % (173/178) of all 178 PNSP isolates. The other five isolates contained two of serotype 15 and one each of serotypes 6A, 9V and 20. Among these PNSP isolates, the proportion of serotypes covered by PCV7, PCV10 and PCV13 was 50.6 %, 50.6 % and 98.3 %, respectively. 19A (51, 70.8 %), 19F (19, 26.7 %) and 23F (2, 2.8 %) were the common serotypes among 72 PRSPs, in which the serotype coverage for PCV7, PCV10 and PCV13 was 29.2 %, 29.2 % and 100 %, respectively. Although 19A was the dominant serotype accounting for 51.3 % (39/76) of IPD isolates during 2000–2008, the proportion of serotype 19A isolates decreased over the following years.

Variations in or close to three conserved motifs (SXXX, SXN and KT/SG) in PBP2b, PBP2x and PBP1a

Three conserved amino acid motifs in PBPs were 385SVVK, 442SSN and 614KTG in PBP2b; 337STMK, 395SSN and 547KSG in PBP2x; and 370STMK, 428SRN and 557KTG in PBP1a. No amino acid substitutions were found in these conserved motifs from 45 isolates with MICs to PEN (0.016–0.25 mg/L). Fewer amino acid alterations (<10/gene) were found in these isolates. The number of amino acid substitutions and variation of conserved motifs forming or surrounding the active PBP binding sites in PBP2b, PBP2x and PBP1a in the other 211 *S. pneumoniae* isolates are shown in Table 3.

Variations of the *pbp 2b* gene

Compared with PSSP isolate R6 (GenBank accession no. X16022), 42 isolates with MICs to PEN (0.016–0.25 mg/L) had highly similar sequence to that of the R6 isolate. In these isolates, no variations were found in the three conserved motifs (385SVVK, 442SSN and 614KTG), and only two amino acid substitutions were identified in the non-conserved domains.

Table 1 Antimicrobial susceptibility to β -lactam antibiotics (BLAs) of 256 *Streptococcus pneumoniae* isolates

Antibiotics	Meningitis (55)				Non-meningitis (201)						
	PSSP (11)		PRSP (44)		PSSP (67)	PISP (69)			PRSP (65)		
	S (%)	S (%)	I (%)	R (%)		S (%)	S (%)	I (%)	R (%)	S (%)	I (%)
AMC	100.00	63.64	6.82	29.54	100.00	53.62	8.7	37.68	1.54	18.46	80.00
CTX	100.00	18.18	22.73	59.09	100.00	26.09	71.01	2.90	7.69	44.62	47.69
CRO	100.00	18.18	22.73	59.09	100.00	34.78	43.48	21.74	3.08	29.23	67.69
MEM	100.00	25.00	52.27	22.73	100.00	2.9	72.46	24.64	3.08	30.77	66.15

PSSP penicillin-susceptible *S. pneumoniae*; PISP penicillin-intermediate *S. pneumoniae*; PRSP penicillin-resistant *S. pneumoniae*; S susceptible; I intermediate; R resistant; AMC amoxicillin; CTX cefotaxime; CRO ceftriaxone; MEM meropenem

The variations were mainly concentrated in 454 amino acids between codons 224 and 678 in the other 214 isolates, and 14.31 % (65/454) of amino acid substitutions were identified in PBP2b. Compared with *pbp2b* sequences of the control R6 isolate, an average of 8.22 ± 7.73 mutations in PSSP, 30.88 ± 14.37 in PISP and 42.94 ± 4.11 in PRSP isolates were found. The number of variations in PRSP and PISP isolates was higher than in PSSP isolates ($p < 0.01$). Although there were no mutations in the three conserved motifs, an Ala618 \rightarrow Gly substitution and a high frequency of substitutions containing 25 amino acids between positions 560 and 675 were identified in 115 PNSP isolates. In addition, the Thr445 \rightarrow Ala next to SSN (442–444) motif and Glu475 \rightarrow Gly mutation were the most common mutations found in 214 isolates.

A total of 214 isolates were grouped into eight different variants according to Baek et al. [16] and Granger et al. [18] based on the amino acid sequences of the transpeptidase binding domain in PBP2b (Table 4). Groups I (58 isolates), II (115 isolates) and III (23 isolates) were the main groups in all the isolates. Eighteen isolates (11 PSSP and 7 PISP) were classified in Baek et al.'s group I, which showed the same 15 amino acid substitutions. Additional amino acid changes of F381L and S411P were found in our group I isolates besides 13

amino acid substitutions described by Granger et al. [18]. Another 40 isolates (14 PSSP and 26 PISP) showed additional amino acid alterations Lys365 \rightarrow Asn, but without Ile360 \rightarrow Leu mutation; they were named as subgroup Ia. Isolates (115) belonging to group II were all PNSP isolates, which contained 97.2 % (70/72) of PRSP (MICs to PEN ≥ 8 mg/L). These isolates showed that not only did the mutations belong to group I, but there were also another 25 amino acid substitutions between positions 560 and 675. Among these isolates, 87 isolates containing the short mosaic sequence VDT between residues 248 and 250 were assembled in subgroup IIa. Their serotypes were all 19A, except for two which were 14. The other 28 isolates were named as subgroup IIb, which did not have the short mosaic sequence VDT but showed the amino acid alteration Val224 \rightarrow Ile and Glu368 \rightarrow Asp. Twenty-three isolates containing the group I mutation block (except Ile360 \rightarrow Leu), Ala515 \rightarrow Ser and Ala314 \rightarrow Ser, belonged to group III, which included 12 PSSP and 11 PISP isolates. Four isolates shared 20 amino acid alterations containing another distinct block (314–365) in addition to the mutation block (421–488) were assembled into group IV. The isolates belonging to groups V, VII and VIII were all PSSP isolates. No isolate with a similar sequence to Baek et al.'s group VI was found in our study.

Table 2 Association between serotypes and antimicrobial susceptibility to penicillin in 256 *S. pneumoniae* isolates

Serotypes	No. of isolates	Meningitis		Non-meningitis			Total PNSP
		S	R	S	I	R	
19A	92	0	9 (100.0 %)**	8 (9.6 %)	27 (32.5 %)	48 (57.9 %)**	84 (91.3 %)**
19F	41	1 (10 %)	9 (90.0 %)*	5 (16.1 %)	10 (32.3 %)	16 (51.6 %)**	35 (85.4 %)**
14	44	0	11 (100.0 %)**	12 (36.7 %)	21 (63.6 %)	0	32 (76.2 %)**
23F	21	1 (16.7 %)	5 (83.3 %)	7 (46.7 %)	6 (40.0 %)	2 (13.3 %)	11 (52.4 %)*
6B	23	2 (25.0 %)	6 (75.0 %)	11 (73.3 %)	4 (26.7 %)	0	10 (43.5 %)*
Others	35	7 (63.6 %)	4 (36.4 %)	22 (91.7 %)	2 (8.3 %)	0	6 (17.2 %)

S susceptible; I intermediate; R resistant; PNSP penicillin-non-susceptible *S. pneumoniae*

* $p < 0.05$, ** $p < 0.01$, the data of this group compared with the data of the 'others' group

Table 3 Association of serotypes, antimicrobial susceptibility, number of altered amino acids and variations of conserved motifs forming or surrounding active penicillin-binding protein (PBP) binding sites in PBP2b, PBP2x and PBP1a in 211 *S. pneumoniae* isolates

Serotypes	n	Penicillin MIC, µg/ml	No. (%) of altered amino acids			Deduced amino acid substitution ^a						
			PBP2b	PBP2x	PBP1a	PBP2b		PBP2x			PBP1a	
			224–678	254–715	316–695	Thr445	Ala618	Thr338	Met339	Leu546	Thr371	Pro432
14	1	0.5	15 (3.30)	16 (3.47)	22 (5.79)	-	-	Ala	-	-	Ser	-
6A	2	0.5	11 (2.42)	2 (0.43)	2 (0.53)	Ala	-	-	-	-	-	-
6B	1	0.5	11 (2.42)	26 (5.59)	17 (4.49)	Ala	-	Ala	-	-	-	-
6B	2	0.5	15 (3.30)	41 (8.89)	53 (13.98)	Ala	-	Ala	-	Val	Ser	Thr
19F	3	1	17 (3.74)	63 (13.67)	43 (11.35)	Ala	-	Ala	-	Val	Ser	Thr
23F	2	1	15 (3.30)	65 (14.07)	44 (11.49)	Ala	-	Ala	-	Val	Ser	Thr
6B	3	1	11 (2.42)	41 (8.89)	53 (13.98)	Ala	-	Ala	-	Val	Ser	Thr
19A	1	1	17 (3.74)	62 (13.44)	43 (11.35)	Ala	-	Ala	-	Val	Ser	Thr
14	1	1	20 (4.41)	40 (8.68)	44 (11.49)	Ala	-	Ala	-	Val	Ser	Thr
14	13	2	15 (3.30)	64 (13.88)	54 (14.25)	Ala	-	Ala	-	Val	Ser	Thr
14	1	2	25 (5.51)	64 (13.88)	54 (14.25)	Ala	-	Ala	-	Val	Ser	Thr
9V	2	2	15 (3.30)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
19F	2	2	16 (3.52)	63 (13.67)	43 (11.35)	Ala	-	Ala	-	Val	Ser	Thr
19A	3	2	17 (3.74)	62 (13.44)	43 (11.35)	Ala	-	Ala	-	Val	Ser	Thr
23F	2	2	15 (3.30)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
23F	1	2	20 (4.41)	40 (8.68)	44 (11.49)	Ala	-	Ala	-	Val	Ser	Thr
23F	2	2	14 (3.08)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
23F	1	2	14 (3.08)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
6B	4	2	15 (3.30)	65 (14.07)	53 (13.98)	Ala	-	Ala	-	Val	Ala	Thr
6B	2	2	18 (3.96)	65 (14.07)	53 (13.98)	Ala	-	Ala	-	Val	Ala	Thr
11	1	2	15 (3.30)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
19A	34	4	44 (9.69)	62 (13.44)	43 (11.35)	Ala	Gly	Ala	-	Val	Ser	Thr
19F	9	4	42 (9.25)	68 (14.75)	43 (11.35)	Ala	Gly	Ala	Phe	Val	Ser	Thr
19F	6	4	16 (3.52)	65 (14.07)	43 (11.35)	Ala	-	Ala	-	Val	Ser	Thr
14	2	4	44 (9.69)	64 (13.88)	54 (14.25)	Ala	Gly	Ala	-	Val	Ser	Thr
14	26	4	15 (3.30)	64 (13.88)	54 (14.25)	Ala	-	Ala	-	Val	Ser	Thr
23F	4	4	15 (3.30)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
23F	2	4	15 (3.30)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
23F	1	4	15 (3.30)	47 (10.17)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
15	1	4	15 (3.30)	65 (14.07)	54 (14.25)	Ala	-	Ala	-	Val	Ala	Thr
6A	1	4	13 (2.86)	65 (14.07)	43 (11.35)	Ala	-	Ala	-	Val	Ser	Thr
6B	2	4	16 (3.52)	65 (14.07)	53 (13.98)	Ala	-	Ala	-	Val	Ala	Thr
6B	1	4	16 (3.52)	65 (14.07)	53 (13.98)	Ala	-	Ala	-	Val	Ala	Thr
23F	2	8	21 (4.63)	68 (14.75)	43 (11.35)	Ala	-	Ala	Phe	Val	Ala	Thr
19A	12	8	44 (9.69)	68 (14.75)	43 (11.35)	Ala	Gly	Ala	Phe	Val	Ser	Thr
19A	24	8	44 (9.69)	62 (13.44)	43 (11.35)	Ala	Gly	Ala	-	Val	Ser	Thr
19F	16	8	42 (9.25)	68 (14.75)	43 (11.35)	Ala	Gly	Ala	Phe	Val	Ser	Thr
19A	14	16	44 (9.69)	68 (14.75)	43 (11.35)	Ala	Gly	Ala	Phe	Val	Ser	Thr
19F	3	16	42 (9.25)	68 (14.75)	43 (11.35)	Ala	Gly	Ala	Phe	Val	Ser	Thr
19A	1	32	44 (9.69)	68 (14.75)	43 (11.35)	Ala	Gly	Ala	Phe	Val	Ser	Thr

^a Deduced amino acid substitutions at positions in or close to each of the three conserved motifs (SXXK, SXN and KT/SG) in PBPs 2b, 2x and 1a (385SVVK, 442SSN and 614KTG for PBP2b; 337STMK, 395SSN and 547KSG for PBP2x; 370STMK, 428SRN and 557KTG for PBP1a). The dashes indicate positions at which no variation was found

Table 4 Deduced amino acid sequences of PBP2b in 214 *S. pneumoniae* isolates

Penicillin MIC, µg/ml	Serotype	n	R6	333344444	444444444	444455555	555555555	666666666	666666666	66666	Group
0.5	14	1	222233333	333344444	444444444	444455555	555555555	666666666	666666666	66666	I
1	19F	3	2445123566	6678122222	2333456677	7888001366	6666778899	0012223455	0012223455	67777	I
1	19A	1	4890412603	5841115678	9017546825	9289275704	5678071216	5884799089	5884799089	33458	I
2	6B	1	VAEAAAESID	KELFSNTQAY	GSFQLGSSE	SGTAVSANDQ	LQPTMDSIAG	NLADQTSNDG	NLADQTSNDG	SQKYM	I
2	19A	3	- - - - - G-L	- - - LPYKL-	- - - EAI -- TG	A - A - - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	I
2	19F	2	- - - - - G-L	- - - LPYKL-	- - - EAI -- TG	A - A - - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	I
4	6A	1	- - - - - G-L	- - - LPYKL-	- - - EAI -- TG	A - A - - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	I
4	19F	6	- - - - - G-L	- - - LPYKL-	- - - EAI -- TG	A - A - - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	I
2	14	14	- - - - - G	N - - LPYKL-	- - - EAI -- TG	A - A - - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	Ia
4	14	26	- - - - - G	N - - LPYKL-	- - - EAI -- TG	A - A - - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	Ia
4	19A	34	- VDT - - G-L	N - - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ila
4	14	2	- VDT - - G-L	N - - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ila
8	19A	36	- VDT - - G-L	N - - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ila
16	19A	14	- VDT - - G-L	N - - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ila
32	19A	1	- VDT - - G-L	N - - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ila
4	19F	9	I - - - - - G-L	- D - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ilb
8	19F	16	I - - - - - G-L	- D - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ilb
16	19F	3	I - - - - - G-L	- D - LPYKL-	- - - EAI -- TG	A - A - - - DEA	IDTKIEA- SP	DTGGENTEKN	DTGGENTEKN	ANQH-	Ilb
2	23F	4	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
2	9V	2	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
2	11	1	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
2	6B	5	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
4	6B	3	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
4	23F	7	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
4	15	1	- - - S - - G - -	- - - LPYKL-	- - - EAI -- TG	A - A - - S - - -	- - - - -	- - - - -	- - - - -	- - - - -	III
1	14	1	I - - - SSTA-N	EDF- PYKL-	- - - EAI -- TG	A - A - - - - - -	- - - - -	- - - - -	- - - - -	- - - - -	IV
2	23F	1	I - - - SSTA-N	EDF- PYKL-	- - - EAI -- TG	A - A - - - - - -	- - - - -	- - - - -	- - - - -	- - - - -	IV
8	23F	2	I - - - SSTA-N	EDF- PYKL-	- - - EAI -- TG	A - A - - - - - -	- - - - -	- - - - -	- - - - -	- - - - -	IV
0.5 ~ 1	6B	5	- - - S - - - - -	- - - - - Y - - -	- - - EA - - - G	- - - S - - - D - -	- - - - -	- A- GEN - - -	- A- GEN - - -	- - - - -	V
0.5	6A	2	- - - S - - - - -	- - - - - Y - - -	- - - EA - - - G	- - - S - - - D - -	- - - - -	- A- GEN - - -	- A- GEN - - -	- - - - -	V
0.125*	23F	2	- - - - - - - - -	- - - LPYKL-	- - - EAI LN - G	- ASSIN- D - -	- - - - - V - -	- - - - -	- - - - -	- NQHI	VII
0.125 ~ 0.53*	6B	2	- - - - - - - - -	- - - - - AFS	VPM- A - - - G	- - - S - I - - D - -	- - - - -	- - - - -	- - - - -	- - - - -	VIII
1 ~ 2	23F	3	- - - - - G - - -	- - - - - AFS	RPM- A - - - TG	- - - S - I - - D - -	- - - - - V - -	- - - - -	- - - - -	- - - - -	VIII

R6 strain (GenBank accession no. X16022) was used as the reference. The dashes indicate positions at which no variation was found

*Strains isolated from CSF

Variations of the *pbp2x* gene

Compared with the *pbp2x* gene sequence of the R6 strain, 45 isolates (MIC to PEN ≤ 0.25 mg/L) were highly prototype-like, and the remaining 211 isolates (MIC to PEN ≥ 0.5 mg/L) had 79 (17.14 %) amino acid mutations concentrated within 461 amino acids between codons 254 and 715. We found an average of 29.26 ± 27.88 mutations in PSSP, 63.37 ± 2.51 in PISP and 65.55 ± 2.93 in PRSP isolates. The numbers of mutations in PISP and PRSP were significantly increased compared with those of PSSP ($p < 0.01$). A Thr338 \rightarrow Ala mutation in the STMK motif (337–340) and Leu546 \rightarrow Val mutation just before the KSG motif (547–549) were found in 208 isolates (MIC to PEN ≥ 0.5 mg/L). Only one isolate of serotype 14 from CSF showed a His394 \rightarrow Leu alteration next to the SSN motif (395–397), without the two mutations mentioned above.

Based on deduced PBP2x amino acid sequences, 211 isolates were grouped referring to the method of Granger et al. [18] (Table 5). Thirty-nine isolates belonged to Granger et al.'s group V, which were all serotype 14 isolates. Another 169 isolates containing one unique amino acid mutation of Asn417 \rightarrow Lys could be classified in Granger et al.'s group VI, which contained 36 PSSP isolates and the majority (133/178, 74.72 %) of PNSP isolates.

To define further the character of amino acid mutations, we classified Granger et al.'s group VI isolates into four subgroups: VIa, VIb, VIc and VI d (Table 5). We named six serotype 6B isolates (MIC to PEN 0.5–1 mg/L) that shared the same 42 amino acid mutations as subgroup VIa. An additional substitution block that contained 20 amino acid alterations was found in 45 isolates (MIC to PEN 1–4 mg/L), which were named as subgroup VIb. Besides these 65 mutations (except M343T), Met339 \rightarrow Phe, Met400 \rightarrow Thr, Tyr595 \rightarrow Phe and Gln629 \rightarrow Lys were also found in 54 PNSP isolates, which were named as subgroup VIc. Among these isolates, 46 were PRSP strains, which accounted for 63.89 % of PRSP isolates. Sixty-four isolates showing an amino acid mutation ratio of 13.45 % (62/461) were named as subgroup VI d, which were all serotype 19A isolates. Compared with other group VI isolates, they had Asn501 \rightarrow Lys, Lys505 \rightarrow Glu, Ala507 \rightarrow Thr, Glu651 \rightarrow Lys, Glu706 \rightarrow Ala, Thr709 \rightarrow Ala and Ala711 \rightarrow Ser mutations, but without Asp506 \rightarrow Glu, Asp648 \rightarrow Glu and Thr678 \rightarrow Lys mutations. Except for four PSSP isolates, most of these isolates were PNSP isolates that contained 36.11 % (26/72) of PRSP. The remaining three isolates (MIC to PEN 0.5 mg/L) could not be classified into any group.

Variations of the *pbp1a* gene

Compared with reference PBP1a, a total of 75 amino acid mutations were found in 256 isolates, which were mainly

concentrated between codons 316 and 695. Forty-seven (58 %) PSSP (MIC to PEN ≤ 0.5 mg/L) isolates almost had no amino acid mutations (< 3 /isolate) compared with the PBP1a of the R6 strain. We found an average of 29.26 ± 27.88 , 63.37 ± 2.51 and 65.55 ± 2.93 amino acid alterations in PBP1a from PSSP, PISP and PRSP isolates, respectively. The numbers of mutations in PISP and PRSP were significantly increased compared with those of PSSP ($p < 0.01$). All of the PNSP isolates had a Thr371 \rightarrow Ala/Ser mutation in the STMK motif (370–373) and a Pro432 \rightarrow Thr mutation next to the SRN motif (428–430). A short mosaic sequence NTGY instead of TSQF between positions 574 and 577 was found in all the PNSP isolates. However, these mutations were also observed in some PSSP isolates. No mutations were found within or close to the KTG motif (557–559).

According to PBP1a, 48 (60.1 %) PSSP isolates characterised by 1–3 amino acid alterations were assembled in Granger et al.'s group I. Six amino acid substitutions of Thr371 \rightarrow Ala, Ile393 \rightarrow Met, His395 \rightarrow Asn, Glu397 \rightarrow Ile, Asn405 \rightarrow Ser and Pro432 \rightarrow Thr were shared by 27 isolates, which were classified within group II (16 PSSP and 11 PISP isolates). Thirteen isolates of serotype 6B showed the same 53 amino acid substitutions as group IIb, and the other isolates were named as group IIa. Most isolates (179), including 29 PSSP and 150 PNSP isolates, had the characteristic mutations of Thr371 \rightarrow Ser, Thr392 \rightarrow Ala, Glu397 \rightarrow Val, Asn405 \rightarrow Asp and Val408 \rightarrow Leu, which belonged to Granger et al.'s group V. According to the characteristic amino acid mutations in our study, we further classified the isolates into three subgroups, Va, Vb and Vc (Table 6). It is worth noting that all the PRSP isolates belonged to subgroup Vc. Glu388 \rightarrow Asp was shown in all the isolates in this study.

Discussion

The morbidity of invasive disease induced by *S. pneumoniae* has shown an increasing trend in the past 15 years, especially for children aged < 5 years, who comprised 84.8 % (217/256) of the patients with IPD in our region. The increasing morbidity of IPD in children may be partly because of their immature organs and immune system, or due to the variation in virulence of *S. pneumoniae* isolates caused by misuse of antibiotics. Vaccination is the most effective and economic way to prevent IPD and reduce its incidence and mortality in children, as well as being one of the important ways to prevent the spread of antibiotic resistance [19].

The serotypes associated with IPD vary with time, geographic regions, race, age and vaccination. In the present study, the most prevalent serotypes were 19A, 19F, 14, 23F and 6B. The results were similar to a multicentre surveillance study from China. That study showed that 19A (22.1 %), 19F (21.7 %), 14 (7.5 %), 3 (7.1 %) and 23F (5.4 %) were the most

Table 5 Deduced amino acid sequences of PBP2x in 211 *S. pneumoniae* isolates

Penicillin MIC, µg/ml	Serotype	n	R6	333333333333	4444444444	5555555555	5555555556	6666666666	6666666666	6666666666	6667777777	Group
0.5	6A	2		-----G-	-----	-----	-----N	-----	-----	-----	-----	
0.5	14	1		SY-V-DT-L	ST-----	-----I--	-L-----N-	-----	-----	-----	-----	
1~2	14	11		SYF-TDTGL-	ST-SLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	V	
4	14	28		SYF-TDTGL-	ST-SLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	V	
0.5~1	6B	6		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	-----	-----	-V-----	Vla	
1~2	23F	8		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
1~2	19F	5		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
2	6B	6		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
2	14	4		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
2	11	1		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
2	9V	2		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
4	15	1		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
4	6A	1		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
4	6B	3		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
4	19F	7		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
4	23F	7		SYF-TDTGL-	S-KSLTNSV	--E-TNHILY	IIVSNVTN-T	ESPND-TTES	AI--AIETVT	LVIK--F-D	Vlb	
4	19F	8		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
4	19F	16		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
8	19A	12		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
8	23F	2		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
16	19F	3		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
16	19A	12		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
32	19A	1		SYF-TDTGL-	TS-KSLTNSV	--E-TNHILY	IIVSNVTNFT	ESPNDKTTES	AI--AIETVT	LVIK--F-D	Vlc	
1~2	19A	4		SYF-TDTGL-	S-KSLTNSV	KE-T----ILY	IIVSNVTN-T	ESPND-TTES	AI-KAIETV-	LVI-AAFS-	Vld	
4	19A	34		SYF-TDTGL-	S-KSLTNSV	KE-T----ILY	IIVSNVTN-T	ESPND-TTES	AI-KAIETV-	LVI-AAFS-	Vld	
8	19A	24		SYF-TDTGL-	S-KSLTNSV	KE-T----ILY	IIVSNVTN-T	ESPND-TTES	AI-KAIETV-	LVI-AAFS-	Vld	
16	19A	2		SYF-TDTGL-	S-KSLTNSV	KE-T----ILY	IIVSNVTN-T	ESPND-TTES	AI-KAIETV-	LVI-AAFS-	Vld	

R6 strain (GenBank accession no. X16022) was used as the reference. Only isolates whose amino acids were different from the reference sequences of the R6 strain are shown. The dashes indicate positions at which no variation was found

Table 6 Deduced amino acid sequences of PPB1a in 211 *S. pneumoniae* isolates

Penicillin MIC, µg/ml	Serotype	n	R6	33333333333	4444444444	4444444555	5555555555	5555555666	6666666666	6666666666	6666666666	66666	Group
0.5	6A	2	—	—	—	—	—	—	—	—	—	—	I
0.5	14	1	—	S-D	—	L	—	YNTGYMVIID	FL	—	MDI	—	—
0.5	6B	2	—	—	D	L	—	YNTGYMV-D	—	—	N	—	D
2	9V	2	SQSDVATAID	-MNIS-A-T	—	DMANQ—K	—	-NTGYMVI-D	FL-IDTH-T	—	MDF-PITEST	—	SSTA- IIa
2	11	1	SQSDVATAID	-MNIS-A-T	—	DMANQ—K	—	-NTGYMVI-D	FL-IDTH-T	—	MDF-PITEST	—	SSTA- IIa
2	23F	3	SQSDVATAID	-MNIS-A-T	—	DMANQ—K	—	-NTGYMVI-D	FL-IDTH-T	—	MDF-PITEST	—	SSTA- IIa
4	15	1	SQSDVATAID	-MNIS-A-T	—	DMANQ—K	—	-NTGYMVI-D	FL-IDTH-T	—	MDF-PITEST	—	SSTA- IIa
4	23F	7	SQSDVATAID	-MNIS-A-T	—	DMANQ—K	—	-NTGYMVI-D	FL-IDTH-T	—	MDF-PITEST	—	SSTA- IIa
0.5~2	6B	10	SQSDVATAID	-MNIS-A-T	—	DMANQIHN-K	D-EAGPAD-K	-NTGYMVI-D	FL-IDTH-T	—	MDF-N	—	IIb
4	6B	3	SQSDVATAID	-MNIS-A-T	—	DMANQIHN-K	D-EAGPAD-K	-NTGYMVI-D	FL-IDTH-T	—	MDF-N	—	IIb
1~2	14	15	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Va
1~2	23F	5	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Va
4	14	28	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVIID	FLGR-DNHGT	—	M-S	—	Vb
1~2	19F	5	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
1~2	19A	4	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
4	6A	1	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
4	19F	15	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
4	19A	34	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
8	23F	2	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
8	19F	16	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
8	19A	36	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
16	19A	14	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
16	19F	3	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc
32	19A	1	SQSDVATS-D	S-VDLHVIT	—	DMAN-IHNI-	DAETGP—K	YNTGYMVI-D	FL	—	—	—	Vc

R6 strain (GenBank accession no. X16022) was used as the reference. Only isolates whose amino acids were different from the reference sequences of the R6 strain are shown. The dashes indicate positions at which no variation was found

common pneumococcal serotypes among 218 IPD strains collected from 2005 to 2011 [20]. Nevertheless, the serotype distribution was completely different from that of the USA, in which 33F, 22F, 15B/15C and 35B instead of 19A and 7F became the most common serotypes after PCV13 implementation during 2011–2013 [21]. The spread of serotype 19A *S. pneumoniae* in the USA was after the large-scale use of PCV7 (including pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F). During 1998–1999, the serotype 19A isolates only accounted for 2.5 % of all isolates causing IPD, but this ratio increased to 36 % in 2005 [22]. Now, serotype 19A has become common in isolates causing IPD and is associated with multidrug resistance [23]. However, some researchers have suggested that the prevalence of serotype 19A was not the result of the introduction of PCV7, but associated with antibiotic selection pressure [24]. A multicentre study [25] showed that, before PCV7 was introduced into China, serotype 19A was the most common serotype in paediatric patients in the cities in Northern China, while it rarely appeared in Southern and Eastern China. Although PCV7 was introduced to China in 2008, it was still a for-fee vaccine. Because of the relatively high price, the coverage of PCV7 remained low [25, 26]. Thus, the potential cause of prevalence of serotype 19A in our region was that 19A, as the multidrug-resistant serotype, was easier to spread under the pressure of antibiotics. Among the 72 PRSP isolates, the serotype coverage rates of PCV7, PCV10 and PCV13 were 29.2 %, 29.2 % and 100 %, respectively. It was obvious that the protection of PCV7 and PCV10 against *S. pneumoniae* IPD was limited. Therefore, PCV13 was urgently needed in our region for protecting more children from *S. pneumoniae* IPD in North-east China.

Correctly identifying pathogens and effective antibiotic treatment at an early stage, which can reduce the hospital stay and mortality of IPD, are important in the prognosis of IPD. All the PRSP isolates belonged to serotypes 19A, 19F and 23F, especially serotype 19A, which accounted for 70.8 % of PRSP isolates. In addition, for all the PRSP from non-meningitis isolates, the susceptibility rates to AMC, CTX, CRO and MEM were all low, which was possibly related to the high frequency of application of these BLAs in this area. It is suggested that CTX, CRO and MEM should be chosen carefully according to the results of susceptibility testing when treating IPD. Our data showed that antibiotic resistance had been a serious problem in Northeast China, but, fortunately, overall, the serotypes of PRSP strains in this study could be covered by PCV13. As confirmed in other countries, PCV13 can prevent IPD in children and control the spread of antibiotic-resistant strains [27–29].

In the present study, the genetic diversity of *pbp2b*, *pbp2x* and *pbp1a* genes were analysed in 256 IPD strains by comparing with the reference sequence of the R6 strain. The mean numbers of amino acid substitutions in PNSP isolates were

significantly higher than those of PSSP isolates in all the PBP genes ($p < 0.01$). This was similar to the results reported previously [10, 16, 18] and confirmed that the increase in resistance to BLAs was associated with the increase in amino acid alterations of PBP2b, PBP2x and PBP1a.

The amino acid mutations of PBP2b were classified into eight subgroups. Some subgroups were associated with a specific serotype, such as subgroup Ia and serotype 14, and subgroup IIb and serotype 19F. In this study, the Thr445 → Ala alteration adjacent to the SSV motif and Glu475 → Gly were the most common mutations found in 214 isolates, including some PSSP isolates. An in vitro study showed that the Thr445 → Ala mutation displayed a 60 % reduction affinity to penicillin G compared with wild-type protein [30], and many researchers have found this mutation in PNSP isolates [31–33]. In contrast, other researchers have also found this mutation in PSSP isolates, as in our study [10, 18]. Whether this mutation plays a key role in PEN resistance is worthy of exploration.

Group II was the most prevalent mutation type in our study, which carried the largest number of amino acid mutations. All the isolates (115) belonging to this group were PNSP isolates. The Ala618 → Gly mutation next to the KTG (614–616) motif was found in these isolates. This mutation has also been reported in PRSP strains previously [16, 18, 30]. This suggests that the mutation is closely related to the mechanism of resistance of *S. pneumoniae* to BLAs. All the subgroup IIa isolates were serotype 19A, except for two that were serotype 14, and all the subgroup IIb isolates were serotype 19F. The amino acid sequence of these isolates was the same as Korean J77, Korean J83 or Canadian 14907 [16, 18]. The spread of these high-level-resistant strains may be due to the limited spread of serotypes 19A and 19F clones. Some antibiotic-resistant isolates could be imported to China with the increasingly frequent international exchanges, which could be one of the major causes of increasing high-level-resistant strains. To the best of our knowledge, Phe 381 → Leu was a novel mutation that was identified in 198 isolates. There are no data demonstrating that the mutation was directly related to PEN resistance, and the biochemical function of this mutation needs further research.

Variation of *pbp2x* is also one of the important causes of BLA resistance in *S. pneumoniae*. Amino acid substitutions of Thr338 → Ala and Met339 → Phe in the STMK motif simultaneously occurred in subgroup VIc isolates, which were not only resistant to PEN but also to AMC, CTX, CRO and MEM. The Thr338 → Ala mutation drastically reduced acylation efficiency for BLAs due to the absence of a hydroxyl group at this position [34]. The T338A variation was frequently observed in clinical BLA-resistant isolates (208) in our study. The crystal structure of the double mutant T338A/M339F showed a distortion of the active site and an alternative conformation of the active site Ser337, which reduced 20-

fold the efficiency of acylation of PEN and CTX [35, 36]. These two mutations were also found in BLA-resistant strains in other countries [37–39]. These isolates also carried amino acid substitutions of Met400 → Thr, Tyr595 → Phe and Gln629 → Lys compared with other isolates, suggesting that these mutations are responsible for the high level of resistance. Met339 → Phe, Met400 → Thr and Tyr595 → Phe substitutions have not been reported in previous studies from our region [33].

The His394 → Leu mutation just before the conserved SSN motif was found in one serotype 14 PRSP isolate from CSF (MIC to PEN 0.5 mg/L), which could not be classified in any group. This isolate did not carry Thr338 → Ala and Leu546 → Val mutations, which is consistent with previous studies [25, 35, 36]. The Leu546 → Val mutation adjacent to the KSG motif was found in 208 isolates, which contained 168 isolates with MICs to CTX and CRO \geq 1 mg/L [39, 40], showing that the Leu546 → Val substitution is associated with high-level BLA resistance, especially with resistance to cephalosporins. However, it was also found in some cephalosporin-sensitive isolates, which suggests that the single mutation is not enough to cause high-level resistance. Granger et al.'s group VIId was another prevalent group with a high level of BLA resistance, which contained 60 PNSP isolates with serotype 19A. This PBP2x pattern may present as a new prognostic indicator of resistance to BLAs in serotype 19A isolates, which could be applied to clinical practice using PCR.

The variation of the PBP1a pattern of 209 isolates was classified into six groups: Granger et al.'s group I and subgroups IIa, IIb, Va, Vb and Vc. Subgroups IIb, Vb and Vc were associated with specific serotypes 6B, 14, and 19A/19F, respectively. In all PNSP isolates (MICs for PEN \geq 4 mg/L and CTX \geq 2 mg/L), a Thr371 → Ala/Ser mutation close to the STMK motif, a Pro432 → Thr substitution next to the SRN motif and TSQF(574–577)NTGY were found, which were identified with high-level resistance to PEN and cephalosporins [18, 39, 41]. A site-directed mutagenesis study demonstrated that the Thr371 → Ala mutation reduced the efficiency of acylation of R6-PBP1a by cefotaxime (2.4-fold) and penicillin G (26-fold), and the decrease of acylation efficiency induced by the TSQF(574–577)NTGY mutation was even greater [42]. An in vivo study confirmed that only the double mutant conferred increased resistance [42]. This suggested that the impact of mutation on antibiotic susceptibility might be more complicated than we expected.

Our data showed that the different serotype isolates with similar levels of BLA resistance often share the same pattern of amino acid substitution in PBP2b, PBP2x and PBP1a, such as serotypes 19A, 19F, 14, 23F and 6. Before 2008, the breakpoints of parenteral PEN for non-meningitis strains were \leq 0.125, 0.125–1 and \geq 2 mg/L for susceptible, intermediate and resistant strains, respectively [43]. In the present study,

CLSI standards (2013) were used as the criteria. Some PSSP isolates (MIC for PEN 0.5–2 mg/L) that belonged to PNSP according to old standards showed the same amino acid substitution pattern as the PNSP isolates.

We investigated variation in *pbp2b*, *pbp2x* and *pbp1a* genes and serotype distribution from IPD *S. pneumoniae* isolates with a large sample size (256) from 2000 to 2014 in Northeast China. We analysed the association among the amino acid substitutions, resistance to BLAs and serotype distribution. This was a serious problem for the widespread prevalence of BLA-resistant *S. pneumoniae* isolates in Northeast China. Inappropriate use of antibiotics may provide the necessary selective advantage for the expansion of drug-resistant serotypes. Based on our data, PCVs, especially PCV13, were necessary in our region to reduce the occurrence of IPD and prevent the spread of antibiotic-resistant strains. The limitation of this study was that we did not analyse the multilocus sequence typing of isolates because of the high costs. So, the prevalent antibiotic-resistant clones could not be definitely identified and the results could not be directly compared with the data of some international studies.

Compliance with ethical standards

Funding No funds were received for the realisation of this work.

Conflict of interest The authors declare that they have no conflicts of interest.

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