Vaccine 37 (2019) 721–731

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Molecular characterization of *Streptococcus pneumoniae* in children living in southwest China and assessment of a potential protein vaccine, rPfbA

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ARTICLE INFO

Article history:
Received 12 September 2018
Received in revised form 28 November 2018
Accepted 11 December 2018
Available online 2 January 2019

Keywords:
Streptococcus pneumoniae
MLST
Serotypes
Antibiotic resistance
Mucosal immunization
rPfbA

ABSTRACT

Background: Few children living in southwest China are vaccinated against *Streptococcus pneumoniae* (*S. pneumoniae*), which is an important pathogen in causing high morbidity and high mortality. This study aimed the molecular characterization of *S. pneumoniae* strains isolated from children and a new vaccine strategy based on a potential protein antigen.

Methods: Molecular characterizations, including serotype, virulence gene and pilus analyses, were performed using PCR. Seven housekeeping genes were sequenced to identify the sequence types (STs), and antibiotic resistance was analysed using the microdilution broth method. In addition, we evaluated the protective effects of recombinant plasmin- and fibronectin-binding protein A (rPfbA) in murine pneumococcal infection models by challenge and passive transfer analyses, and assessed cytokine changes after immunization.

Results: The prevalent serotypes were 19F (31.4%), 19A (21.6%), 6B (13.7%), 14 (11.8%) and 23F (9.8%), and the coverage rates of the 13-valent pneumococcal conjugate vaccine (PCV13) were high in 93.3% of the isolates. The predominant STs were ST271 (23.5%), ST320 (21.6%) and ST876 (11.8%). Most of the *S. pneumoniae* isolates were resistant to erythromycin (95.1%) and clindamycin (90.2%). The molecular distributions and antibiotic resistance rates of the *S. pneumoniae* isolates differed between the plateau and the basin regions. More than 93% of the *S. pneumoniae* isolates carried *ply*, *cbpA*, *phtD* and *nanA*, and over half of the isolates carried pilus-1, pilus-2 and *pfbA*. Mucosal immunization with rPfbA induced pneumococcal specific antibody responses which provided to eliminate colonization in lung and nasopharynx, and protection against pneumococcal challenge.

Conclusion: Vaccine strategies based on epidemiological surveillance can be more adaptive to specific areas, reduce costs and protect against changing antigenic sites. We advise that children currently living in southwest China be vaccinated with PCV13.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is the prominent pathogen underlying bacterial pneumonia, meningitis, and septicaemia [1] and causes high morbidity and high mortality, especially in children [2]. According to figures, nearly 500,000 children worldwide under 5 years of age are infected annually, and most infections occur in developing countries. In China, approximately 35,000 children die of *S. pneumoniae*-related pneumonia annually [3]. In addition to pneumonia, invasive pneumococcal diseases (IPDs) also have a substantial burden on public health.

The polysaccharide capsule is the major *S. pneumoniae* virulence factor and the target of current mainstream pneumococcal vaccines [4]. According to differences in the polysaccharide capsule, *S. pneumoniae* are divided into more than 90 capsule types, which

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https://doi.org/10.1016/j.vaccine.2018.12.021
0264-410X/

are also called serotypes. The distribution of serotypes varies by region and is related to the native strains and vaccination. For example, the prevailing serotypes are 19A, 7F, 3 and 1 in Europe [5] and 14, 1, 5 and 19F in India [6]. In China, the serotype distribution varies in the main cities (Beijing: 19F, 19A, 23F; Shanghai: 19F, 14, 23F; Guangzhou: 19F, 23F, 6B) [7].

In 2017, the 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in mainland China. However, few impoverished people, especially those in southwest China, can afford this vaccination. This area contains the Tibet Plateau (elevation > 3000 m) and the Sichuan Basin (elevation < 500 m) and has a population greater than 80 million people. The plateau is mainly inhabited by ethnic minorities, such as Tibetans, who live in a low economic situation. In addition to its high cost, PCV can also lead to serotype replacement and morbidity rebounds. Pneumococcal protein-based vaccines primarily include protein virulence factors and surface enzymes, which are not restricted by serotypes and mediate adhesion and invasion. Plasmin- and fibronectin-binding protein A (PfbA) contains an LPXTG anchoring motif, which is an extracellular *Streptococcus pneumoniae* cell-wall-attached surface protein that binds to fibronectin, plasmin, and plasminogen. The crystal structure of the PfbA core domain is an elongated 12-stranded parallel beta-helix fold similar to proteins with carbohydrate modifying activity [8].

In this study, we evaluated the relationships among *S. pneumoniae* sources, diseases, serotype distributions, sequence type (ST) distributions, antibiotic resistance rates and virulence gene prevalence. In addition, we assessed a new pneumococcal vaccine protein candidate, recombinant PfbA (rPfbA).

2. Materials and methods

2.1. Study area and population

The study was conducted from February 2016 to February 2018 at West China Second University Hospital, which is one of the largest specialty hospitals for children and women in China. The hospital clinical laboratory has been accredited by both the College of American Pathologists (CAP) [9] and the China National Accreditation Service for Conformity Assessment (CNAS) under the ISO15189 accreditation standard.

The targeted subjects were children from southwest China presenting with a *S. pneumoniae* infection who were admitted to our hospital. The participant eligibility criteria included the following: (1) younger than 12 years old; (2) not vaccinated against *S. pneumoniae*; (3) gave clinical specimens from which *S. pneumoniae* was isolated and positively cultured; (4) had respiratory, neural, circulatory or local infectious manifestations. In addition, we collected antibiotic susceptibility test (AST) data for IPD inpatients who met the participant eligibility criteria from January 2014 to January 2016.

2.2. Specimen collection, isolate culture and identification

Specimens were collected by specialized sample collection personnel or physicians. If *S. pneumoniae* was isolated from multiple specimens from the same patient, only the first sample (acute phase) of the normally sterile body site was included, and consecutive samples from the same source and the same patient were ignored.

Cerebral spinal fluid (CSF) and blood specimens were cultured in vials using the BD BACTEC™ FX system and then subcultured onto Columbia Agar + 5% sheep blood plates (BD Medical Technology, NJ, USA). Other specimens were cultured directly onto Columbia Agar + 5% sheep blood plates. All culture plates were incubated at 37 °C for 24–48 h in a 5% carbon dioxide environment. Bacterial isolates were identified using the Vitek MS system (BioMerieux, Rhône, France).

2.3. Molecular serotyping

Bacterial DNA was extracted using TIANamp Bacteria DNA Kits (TIANGEN, Beijing, China) according to the manufacturer's instructions. Molecular serotypes were identified by multiplex polymerase chain reactions (PCRs) according to methods described by the Center for Disease and Prevention (CDC) and previous reports [10–12]. Forty primers were grouped into nine multiplex reactions according to the serotype distribution in China, and a primer pair targeting *cpsA* was used in each reaction as the positive control. The PCR products were analysed by gel electrophoresis with the Gel Doc XR System (Bio-Rad, Hercules, USA). The sizes of the PCR products were determined using a molecular size standard (Qingke, Beijing, China).

2.4. Virulence genes and pilus

Virulence genes, including pneumolysin (*ply*), choline-binding protein A (*cbpA*), neuraminidase A (*nanA*), polyhistidine triad protein D (*phtD*), plasmin- and fibronectin-binding protein A (*pfbA*) and pilus (*rlrA* for pilus-1 and *sipA* for pilus-2), were detected using PCR assays [13](Table S1), and the PCR products were analysed by gel electrophoresis with the Gel Doc XR System (Bio-Rad, Hercules, USA). The sizes of the PCR products were determined using a molecular size standard (Qingke, Beijing, China). *pfbA* genes from all clinical isolates were sequenced using the Sanger method (ABI3730XL, Sangon Biotech, Shanghai, China).

2.5. Multilocus sequence typing

Multilocus sequence typing (MLST) was performed to determine the STs of the isolates. Seven housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, *ddl*) were amplified by PCR and sequenced by the Sanger method (ABI3730XL, Sangon Biotech, Shanghai, China) according to the described protocols [14]. Allelic and ST profiles were confirmed by querying the pneumococcal MLST database (https://pubmlst.org/spneumoniae) and grouping the STs sharing six identical alleles of seven loci into a clonal complex (CC).

2.6. Antibiotic susceptibility tests

ASTs were performed based on turbidimetry using AST cards (BioMerieux, Rhône, France). The antimicrobial agents included penicillin, amoxicillin, cefotaxime, erythromycin, quinupristin/dalfopristin (Synercid), clindamycin, tetracycline, levofloxacin, chloromycetin, vancomycin and trimethoprim-sulfamethoxazole. Quality control analysis was performed using *S. pneumoniae* ATCC49619. The operational processes and result interpretations were performed according to the manufacturer's instructions and the CLSI 2016 standard [15].

2.7. Protein cloning and purification

Truncated rPfbA (amino acids 139-560) was amplified from *S. pneumoniae* TIGR4 genomic DNA and expressed from pET-28b(+)-pfbA with N- and C-terminal 6 × His tags in *E. coli* BL21 (DE3) cells. Next, rPfbA was purified using a Ni-NTA column, cleared of endotoxins using a polymyxin B column (GenScript, New Jersey, USA) to ensure low endotoxin levels (<10 EU/mg), and identified by SDS-PAGE (Fig S1) and MALDI-TOF/TOF (ABI5800, ThermoFisher, Waltham, USA). Finally, rPfbA buffer was replaced by PBS via ultrafiltration (Millipore, Bedford, USA) and sterilized by a 0.22-μm

microfiltration membrane (Millipore, Bedford, USA) for in vivo and in vitro experiments.

2.8. Mice immunization

Female BALB/c mice (6–8 weeks at the start of the experiments, obtained from the laboratory animal centre of Sichuan University) were immunized with rPfbA by mucosal immunization. Mice were prepared for antibody titre evaluation (n = 6), colonization modelling (n = 16) and lethal challenge modelling (n = 24). In treatment groups, 15 μg of protein was mixed with 5 μg of cholera toxin (CT) adjuvant (Sigma-Aldrich, CA, USA) in sterile PBS to a total volume of 30 μl. After the mice were anaesthetized with 1.5% pentobarbital sodium intraperitoneal, rPfbA with CT adjuvant was administered into the nasal cavity. The mice were immunized four times, with a 7-day interval between each immunization. In the control groups, mice were immunized with 5 μg CT adjuvant dissolved in 30 μl sterile PBS. Caudal blood sampling of the immunized mice was used to derive serum samples. Specific IgG/IgA titres in serum and specific IgA titres in saliva were evaluated. Indirect ELISA was used to evaluate the level of specific antibody response as previously described [16]. Briefly, 96-well ELISA plates were coated with rPfbA overnight at 4 °C and blocked with 10% fetal calf serum (Sigma-Aldrich) in PBS. Samples were added and serially diluted in PBS for incubation (37 °C). After the plates were washed, titres of IgG/IgA were determined by the addition of peroxidase conjugated goat anti-mouse IgG/IgA (Sigma-Aldrich); following incubation, the colour reaction for this ELISA was developed by adding tetramethylbenzidine (Sigma). The colour reaction lasted 20 min and was stopped with H_2SO_4. Antibody titres were determined as the reciprocal of the dilution of serum yielding 50% of the maximum A405 above the background. The background for all of the ELISA results was defined as the mean absorbance value for sera obtained from the mice before immunization with the antigens.

2.9. Murine challenge study

To confirm the broadness of the immune response, we selected different serotypes for a murine challenge study. In the colonization model (n = 16), each immunized postanaesthetic mouse was administered a 6B bacterial suspension (CMCC[B]31207, 1.0×10^7 colony-forming units [CFU], resuspended in PBS) via their nasal cavity. The challenged mice were carefully observed until their righting reflexes were regained. The mice were sacrificed on the third day after challenge, and nasal wash and lung tissue homogenization samples were collected and plated onto blood agar to determine the colony counts. In the lethal challenge model (n = 24), we challenged immunized mice with a serotype 3 bacterial suspension (CMCC[B]31436, 5.0×10^7 CFU, resuspended in PBS) as described above. The challenged mice were continuously monitored once a day by an expert person, and signs of sickness and death were recorded. Mice alive after 21 days were considered to have survived the lethal infection since all of the control mice died in less than 1 week in this experiment [17].

2.10. Murine passive transfer study

Anti-rPfbA serum was prepared by subcutaneous immunization. In brief, female BALB/c mice were prepared for antibody titre evaluation (n = 6) and anti-rPfbA serum collection (n = 24). For the treatment group, 10 μg of protein was mixed with 5 μg of aluminium hydroxide adjuvant (Sigma-Aldrich) in sterile PBS at a total volume of 50 μl, which was then subcutaneously injected. In the control groups, mice were immunized with 5 μg aluminium hydroxide adjuvant dissolved in 50 μl sterile PBS. The mice were immunized three times, with a 14-day interval between each immunization. Caudal blood sampling of the immunized mice was used to derive serum samples to confirm that the immunization resulted in a specific IgG response of appropriate magnitude (IgG titre = 2.0×10^6) in the treatment group. The immunized mice were sacrificed, and the treatment serum and control serum were collected.

Afterwards, postanaesthetic female BALB/c mice (n = 24) were intraperitoneally injected with 200 μl of prepared serum, and then, the serotype 3 bacterial suspension (CMCC[B]31436, 5.0×10^7 CFU, resuspended in PBS) was administered to establish a lethal challenge model and to evaluate the survival time as a murine challenge study [18].

2.11. Cytokine assays

Seven days after the last mucosal immunization, murine single-splenocyte suspensions were obtained as described previously [19]. Splenocytes were cultured in 24-well plates at 1.0×10^6 -cells/ml in RPMI 1640 (Hyclone, IL, USA) supplemented with 10% FBS and stimulated by 10 μl of rPfbA (1 mg/ml, PBS as a control). The culture clusters were placed in an incubator (37 °C, 5% CO_2) for 72 h, and the supernatants were collected to detect the cytokines IFN-γ, IL-10 and IL-17A using an ELISA kit (Biolegend, CA, USA) [20].

2.12. Statistics

Statistical Package for Social Science (SPSS) software for Windows was used to assess the statistical significance of the data (version 17.0, SPSS, Chicago, IL, USA). The Chi-square test, Fisher's exact test, T-test and Mantel-cox test were used, and P values < 0.05 were considered statistically significant. We serialized the seven housekeeping gene sequences to illustrate the minimum-evolution tree by molecular evolutionary genetics analysis (MEGA, version 7.0, http://www.megasoftware.net). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are given as the number of base substitutions per site. The ME tree was searched using the close-neighbour-interchange (CNI) algorithm at a search level of 1. The neighbour-joining algorithm was used to generate the initial tree [21]. The minimum spanning tree was illustrated by PHYLOVIZ software (version 2.0, http://www.phyloviz.net), computed by goeBURST Full MST (goeBURST distance) at level 6 [22].

3. Results

3.1. Demographic and clinical characteristics

A total of 102 patients from 14 cities infected with *S. pneumoniae* were included, and their ages ranged from 1 to 12 years, with a median age (P25-P75) of 1.4 (0.8–3.1) years (Table 1, Fig S2). Thirty-one patients (30.4%) were from the plateau (Tibetan 87.1%), and 71 patients were from the basin (Han 98.6%). The diseases caused by *S. pneumoniae* infection included pneumonia, bacteraemia, meningitis, upper respiratory infections, bronchitis, pleural & peritoneal inflammation, otitis media and conjunctivitis. In total, 96 patients (94.1%) had a favourable prognosis.

3.2. Molecular serotyping

All of the isolates were successfully serotyped. Briefly, 13 different serotypes were identified in this study, and the proportions of each serotype among all isolates are as follows: 19F (31.4%), 19A (21.6%), 6B (13.7%), 14 (11.8%), 23F (9.8%), 18C (2.0%), 3 (2.0%), 7C (2.0%), 10A (2.0%), 5 (1.0%), 2 (1.0%), 12F (1.0%) and 23A

Table 1
Demographic and clinical characteristics.

Characteristics	No. of patients	%
Total	102	100.0
Gender		
Male	66	64.7
Female	36	35.3
Age (years)		
<1	35	34.3
1–2	33	32.3
3–5	22	21.6
5–12	12	11.8
Patients from		
Basin	71	69.6
Plateau	31	30.4
Primary discharge diagnosis		
Invasive pneumococcal disease	20	19.7
Meningitis	8	7.8
Bacteraemia	9	8.8
Pleural inflammation	2	2.0
Peritoneal inflammation	1	1.0
Non-invasive pneumococcal disease	82	80.3
Pneumonia	72	70.6
Upper respiratory infections	4	3.9
Bronchitis	4	3.9
Otitis media	1	1.0
Conjunctivitis	1	1.0
Prognosis		
Cured	69	67.6
Improved and transferred	27	26.5
Sequela	1	1.0
Dead	5	4.9

(1.0%) (Fig. 1A). Classified by region, the predominant serotypes were 19F in the basin patients and 19A and 6B in the plateau patients. The proportions of each serotype for the basin patients were 19F (38.0%), 6B (9.9%), 14 (12.7%), 23F (9.9%), 18C (1.4%), 19A (21.1%), 7C (2.8%), 10A (2.8%) and 2 (1.4%); the proportions of each serotype for the plateau patients were 19A (22.6%), 6B (22.6%), 19F (16.1%), 14 (9.7%), 23F (9.7%), 3 (6.5%), 18C (3.2%), 5 (3.2%), 12F (3.2%) and 23A (3.2%). The proportion of 19F was significantly higher in the basin than in the plateau group (38.0% vs. 16.1%, p = 0.037, Fig. 1B). Classified by disease, the predominant serotypes were 19F (36.6%) in non-IPD patients and 19A (25.0%), 6B (20.0%) and 14 (20.0%) in IPD patients, and the proportion of 19F was significantly higher in non-IPD patients than in IPD patients (36.6% vs. 10.0%, p = 0.022, Fig. 1C).

The overall coverage rates of PCV7, PCV10 and PCV13 were 68.7%, 69.7% and 93.3%, respectively. The coverage rates of each PCV between different regions and different diseases were not significantly different.

3.3. Virulence genes and pilus

The detailed virulence gene and pilus data are shown in Table 2. In general (Fig. 2A), most of the isolates carried *ply* (100%), *cbpA* (97.1%), *nanA* (93.1%) and *phtD* (96.1%). Approximately 65.7% and 52.9% of the isolates carried pilus-1 and pilus-2, respectively, and more basin patient isolates carried pilus-1 compared to plateau patient isolates (73.2% vs. 48.4%, p = 0.023, Fig. 2B). In addition, 52.9% of the isolates carried *pfbA*, and more isolates from IPD patients carried *pfbA* compared to those from non-IPD patients (80.0% vs. 46.3%, p = 0.011); all of the non-19F/19A isolates carried *pfbA* (Fig. 2C). The similarity in *pfbA* sequences was > 99.4% among the *pfbA*-positive isolates.

3.4. Relationship among pfbA, pilus, serotypes and STs

We identified 24 different types of STs, including 9 novel STs (ST13962, ST14094-14098, ST14216 and ST14290-14291) and 4 novel sequences (*recP*437, *ddl*891 and sip614-615). All of the STs contained 2 CCs (CC271, CC81) and 19 singletons, with CC271 being predominant. Among these isolates, ST271 (23.5%), ST320 (21.6%) and ST876 (11.8%) were the most common STs. The predominant ST in the basin was ST271, while the predominant ST in the plateau was ST320. The predominant ST from non-IPD patients was ST271, while the predominant ST from IPD patients was ST320.

The minimum-evolution tree is shown in Fig. 3A, where the optimal tree with a branch length sum of 0.12 is shown, and the percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) are shown next to the branches. We associated the presence of *pfbA* and pilus with the branches. The red area indicates branches carrying *pfbA*, and the green area indicates branches carrying pilus. The minimum spanning trees also show the relationship between clusters and the presence of pilus-1 (Fig. 3D) and *pfbA* (Fig. 3E). All of the CC271 (ST271, ST320, ST236) isolates carried pilus-1 and pilus-2, while few of them carried *pfbA*. In addition, ST271, ST320 and ST876 were specifically associated with serotypes 19F, 19A and 14, respectively (Fig. 3B).

3.5. Antibiotic susceptibility tests

In general, all of the isolates identified were susceptible to vancomycin and levofloxacin. Most of the isolates were susceptible to penicillin, cefotaxime, amoxicillin, quinupristin & dalfopristin and

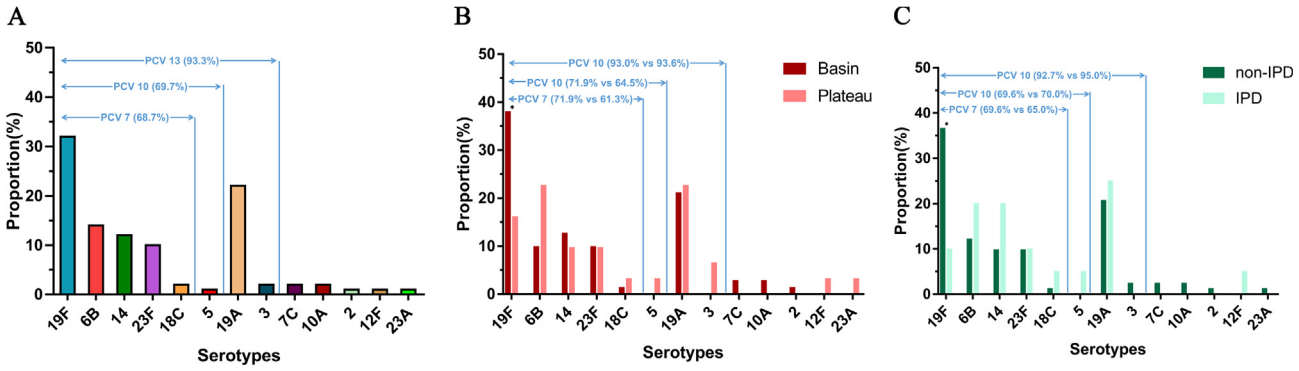

Fig. 1. Serotype distribution and coverage of polysaccharide conjugate vaccines (PCVs) among *S. pneumoniae* isolates stratified by region and disease. A. Proportions of each serotype in all isolates: 19F (31.4%), 6B (13.7%), 14 (11.8%), 23F (9.8%), 18C (2.0%), 5 (1.0%), 19A (21.6%), 3 (2.0%), 7C (2.0%), 10A (2.0%), 2 (1.0%), 12F (1.0%) and 23A (1.0%). B. Proportions of each serotype in different regions. The proportion of 19F was significantly higher in the basin than in the plateau (38.0% vs 16.1%, p = 0.037). C. Proportions of each serotype in different diseases. The proportion of 19F was significantly higher in non-IPD patients than in IPD patients (36.6% vs 10.0%, p = 0.022).

Table 2
Virulence genes and pilus among regions, diseases, serotypes and clonal complexes.

Categories	*ply*		*cbpA*		*nanA*		*phtD*		*pfbA*		Pilus-1		Pilus-2		Total	
	n[a]	%[b]	n	%	n	%	n	%	n	%	n	%	n	%	n[c]	%[d]
Regions																
Basin	71	100.0	69	97.2	64	90.1	67	94.4	35	49.3	**52**	**73.2**	42	59.2	71	69.6
Plateau	31	100.0	30	96.8	31	100.0	31	100.0	19	61.3	**15**	**48.4**	12	38.7	31	30.4
		p = 1.000[e]	p = 0.098	p = 0.311	p = 0.289	***p = 0.023**	p = 0.084									
Diseases																
IPD	20	100.0	20	100.0	19	95.0	19	95.0	**16**	**80.0**	11	55.0	8	40.0	20	19.7
Non-IPD	82	100.0	79	96.3	76	92.7	79	96.3	**38**	**46.3**	56	68.3	46	56.1	82	80.3
		p = 1.000	p = 1.000	p = 1.000	***p = 0.011**	p = 0.299	p = 0.220									
Serotypes																
19F	32	100.0	32	100.0	32	100.0	32	100.0	**3**	**9.4**	**31**	**96.9**	**31**	**96.9**	32	31.4
		p = 0.550[f]	p = 0.095	p = 0.306	***p = 0.000**	***p = 0.000**	***p = 0.000**									
19A	22	100.0	22	100.0	19	86.4	22	100.0	**3**	**13.6**	**22**	**100.0**	**22**	**100.0**	22	21.6
		p = 1.000	p = 0.169	p = 0.575	***p = 0.000**	***p = 0.000**	***p = 0.000**									
6B	14	100.0	14	100.0	14	100.0	14	100.0	**14**	**100.0**	6	42.9	**0**	**0.0**	14	13.7
		p = 1.000	p = 0.589	p = 1.000	***p = 0.000**	p = 0.07	***p = 0.000**									
14	12	100.0	12	100.0	12	100.0	12	100.0	**12**	**100.0**	**3**	**25.0**	**1**	**8.3**	12	11.8
		p = 1.000	p = 1.000	p = 1.000	***p = 0.000**	***p = 0.003**	***p = 0.001**									
Clonal complexes																
CC271	48	100.0	48	100.0	48	100.0	48	100.0	**5**	**10.4**	**48**	**100.0**	**48**	**100.0**	48	47.1
		p = 0.245[g]	p = 1.000	p = 0.120	***p = 0.000**	***p = 0.000**	***p = 0.000**									
Total	**102**	**100.0**	**99**	**97.1**	**95**	**93.1**	**98**	**96.1**	**54**	**52.9**	**67**	**65.7**	**54**	**52.9**	**102**	

[a] Number of positive isolates;
[b] Percentage of positive isolates;
[c] Number of total isolates in each group;
[d] Percentage of total isolates in each group;
[e] T < 5, Fisher's exact test;
[f] Comparison of each serotype with all other isolates;
[g] Comparison of each clonal complex with all other isolates.

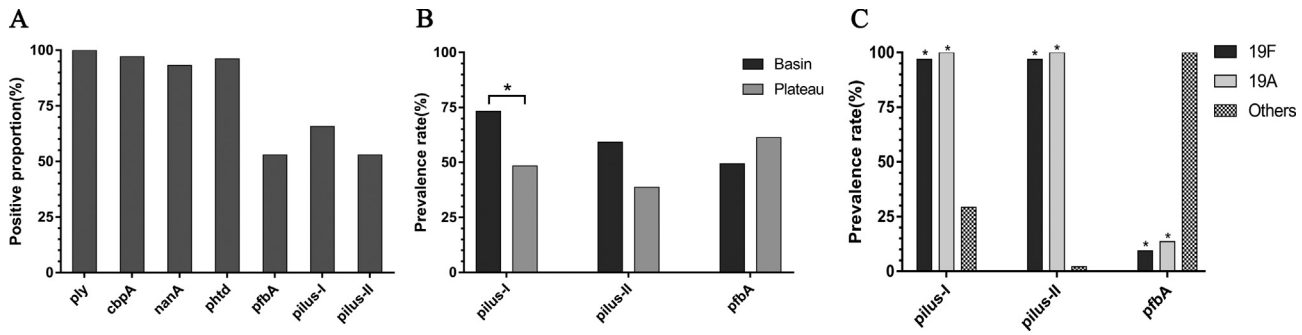

Fig. 2. Prevalence of virulence genes and pilus. A. Positive isolates of virulence genes and pilus in general. B. Positive isolates of pilus and *pfbA* in different regions, "*" indicates a significant difference. C. Positive isolates of pilus and *pfbA* in serotype 19, "*" indicates a significant difference between the specific serotype and the others serotype.

chloromycetin but resistant to erythromycin and clindamycin. The antibiotic resistance varied by region, disease and serotype; the detailed data are shown in Table 3 and Fig. 3C.

To compare the antibiotic resistance rates between IPD and non-IPD patients, we incorporated IPD inpatient AST data from January 2014 to January 2016. Statistical analysis showed that the isolates from IPD patients were more resistant to penicillin, cefotaxime, tetracycline and trimethoprim-sulfamethoxazole compared to those from non-IPD patients. Classified by region, regardless of whether historical inpatient data were incorporated, the resistance rates of erythromycin and clindamycin in the basin were significantly higher than those in the plateau.

3.6. Murine studies

Seven days after the last mucosal immunization with rPfbA, the antibody titre responses in saliva and serum were determined. In saliva (Fig. 4A), both the IgG and IgA levels were significantly elevated in the treated groups ($p = 0.008$; $p = 0.02$). In serum (Fig. 4B), the IgG level was also significantly elevated in the treated groups ($p = 0.02$).

In the colonization model (serotype 6B) for the murine challenge study, mucosal immunization with rPfbA reduced the colonization of *S. pneumoniae* in both the nasopharynx (Fig. 5A, $p < 0.001$) and the lung (Fig. 5B, $p < 0.001$) on the third day after challenge. In the lethal challenge model (serotype 3) for the murine challenge study, mucosal immunization with rPfbA significantly prolonged the murine survival on the 21st day after challenge (58.3% vs. 0.0%, $p < 0.001$, Fig. 5C). In the murine passive transfer study, anti-PfbA serum (antibody titre = 2.0×10^6) prolonged murine survival in the lethal challenge model (serotype 3) on the 21st day after challenge (41.7% vs. 0.0%, $p = 0.002$, Fig. 5D).

3.7. Cytokine assays

Cytokine assays were administered in splenocyte supernatants to investigate the T-cell subtype of the immune responses elicited

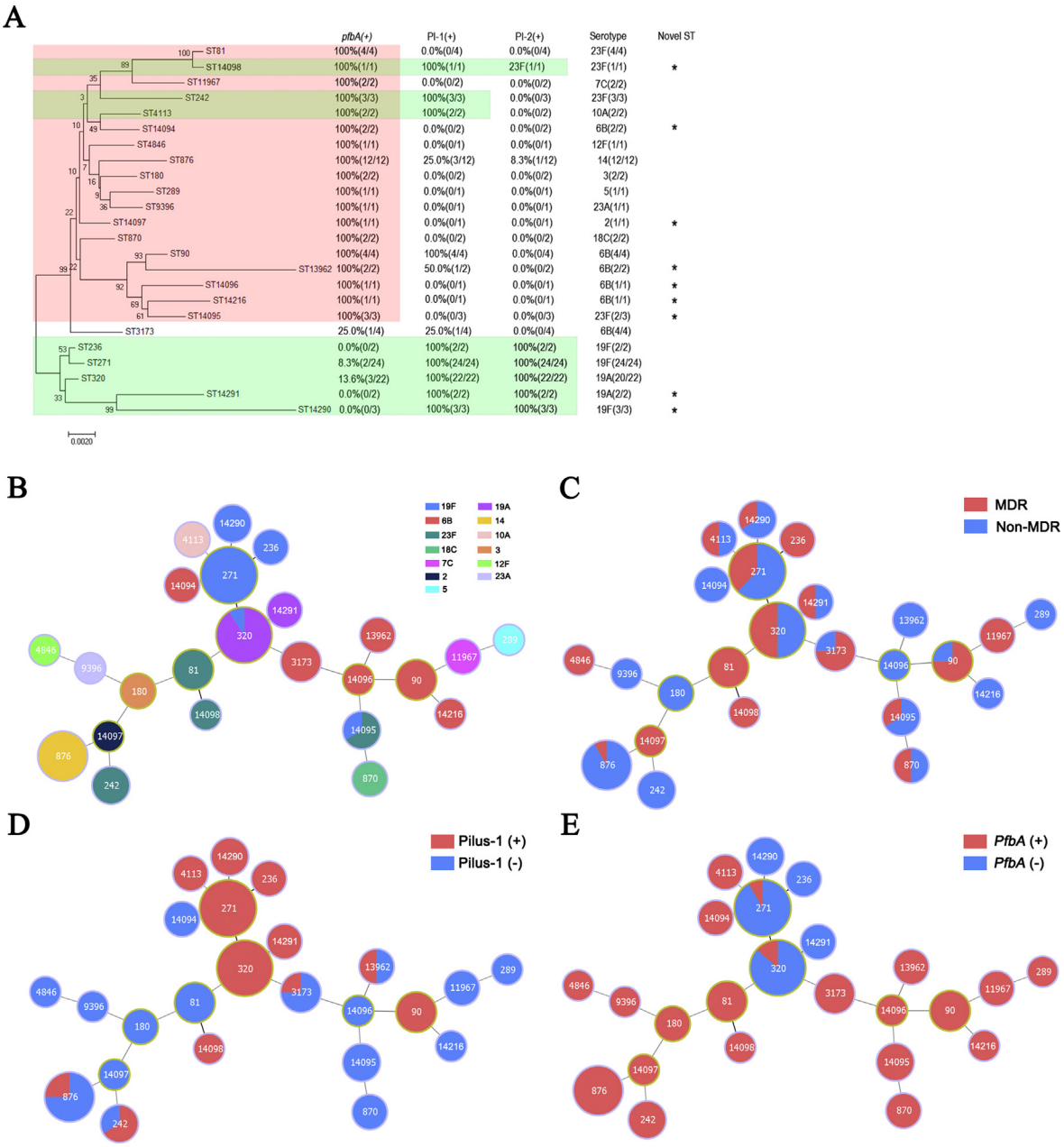

Fig. 3. Minimum-evolution tree and minimum spanning tree. A. Minimum-evolution tree, calculated by serializing the seven housekeeping gene sequences of *S. pneumoniae*, illustrated using MEGA. The red area indicates branches carrying *pfbA*, and the green area indicates branches carrying pilus. B. Minimum spanning tree showing the relationship between STs and serotypes, illustrated using PHYLOVIZ. Each disk represents an ST, and each colour represents a serotype. C. Relationship between STs and MDR. D. Relationship between STs and pilus-1-positivity. E. Relationship between STs and *pfbA*-positivity.

by mucosal immunization with rPfbA. The cytokine levels of IFN-γ (Fig. 6A, $p = 0.02$), IL-10 (Fig. 6B, $p < 0.001$) and IL-17A (Fig. 6C, $p < 0.001$) were significantly elevated in the treated groups.

4. Discussion

The demographics, economies and environments of the plateau and basin regions analysed in our study are significantly different. First, in China, Han Chinese comprise the major ethnic group, accounting for more than 90% of the national population, while Tibetans comprise the majority ethnic group on the Tibetan Plateau. Second, plateau inhabitants have a much lower economic status, and the gross domestic product (GDP) per capita on the plateau is approximately half of that in the basin. The demographic and economic differences in the two regions lead to differences in health care, education, customs and individual immune conditions. Third, the average altitude of the plateau is > 3000 m, and these areas thus have a lower oxygen content and atmospheric pressure than lower altitude regions. All of these differences have an overall effect on the distribution, prevalence and other characteristics of *S. pneumoniae* and *S. pneumoniae*-related disease.

The serotype distributions of *S. pneumoniae* vary among regions and diseases. In general, the most common serotypes of isolates that infected children under 12 years old in southwest China were 19F, 19A, 6B, 14 and 23F; these serotypes were similar to those from studies on other southern regions of China, such as Liuzhou

Table 3
Antibiotic resistance among regions, diseases and serotypes.

Categories	PEN[a]		AMX		CTX		ERY		QD		CLI		TET		LEV		CHL		VAN		SXT		Total	
	n[b]	%[c]	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n[d]	%[e]
Regions																								
Basin	4	5.6	3	4.2	4	5.6	**70**	**98.6**	0	0.0	**69**	**97.2**	10	14.1	0	0.0	2	2.8	0	0.0	30	42.3	71	69.6
Plateau	1	3.2	1	3.2	1	3.2	**27**	**87.1**	0	0.0	**23**	**74.2**	9	29.0	0	0.0	2	6.5	0	0.0	13	41.9	31	30.4
	p = 1.000[f]	p = 1.000	p = 1.000	***p = 0.029**		***p = 0.001**	p = 0.098		p = 0.583		p = 1.000													
Diseases																								
IPD	**14**	**27.5**	4	7.8	**8**	**15.7**	49	96.1	1	2.0	42	82.4	**23**	**45.1**	0	0.0	2	3.9	0	0.0	**32**	**62.7**	51[g]	38.3
Non-IPD	**2**	**2.4**	3	3.7	**3**	**3.7**	78	95.1	0	0.0	74	90.2	**14**	**17.1**	0	0.0	3	3.7	0	0.0	**34**	**41.5**	82	61.7
	***p = 0.000**	p = 0.428	***p = 0.022**	p = 1.000	p = 0.383	p = 0.195	***p = 0.001**		p = 1.000		***p = 0.021**													
Serotypes																								
19F	1	3.1	2	6.3	2	6.3	32	100.0	0	0.0	**32**	**100.0**	5	15.6	0	0.0	1	3.1	0	0.0	13	40.6	32	31.4
	p = 1.000[h]	p = 0.588	p = 0.648	p = 0.322		***p = 0.029**	p = 0.785		p = 1.000		p = 1.000													
19A	2	9.1	0	0.0	1	4.5	22	100.0	0	0.0	19	86.4	**0**	**0.0**	0	0.0	0	0.0	0	0.0	9	40.9	22	21.6
	p = 0.294	p = 0.575	p = 1.000	p = 0.582		p = 0.449	***p = 0.011**		p = 0.575		p = 1.000													
6B	0	0.0	0	0.0	0	0.0	**10**	**71.4**	0	0.0	**9**	**64.3**	**9**	**64.3**	0	0.0	2	14.3	0	0.0	9	64.3	14	13.7
	p = 1.000	p = 1.000	p = 1.000	***p = 0.001**		***p = 0.004**	***p = 0.001**		p = 0.090		p = 0.086													
14	0	0.0	0	0.0	0	0.0	12	100.0	0	0.0	12	100.0	1	8.3	0	0.0	0	0.0	0	0.0	**0**	**0.0**	12	11.8
	p = 1.000	p = 1.000	p = 1.000	p = 1.000		p = 0.602	p = 0.456		p = 1.000		***p = 0.001**													
Total	**5**	**4.9**	**4**	**3.9**	**5**	**4.9**	**97**	**95.1**	**0**	**0.0**	**92**	**90.2**	**19**	**18.6**	**0**	**0.0**	**4**	**3.9**	**0**	**0.0**	**43**	**42.2**	**102**	

[a] PEN (penicillin), AMX (amoxicillin), CTX (cefotaxime), ERY (erythromycin), QD (quinupristin/dalfopristin), CLI (clindamycin), TET (tetracycline), LEV (levofloxacin), CHL (chloromycetin), VAN (vancomycin), SXT (sulfamethoxazole/trimethoprim);
[b] Number of resistant isolates in each antibiotic category;
[c] Percentage of resistant isolates in each antibiotic category;
[d] Number of total isolates in each group;
[e] Percentage of total isolates in each group;
[f] T < 5, Fisher's exact test;
[g] Incorporated IPD inpatient antibiotic susceptibility test data from January 2014 to January 2016;
[h] Comparison of each serotype with all other isolates.

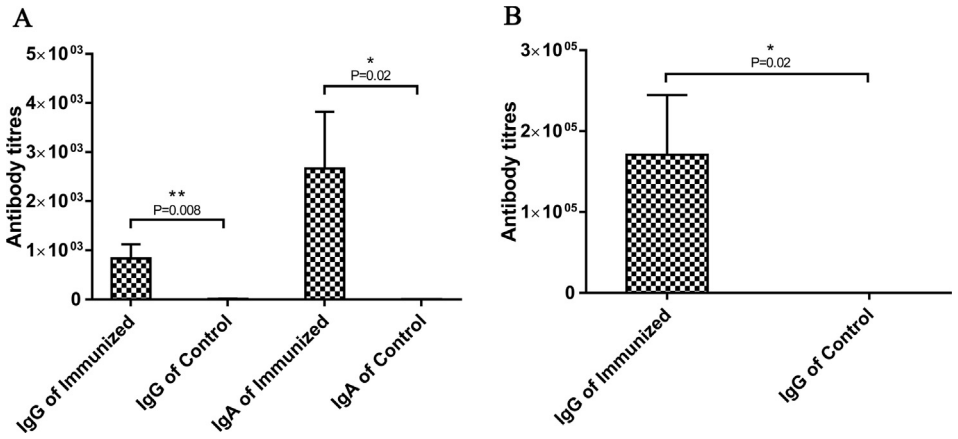

Fig. 4. Anti-rPfbA titres in murine studies. A. Specific antibody titres in saliva obtained from mucosally immunized mice prior to challenge (n = 3). B. Specific antibody titres in sera obtained from mucosally immunized mice prior to challenge (n = 3).

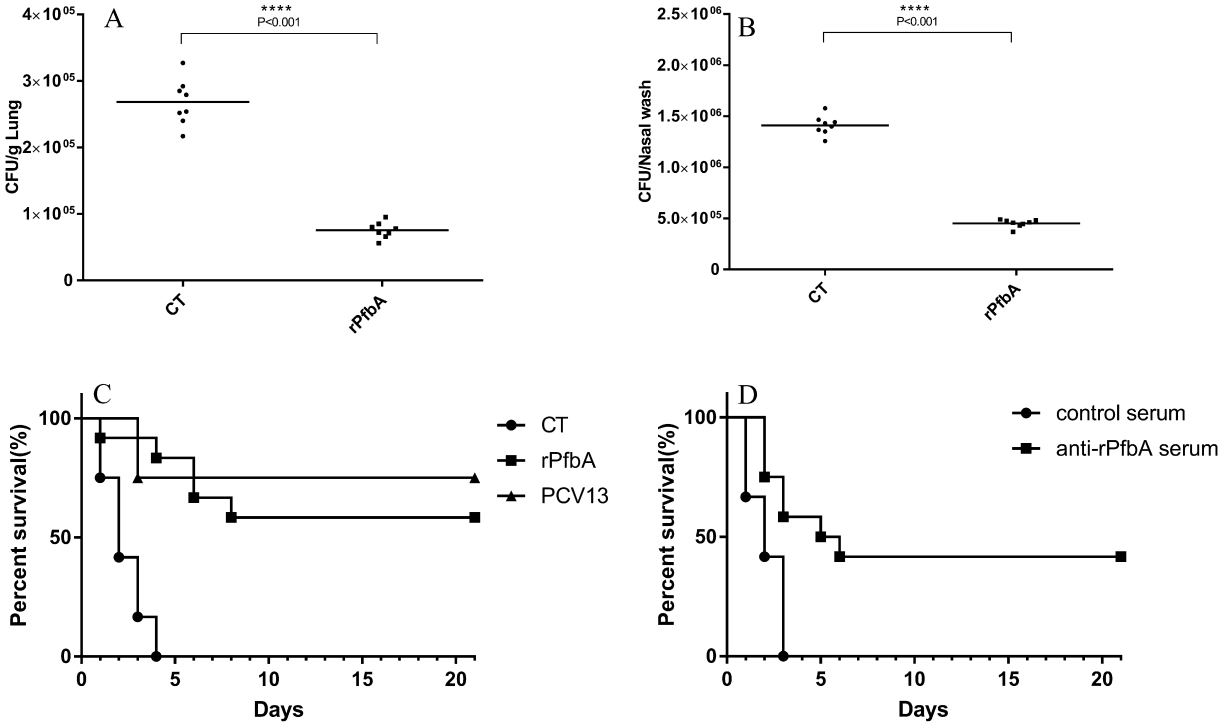

Fig. 5. Murine challenge studies and passive transfer studies. A, B. Effects of mucosal immunization with rPfbA on the colonization of serotype 6B. Colonization in the nasopharynx and lung (n = 8) and the mice were sacrificed on the third day after challenge; CT served as the adjuvant control. C. Survival times of mucosal immunized mice after lethal challenge with serotype 3. Mice (n = 12) were challenged seven days after the last immunization and continuously monitored for 21 days. Intramuscular PCV13 served as a positive control, and CT served as an adjuvant control. Survival rates: rPfbA 58.3% ($p < 0.001$, compared with CT), PCV13 75.0% ($p < 0.001$, compared with CT), CT 0.0%. D. Survival times of passively immunized mice after lethal challenge with serotype 3. Mice (n = 12) were challenged immediately upon serum transfer intraperitoneally and continuously monitored for 21 days. Murine serum injected with an adjuvant served as a control. Survival rates: rPfbA 41.7% ($p = 0.002$, compared with control), control 0.0%.

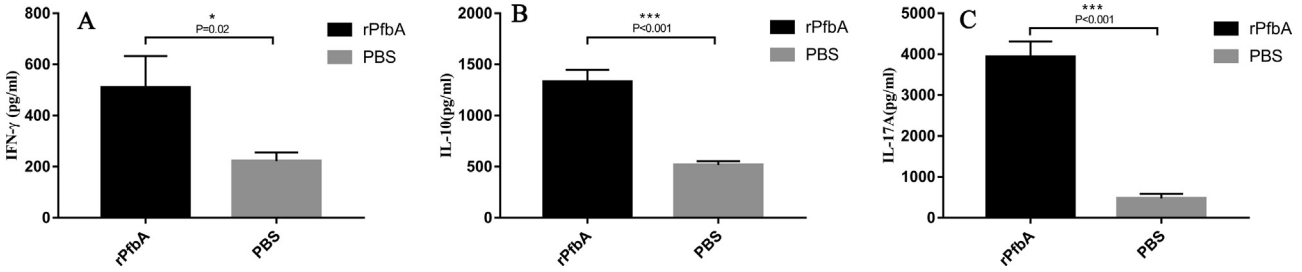

Fig. 6. Cytokine produced by splenocytes from mucosally immunized mice 7 days after the last immunization (n = 3).

(19F, 6B, 19A, 23F, 14) [23], Suzhou (19F, 6B, 19A, 23F) [24] and Shanghai (19F, 14, 23F, 6B, 19A) [25], but the ranking orders varied. Furthermore, the distributions of serotypes were different from those in northern China (19F, 23F, 14, 15, 6A) [26], southeastern Asia (23F, 19F, 6A, 19A, 15B/C) [13] and America (14, 1, 5) [27]. The predominant serotypes from the Sichuan Basin and the Tibet Plateau differed significantly, which suggests that the cluster distributions of *S. pneumoniae* strains are different between the two regions. Possible explanations for this difference are as follows: 1. *S. pneumoniae* have different evolutionary directions in the two regions. 2. *S. pneumoniae* lack a mutual spread between the two regions. 3. The susceptibility of the ethnic population to *S. pneumoniae* differs between the two regions. In addition, the serotype distribution was also related to disease. The predominant serotype of isolates from non-IPD patients was 19F, while this serotype was isolated from a significantly lower proportion of IPD patients, which suggests that the difference in invasiveness varies among serotypes.

MLST is a bacterial molecular typing method, and its comparability, repeatability and definition are superior to pulsed field gel electrophoresis (PFGE) and ribotyping. MLST yields typing information for classification data rather than image data, and the sequence data of seven housekeeping genes can provide more details regarding floral genetic information. We mapped phylogenetic trees using not only profiling data (minimum-spanning tree) but also sequencing data (minimum-evolution tree) to describe the strain relationship and to calculate genetic distances.

Herein, the prevalent STs were ST271, ST320 and ST876 in southwest China, and the ST distributions varied among regions and diseases. In the minimum-evolution tree, a bootstrap test value > 80 is considered to be significantly different on an evolutionary branch. When we associated STs with *pfbA* and pilus, these strains were primarily divided into two groups: 1. carrying *pfbA* but lacking pilus (36.3%); 2. carrying pilus but lacking *pfbA* (47.1%). These molecular differences may be explained by the different choices of the strains at the evolutionary node. In the minimum-spanning trees, we associated STs with MDR, pilus-1 and *pfbA* to display the proportion of each characteristic in the different STs. The same ST has a good homogeneity in serotype, pilus-1 and *pfbA*, which indirectly confirms that the differences in molecular characteristics of the strains are related to evolution. However, we mapped the minimum-evolution tree using seven housekeeping gene sequences instead of the whole genome sequence, causing limitations in the precision of the evolutionary branch.

PfbA, which is expressed on the pneumococcal cell surface and contains an LPXTG anchoring motif, contributes to fibronectin-dependent adhesion and antiphagocytosis [28]. According to Löfling [29], *pfbA* is widely conserved among 32 different strains of *S. pneumoniae* whose sequences are available (conservation > 99%). The protein expression sites, function and high conservation of *PfbA* make it an ideal vaccine protein. In this study, we found that the prevalence rate of *pfbA*-expressing isolates was 52.9% in southwest China, and the sequence similarities of *pfbA* were > 99.4%. All of the isolates that lacked *pfbA* pertained to CC271, which was the main evolutionary branch lacking *pfbA* and the predominant CC in this region, explaining the relatively low prevalence of *pfbA* observed in this study.

The pilus participates in host cell invasion, biofilm formation, cell aggregation, DNA transfer and twitching motility [30]. In this study, the total carriage rates of pilus-1 and pilus-2 were 65.7% and 52.9%, respectively, which are significantly higher than those in other regions (pilus-1: 14.4%-35.2%, pilus-2: 16%-21% [23]). In the Sichuan Basin, the positive rates were even higher, with the following possible explanations: 1. *S. pneumoniae* has different evolutionary trends in pilus-1 between the two regions. 2. *S. pneumoniae* in the basin has a stronger selection pressure (better health and medical conditions). 3. The invasiveness of pilus-1 varies with ethnic population. In sum, the difference found in the distribution of pilus-1 between the two populations is caused by the difference in epidemic strains.

Antibiotic resistance has gradually become a major global health issue. In this study, 12.0% of the isolates identified were resistant to penicillin, including 2.9% of isolates from non-meningitis patients and 46.4% of isolates from meningitis patients. The results regarding non-meningeal antibiotic resistance were consistent with those of recent studies from southern China (S% =99.3%) [23], Japan (S%=97.8%) [31] and India (S%=96%) [6], indicating that penicillin was still the first choice for the treatment of non-meningeal *S. pneumoniae* infection.

S. pneumoniae isolates from IPD patients were more resistant to penicillin, cefotaxime, tetracycline and SXT, and MDR isolates were found in a greater proportion of IPD patients. This finding indicated the increased difficulty of treating IPDs, especially pneumococcal meningitis. The most recent antibiotic treatment guidelines for pneumococcal meningitis recommend the use of vancomycin combined with cefotaxime or ceftriaxone to treat pneumococcal meningitis when the isolate is non-susceptible to both penicillin and cefotaxime [32–35]. The high rate of penicillin resistance to among IPD patient isolates suggests that vancomycin can be added to ceftriaxone for the initial treatment of severe IPD infection until culture reports become available, after which antibiotics can be administered based on the susceptibility test results.

The antibiotic resistance rates differed between the plateau and basin regions, and the resistance rates of erythromycin and clindamycin in the basin were higher than those in the plateau. Unlike those in the basin, residents on the plateau lack transportation, and the population in the plateau region is more stagnant. Thus, there is less chance for *S. pneumoniae* strains to obtain resistant genes via bacterial conjugation or transformation. Second, antibiotic abuse is more serious in the basin, especially for macrolides, which cause antibiotic selective pressure and lead to a higher resistance rate.

PCV is currently an effective method for preventing pneumococcal infection; PCV7 was introduced in China in 2008 and replaced by PCV13 in 2017. However, because PCV was not included in the national immunization plan, people must pay for the vaccine themselves. Many people in southwest China cannot afford the vaccine, and thus, the vaccination rate in this area is extremely low. In this study, the serotype coverages of PCV13 in both the basin and plateau were > 90%, which is higher than the rates in other regions of China (average = 58.3%) [36]. In America, the incidence of IPD caused by serotypes covering PCV7 declined to almost zero after the widespread use of PCV7 [37], demonstrating the efficacy of PCV. Thus, widespread vaccination with PCV13 most likely eases the burden of pneumococcal infection in high serotype coverage regions.

However, serotype replacement occurred in some developed country decades after PCV vaccination [38,39], causing a morbidity rebound and an increased prevalence of non-PCV serotypes. Furthermore, reducing vaccine costs is an alternative method for increasing the rate of vaccination. The costs of protein-based vaccines are significantly lower than those of conjugate vaccines, making them more acceptable. In addition, protein-based vaccines have a stronger immunogenicity and have antigenic targets that are widely conserved among *S. pneumoniae* strains without the limitation of serotype [40]. In this study, we evaluated the prevalence rates of some potential protein antigenic targets, such as Ply, CbpA, NanA and PhtD, whose immunoprotection abilities have been confirmed [41–43]. The carriage rates of *ply, cbpA, nanA* and *phtD* were extremely high in this region (>93%), indicating the pervasiveness of immune protection and confirming the vaccinal potential.

Mucosal immunity can induce sIgA [44] antibody production and a Th17-mediated immune response [45], which plays a role

in eliminating the nasopharyngeal colonization of *S. pneumoniae* and is noninvasive and painless compared to other immunization methods. It is generally believed that the vaccine can eliminate the colonization of *S. pneumoniae* and can also protect against invasive *S. pneumoniae* infection. In this study, we measured antibody levels in the saliva and sera of immunized mice and found specific antibody production against rPfbA. The opsonophagocytosis of antibodies is the main mechanism against *S. pneumoniae* infection, and high-titre antibody production is required for the protective effect of vaccine proteins. We selected serotype 6B to construct murine colonization models and serotype 3 to construct murine lethal challenge models, in order to assess the nonserotype dependence of protein protection. Immunization with rPfbA eliminated colonization in the nasopharynx and lung and prolonged murine survival, indicating that rPfbA indeed protected the hosts against *S. pneumoniae* infection for serotype 6B and 3. In the murine passive transfer study, anti-PfbA serum also prolonged murine survival. Both the high titre of the specific antibody and the murine passive transfer study indicated that humoural immunity plays an important role in the immune process mediated by rPfbA. In addition, increased IFN-γ, IL-10 and IL-17A levels indicate that Th cells, particularly Th1 and Th17 cells, were activated by PfbA. IFN-γ treatment can significantly increase survival rates after *S. pneumoniae* challenge [46], and IL-17A is implicated in the antibody response to *S. pneumoniae* capsular polysaccharide and can also effectively enhance bacterial phagocytosis and killing functions to resist lethal infection [47,48]. Together, these results show that mucosal immunization with rPfbA can protect mice against *S. pneumoniae* infection via both humoural and cellular immunity mechanisms.

Pili are also vaccine candidate antigen targets; pilus-1 is composed of the structural proteins RrgA, RrgB and RrgC [49]. Nelson [50] and Harfouche [51] confirmed the protective effects of both RrgA and RrgB against *S. pneumoniae* infection in mice. Herein, all of the clinical *S. pneumoniae* isolates carried either *rlr-A* or *pfbA*, which indicates that pilus-1 and PfbA can be combined as an alternative protein vaccine strategy to protect against *S. pneumoniae* infection. However, this study did not determine the allelic variants of pilus-1; thus, fusion proteins with different clades will be considered if pilus is used as a component of the combined protein vaccine.

To the best of our knowledge, this is the first study to investigate the strain distribution and characteristics of *S. pneumoniae* in the Tibet Plateau region as well as the first study to evaluate the protective effect of rPfbA by mucosal immunization and serum passive immunization in vivo and vitro. However, our study does have some limitations. First, to thoroughly reveal the distribution and prevalence of *S. pneumoniae* in southwest China, the sample capacity, especially that in the plateau region, needs to be further expanded. Second, all of the patients were admitted to the top national hospital, which may have caused sampling bias. However, the study still successfully depicted the prevalence of *S. pneumoniae* and helped advance research in this region. As a next step, we have planned a multicentre investigation that incorporates rural primary health centres and first-class hospitals. In addition, as an alternative *S. pneumoniae* vaccine strategy, we will further evaluate the combined protective effects and mechanism of PfbA and pilus-1.

In conclusion, we found that in southwest China, the predominant serotypes of the isolates were 19F and 19A, the predominant STs of the isolates were ST271 and ST320, and the distributions varied among regions and diseases. The virulence genes *ply*, *cbpA*, *nanA* and *phtD* were detected in more than 93% of the isolates, and more than half of the isolates carried puils-1, puils-2 and *pfbA*. Mucosal immunization of rPfbA is effective against *S. pneumoniae* infection, and the immune effect of rPfbA is exerted by humoural and cellular immune mechanisms.

Conflict of interest

The authors have no conflicts of interest to disclose.

6. Funding source

This work was supported by the Chinese National Natural Science Foundation (No. 81301399), and the Science & Technology Department of Sichuan Province (No. 2017SZ0122, No. 2016SZ0021). The funders had no role in study design, data collection and analysis and interpretation of the data.

7. Ethics statement

The murine experimental plan was approved by the Laboratory Animal Management and Ethics Committee of West China Second University Hospital, Sichuan University (No. 2018-50). All animal procedures were conducted in accordance with the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act (1986) and associated guidelines, and the EU Directive 2010/63/EU for animal experiments.

The clinical experimental plan was approved by the Clinical Trial Ethics Committee of West China Second University Hospital, Sichuan University (No. 2018021). Before enrolment, written informed consent was obtained from legal guardians on behalf of children involved in the study. The work was carried out in accordance with the Declaration of Helsinki.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.021.

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