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RESEARCH ARTICLE

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Identification and molecular epidemiology of routinely determined *Streptococcus pneumoniae* with negative Quellung reaction results

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Abstract

Background: Some streptococci strains identified as *Streptococcus pneumoniae* (*S. pneumoniae*) by routine clinical methods exhibiting negative Quellung reaction results may belong to other species of viridans group streptococci or non-typeable *S. pneumoniae*. The purpose of this study was to investigate the identification and molecular characteristics of *S. pneumoniae* with negative Quellung reaction results.

Methods: One hundred and five isolates identified as *S. pneumoniae* using routine microbiological methods with negative Quellung reaction results were included. Multilocus sequence analysis (MLSA) was used as a gold standard in species identification, and the capacity of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) in identification was evaluated. Capsular genes and sequence types of *S. pneumoniae* isolates were determined by sequential multiplex PCR and multilocus sequence typing. Antimicrobial susceptibility patterns were determined via broth microdilution with a commercialized 96-well plate.

Results: Among the isolates, 81 were identified as *S. pneumoniae* and 24 were *S. pseudopneumoniae* by MLSA. MALDI-TOF MS misidentified six *S. pneumoniae* isolates as *S. pseudopneumoniae* and nine *S. pseudopneumoniae* isolates as *S. pneumoniae* or *S. mitis*/*S. oralis*. Thirty-one sequence types (STs) were detected for these 81 *S. pneumoniae* isolates, and the dominant ST was ST-bj12 (16, 19.8%). The non-susceptibility rates of *S. pseudopneumoniae* were comparable to those of NESp strains.

Conclusions: Some *S. pneumoniae* isolates identified by routine methods were *S. pseudopneumoniae*. Most NESp strains have a different genetic background compared with capsulated *S. pneumoniae* strains. The resistance patterns of *S. pseudopneumoniae* against common antibiotics were comparable to those of NESp.

KEYWORDS
antimicrobial susceptibility, identification, MLSA, MLST, *Streptococcus pneumoniae*

Ju Jia and Wei Shi contributed equally to this work.

1 | INTRODUCTION

The routine methods for identifying *Streptococcus pneumoniae* (*S. pneumoniae*) in most clinical laboratories are mainly based on phenotypic characteristics, including morphology and biochemical reactions, such as optochin susceptibility test and bile solubility test.[1] However, the bile solubility test is often neglected in daily laboratory analysis.[2] Optochin-susceptible non-pneumococcal alpha-hemolytic streptococci have been reported[3]; thus, it is not reliable to identify *S. pneumoniae* using optochin susceptibility test results alone.

The Quellung reaction is the gold standard for serotyping of *S. pneumoniae*.[4] In previous studies, we noticed that some isolates identified as *S. pneumoniae* using routine methods displayed negative results in the Quellung reaction test, and such isolates were called non-typeable *S. pneumoniae* (NTSp). Most NTSp strains lack a capsule, that is, they are non-encapsulated *S. pneumoniae* (NESp).[5,6] It is important to make clear whether *S. pneumoniae* identified by routine methods with negative Quellung reaction result is true *S. pneumoniae* or other alpha-hemolytic streptococci.

Some molecular methods targeting at specific genes such as 16S rRNA and lytA (encoding autolysin) have proven ineffective in differentiating *S. pneumoniae* from other viridans group streptococci (VGS), especially *S. mitis*.[4,7] Multilocus sequence analysis (MLSA) eliminates the potential problems resulting from homologous recombination, and it has been applied in the identification of several bacterial species that are difficult to identify using other methods.[8] MLSA for VGS, developed by Bishop et al., could accurately differentiate VGS at the species level.[9] Due to its rapid, simple, cost-effective, and high-throughput performance, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been widely used in clinical laboratories.[10,11] In addition, multilocus sequence typing (MLST) and whole-genome sequencing (WGS) are reliable in the identification of VGS.[4] Hence, MLSA was used to accurately differentiated *S. pneumoniae* and to evaluate the ability of MALDI-TOF MS.

Recently, NTSp isolates were categorized into Group I and Group II (null capsule clade1 [NCC1] and NCC2) based on the *cps*, *aliB*, and *pspK* genes.[12] To date, little is known about the genetic background or antibiotic resistance characteristics of NTSp strains.

In this study, 105 clinical isolates identified as *S. pneumoniae* using routine microbiological methods with negative Quellung reaction results were included. MLSA was used as a gold standard in species identification, and the capacity of MALDI-TOF MS for identification was evaluated. Molecular epidemiological characteristics and antibiotic susceptibility patterns of these isolates were detected.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains

The strains were isolated from children's respiratory tract specimens collected during daily work in the Microbiology Laboratory of Beijing Children's Hospital from 2013 to 2019. These isolates were identified as *S. pneumoniae* using a standard optochin susceptibility test, but showed negative results when they were serotyped via Quellung reaction test.

2.2 | Optochin susceptibility and bile solubility tests

A disk (DD0001B 5 μg of optochin, Oxoid Ltd) was placed on a blood agar plate with the inoculated isolate. Then, the plate was incubated in 37℃ for 24 h. An inhibition zone diameter of ≥14 mm denoted a positive result.

The bile solubility assay was performed according to the standard procedures described by Arbique et al.[13] Visible clearing of bacterial suspensions in the deoxycholate [DZ0240 sodium deoxycholate (10%, pH 7), Leagene Biotechnology] was interpreted as a positive result.

In both experiments, strains with known positive and negative reactions kept in our laboratory were used as positive control and negative control strains, respectively.

2.3 | Quellung reaction test

Omni serum (SSI), which contains antibodies to all known pneumococcal serotypes, was used to do the Quellung reaction test for all the isolates. The capsule became visible because of an in situ immunoprecipitation leading to changes in its refractive index, as well as agglutination of the bacterium.[14] Negative Quellung reaction did not have these features.

2.4 | MLSA for VGS

Bacterial DNA was extracted using a DNA extraction kit (SBS Genetic Co. Ltd) according to the manufacturer's protocol. The seven housekeeping genes *map*, *pfl*, *ppaC*, *pyk*, *rpoB*, *soda*, and *tuf* were amplified as previously described.[15] Polymerase chain reaction (PCR) products were sequenced at Tianyihuiyuan Biotechnology Company. The identification of the isolates was performed following the published procedure.[9] Briefly, the sequences of the seven genes were aligned, trimmed, edited, and concatenated using the MEGA 4.0 software (http://www.megasoftware.net). Phylogenetic analysis of the concatenated sequences (*map–pfl–ppaC–pyk–rpoB–soda–tuf*), including isolates in this study and those reported in the MLSA database (download from http://www.emlsa.net/), was conducted using MEGA 4.0 and the maximum likelihood method with 1000 bootstrap replicates to build a neighbor-joining phylogenetic tree.

2.5 | MALDI-TOF MS

All isolates were tested using the Vitek MS system (bioMérieux). Plate preparation, mass spectrum generation, and processing were performed using MYLA software affiliated with IVD version 3.0.

2.6 | Sequential multiplex PCR for molecular serotyping

The molecular serotypes of *S. pneumoniae* isolates were determined with multiplex PCR. Twenty-eight previously described oligonucleotide primers[15] were divided into seven groups and used to detect the serotypes. *S. pneumoniae* American Type Culture Collection strain 49619 (ATCC49619) was used as the quality-control strain and included in each set of tests.

2.7 | MLST for *S. pneumoniae*

The housekeeping genes *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* were amplified via PCR.[16] Each sequence of the seven loci was compared with those of all known alleles at the loci and with the sequence types (STs) in the database of the MLST website (http://spneumoniae.mlst.net). eBURST v3 software (http://spneumoniae.mlst.net/eburst/) was used to determine the relationships between isolates and to assign a clonal complex (CC) based on the stringent group definition of six of seven shared alleles.

2.8 | NTSp grouping by PCR

NTSp can be divided into several groups using the *cpsA*, *pspK*, *aliB-like ORF1*, and *aliB-like ORF2* genes.[12] Among these genes, *cpsA* is a conserved pneumococcal capsular polysaccharide gene, and *aliB-like ORF1* and *aliB-like ORF2* are frequently present in the capsular region of non-capsulated pneumococci. PCR were performed following the protocol described in previous reports.[15,17,18]

2.9 | Antimicrobial susceptibility test

Susceptibility patterns to penicillin, amoxicillin/clavulanic acid, ceftriaxone, cefotaxime, cefuroxime, meropenem, erythromycin, azithromycin, chloramphenicol, trimethoprim/sulfamethoxazole, tetracycline, vancomycin, levofloxacin, and moxifloxacin were tested for all isolates. Minimum inhibitory concentrations (MICs) were determined via broth microdilution method with a commercialized 96-well plate (B8283A, STP6F, Trek Diagnostic Systems Ltd), and the result was read by the Sensititre™ ARIS™ 2X ID/AST system (Thermo Fisher Scientific Inc.). The susceptibility rate was interpreted in accordance with the Clinical and Laboratory Standards Institute 2019 guidelines.[19] Quality control was performed using *S. pneumoniae* ATCC 49619.

2.10 | Data analysis

The phylogenetic tree was built and analyzed using MEGA 4.0 software. Antimicrobial susceptibility and MLST data were analyzed using the WHONET 5.6 software as recommended by the World Health Organization.

3 | RESULTS

3.1 | Identification by MLSA

We observed that most of the isolates displayed small, shiny, smooth, and domed colonies without depressed centers as typical *S. pneumoniae* strains.

The 105 strains in this study and strains in the MLSA database (http://www.emlsa.net/) were analyzed together in the phylogenetic tree, and the results were presented in Figure 1 and Figure 2. As shown in Figure 1, the 105 isolates in the present study were significantly divided into *S. pneumoniae* ($n = 81$) branch and *S. pseudopneumoniae* ($n = 24$) branch. No other VGS were detected.

3.2 | Other identification methods

The results of MLSA analysis were used as a standard to evaluate the other identification methods. The optochin susceptibility test showed that the inhibition zone diameters caused by optochin ranged from 16 to 25 mm for the 81 *S. pneumoniae* isolates (median, 19 mm), whereas the diameters ranged from 14 to 22 mm for the 24 *S. pseudopneumoniae* isolates (median, 15 mm).

All 81 *S. pneumoniae* isolates exhibited positive results in the bile solubility test. For the 24 *S. pseudopneumoniae* strains, 19 showed negative results, and the other 5 showed positive results (Table 1).

Vitek MS could not accurately identify the species for 9 of 24 *S. pseudopneumoniae* isolates and 6 of 81 *S. pneumoniae* isolates (Table 1). The sensitivity and specificity of Vitek MS in the identification of *S. pneumoniae* were 91.7% and 71.4%, respectively. Besides, the isolates mistakenly identified by Vitek MS were distantly separated from each other and located in different sub-branches in the phylogenetic tree, as presented in Figure 2.

3.3 | Multiplex PCR for molecular serotyping and NTSp grouping

None of the isolates could be serotyped by the molecular method. The *cpsA* gene, which served as the internal control, could not be detected in any of the 105 isolates, although it was identified in the ATCC49619 strain, indicating that all isolates belonged to Group II.

Different NTSp types of these isolates were further identified. Seventeen isolates carrying both *aliB-like ORF1* and *aliB-like ORF2* genes were detected, and thus, the isolates were designated as NCC2. Forty-six isolates carried the *pspK* gene, and they were designated as NCC1.

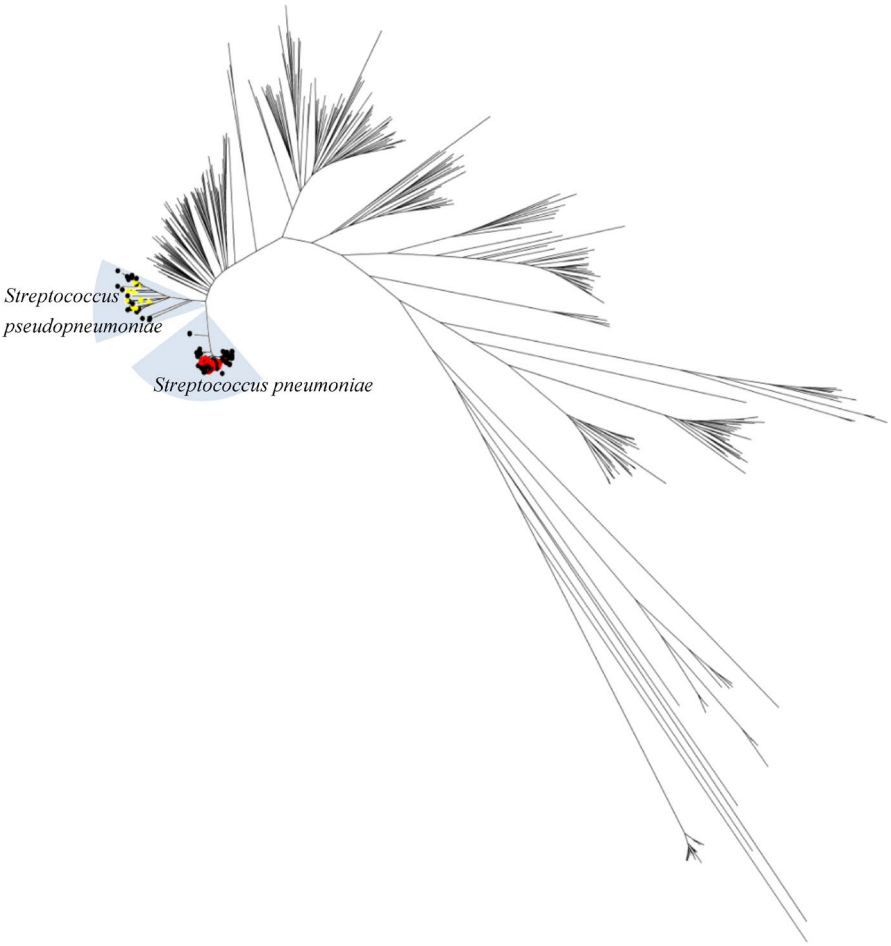

FIGURE 1 Genetic relationships of the 105 isolates determined by multilocus sequence analysis (MLSA). The symbols indicate the following: ● isolates obtained in this study; ● *Streptococcus pneumoniae* strains from the MLSA database; ● *S. pseudopneumoniae* strains from the MLSA database

3.4 | MLST

As shown in Figure 3, thirty-one STs belonging to four CCs were detected in the 81 *S. pneumoniae* isolates, 20 of which were newly identified (named ST-bj01 to ST-bj20) with known alleles. CC-bj12 was the most common CC, being identified in 32.1% of the isolates (26/81). The other three CCs were CC448 (12.3%, 10/81), CC2218 (6.2%, 5/81), and CC10236 (3.7%, 3/81).

Housekeeping genes of the 24 *S. pseudopneumoniae* isolates were also amplified. No ST could be defined for any of the *S. pseudopneumoniae* isolates because at least one sequence of the seven loci did not match the known alleles in the database.

3.5 | Antimicrobial susceptibility test

The antibiotic susceptibility patterns of the present 105 isolates against 14 antimicrobials are presented in Table 2. All of the isolates were susceptible to vancomycin, levofloxacin, and moxifloxacin. The non-susceptibility rates (intermediate and resistant) to amoxicillin/clavulanic acid were 8.6% and 8.4%, respectively, for *S. pneumonia* and *S. pseudopneumoniae*. The resistance rate of *S. pseudopneumoniae* to erythromycin and azithromycin was 95.8%. The resistance rate of *S. pneumoniae* to erythromycin was 74.1% and to azithromycin was 66.7%. The MIC distributions of *S. pseudopneumoniae* and *S. pneumonia* isolates were similar.

4 | DISCUSSION

Our present study showed that MLSA could differentiate *S. pseudopneumoniae* from *S. pneumoniae* well, while the bile solubility test and MALDI-TOF MS could not always completely and accurately distinguish *S. pseudopneumoniae* and *S. pneumoniae*.

S. pseudopneumoniae was first described by Arbique et al.[13] in 2004. Arbique et al.[13] believed that *S. pseudopneumoniae* was resistant or intermediate resistant to optochin when conducting the optochin test in CO_2 atmosphere, and was negative in the bile solubility test. In 2010, Leegaard et al.[20] reported one *S. pseudopneumoniae* isolate with positive result in bile solubility test. A Spanish study examining 61 *S. pseudopneumoniae* strains reported that only 50.8% of the strains exhibited a typical optochin phenotype and 36.1% were bile-soluble.[21] The findings of the above studies, combined with the results of our present study, suggested that the optochin test or the bile solubility test alone is not enough to identify *S. pseudopneumoniae*.

In recent years, MALDI-TOF MS has shown a promising application prospect in the identification of species within the mitis

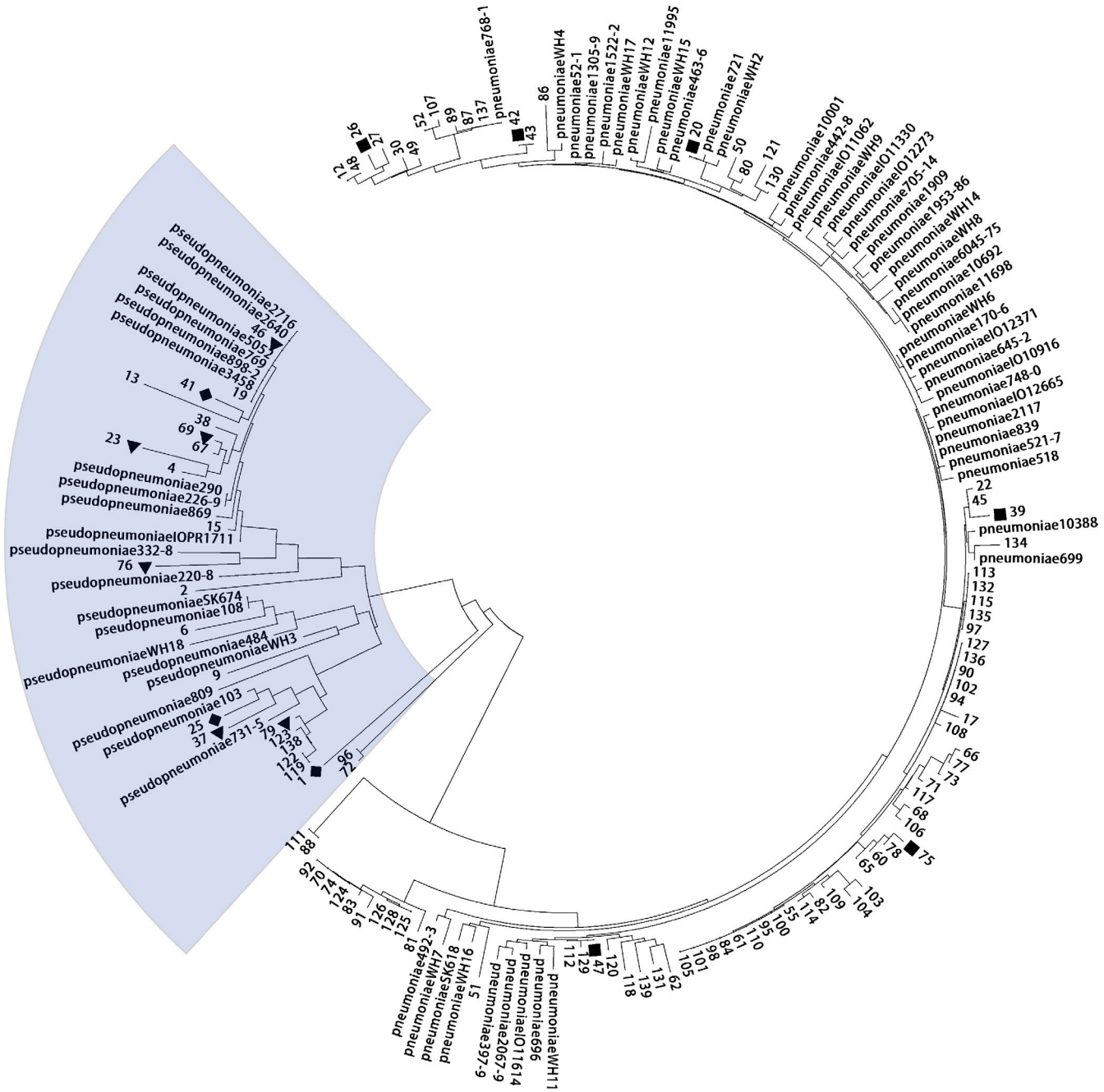

FIGURE 2 Phylogenetic tree of 105 isolates in this study with *Streptococcus pneumoniae* and *S. pseudopneumoniae* strains from the multilocus sequence analysis (MLSA) database. ■ *S. pneumoniae* isolates that were wrongly identified as *S. pseudopneumoniae* by Vitek MS. ▲ *S. pseudopneumoniae* isolates that were wrongly identified as *S. pneumoniae* by Vitek MS. ◆ *S. pseudopneumoniae* isolates that were wrongly identified as *S. mitis*/*S. oralis* by Vitek MS

TABLE 1 Results of the bile solubility test and Vitek MS in this study

	Bile solubility test		Vitek MS		
Species	**Positive**	**Negative**	***S. pneumoniae***	***S. pseudopneumoniae***	***S. mitis*/*S. oralis***
S. pneumoniae ($n = 81$)	81	0	75	6	0
S. pseudopneumoniae ($n = 24$)	5	19	6	15	3

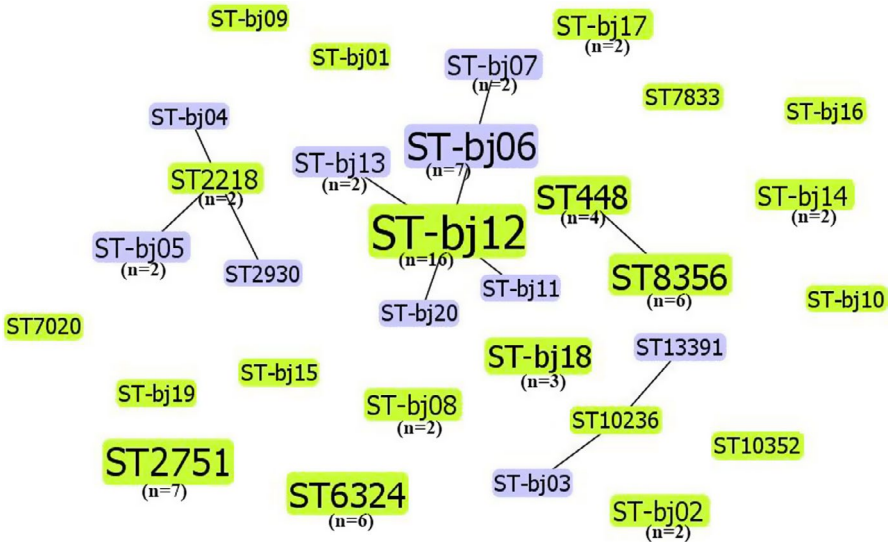

FIGURE 3 Population snapshot of the eBURST analysis for 81 *Streptococcus pneumoniae* isolates. The numbers in parentheses were the corresponding quantities of isolates for different sequence types (STs). STs without parentheses were linked to only one isolate

TABLE 2 Non-susceptibility rates and MIC distribution of the *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* isolates for 14 antimicrobials

	S. pneumoniae (*n* = 81)					*S. pseudopneumoniae* (*n* = 24)				
	Non-susceptibility		MIC (μg/ml)			Non-susceptibility		MIC (μg/ml)		
Antibiotics	I%	R%	MIC50	MIC90	Range	I%	R%	MIC50	MIC90	Range
Penicillin										
Oral	56.8	13.6	0.25	2	≤0.03–>4	54.2	16.6	0.5	2	≤0.03–>4
Meningitis-parenteral	-[a]	70.4	0.25	2	≤0.03–>4	-[a]	70.8	0.5	2	≤0.03–>4
Nonmeningitis-parenteral	3.7	3.7	0.25	2	≤0.03–>4	4.2	4.2	0.5	2	≤0.03–>4
Amoxicillin/clavulanic acid	3.7	4.9	≤2	≤2	≤2–16	4.2	4.2	≤2	≤2	≤2–8
Ceftriaxone										
Meningitis	7.4	9.9	≤0.12	1	≤0.12–>2	8.4	8.4	0.25	1	≤0.12–>2
Nonmeningitis	3.7	6.2	≤0.12	1	≤0.12–>2	0	8.4	0.25	1	≤0.12–>2
Cefotaxime										
Meningitis	4.9	6.2	≤0.12	1	≤0.12–>4	8.4	8.4	0.25	1	≤0.12–>4
Nonmeningitis	2.5	3.7	≤0.12	1	≤0.12–>4	0	8.4	0.25	1	≤0.12–>4
Cefuroxime (parenteral)	8.6	19.8	≤0.5	≥4	≤0.5–>4	8.4	50	1	4	≤0.5–>4
Meropenem	9.9	7.4	≤0.25	0.5	≤0.25–2	20.8	8.4	≤0.25	0.5	≤0.25–2
Erythromycin	3.7	74.1	>2	>2	≤0.25–>2	0	95.8	>2	>2	≤0.25–>2
Azithromycin	8.6	66.7	>2	>2	≤0.25–>2	0	95.8	>2	>2	≤0.25–>2
Chloramphenicol	-[a]	39.5	2	16	≤1–16	-[a]	4.2	2	4	≤1–8
Trimethoprim/sulfamethoxazole	44.4	28.4	2	4	≤0.5–>4	8.4	70.8	>4	>4	≤0.5–>4
Tetracycline	4.9	82.8	>8	>8	≤1–>8	0	91.6	>8	>8	≤1–>8
Vancomycin	-[a]	-[a]	≤0.5	≤0.5	≤0.5	-[a]	-[a]	≤0.5	≤0.5	≤0.5
Levofloxacin	0	0	≤0.5	1	≤0.5–1	0	0	1	1.5	≤0.5–2
Moxifloxacin	0	0	≤1	≤1	≤1	0	0	≤1	≤1	≤1

Abbreviation: MIC, minimum inhibitory concentration.

[a]No breakpoints listed.

group.[22] In the present study, we found that MALDI-TOF MS could not always distinguish *S. pneumoniae* from *S. pseudopneumoniae* accurately. In accordance with our finding, Van Prehn et al.[23] reported one *S. pneumoniae* isolates mistakenly identified as *S. mitis*/*S. oralis* group using the Vitek MS platform. A recent study reported that the Vitek MS only identified 2 of 17 *S. pseudopneumoniae* isolates.[22] The discrepancy may be due to different databases or software versions used for MALDI-TOF MS analysis. Improvement of database entries with multiple spectra of well-characterized species has yielded high identification rates for *Mycobacterium spp*.[24] and *Helicobacter pylori*.[25]

MLSA, a method in prokaryotic taxonomy considering the internal fragments of several genes,[26] is increasingly applied to obtain a higher resolution between species within a genus. The phylogenetic trees of MLSA were based on the concatenated aligned gene sequences and can reflect the accurate relationship of bacterial taxa.[27] Now, MLSA has been successfully used in the identification of *S. anginosus*,[28] *Pseudomonas* spp.,[29] and other species.[30,31] MLSA has also been validated as a tool for the identification of reliable species among VGS,[32] and thus, it served as the standard in the present study. Most of the isolates in this study were identified as *S. pneumoniae* by MLSA. Combined with the negative Quellung reaction result and molecular biological characteristics, these strains were determined as NTSp. Our sequential multiplex PCR results showed that these 81 *S. pneumoniae* isolates did not carry *cpsA* gene fragment. It is important to realize that the regulatory and processing genes *cpsABCD* (also known as *wzg*, *wzh*, *wzd*, and *wze*) are conserved with high sequence identity in all *S. pneumoniae* isolates for the generation of capsular polysaccharides (CPSs).[33] They are common to all pneumococcal serotypes, excluding serotypes 3 and 37, and deletion of any of these genes will affect the production of CPSs.[34] Thus, we considered that the NTSp isolates in our present study may lack the capsule and could be designated as NESp.

NTSp can be divided into two groups based on the capsular genes. Group I strains carry the *cps* locus sequences of conventional capsule types but with disrupted ability to produce CPSs. Group II strains, lacking all of the genes usually found in the *cps* sequences in encapsulated *S. pneumoniae* isolates,[35] can be further divided into different *cps* types (NCC1, NCC2, and NCC3). NCC1 strains carry the *pspK* gene, which encodes a novel pneumococcal surface protein with several features playing a role in cell adhesion and enhanced colonization. NCC2 strains carry both *aliB-like ORF1* and *aliB-like ORF2* genes, which are predicted to encode lipoproteins. NCC3 strains carry *aliB-like ORF2* but not *aliB-like ORF1*, and they were revealed to be not pneumococci.[12] All of the 81 *S. pneumoniae* isolates in our present study were categorized into Group II. Of these isolates, 17 isolates were classified as NCC2, and 46 isolates were classified as NCC1. The remaining 18 isolates failed to generate any production of *PspK*, *aliB-like ORF1*, or *aliB-like ORF2*. A recent study described a Group II isolate with only transposable elements in its *cps* locus and proposed that NCC4 be named for this isolate.[36] The 18 strains in this study may be belonged to NCC4.

The common STs of NTSp found in the present study were different from those described in a similar study from Taiwan, which reported that the three dominant STs were ST1106 (19, 48.7%), ST7494 (6, 15.4%), and ST7502 (2, 5.1%) among 39 NTSp.[37] A study from South Korea reported that the three dominant STs among 26 NTSp strains were ST271 (3, 11%), ST320 (6, 23%), and ST1464 (6, 23%).[38] The variation of ST prevalence among different reports was not only related to the region, but also indicated that the homology of NTSp strains was very different.

Antimicrobial susceptibility data on *S. pseudopneumoniae* are relatively limited, and there are no judgment criteria in the Clinical and Laboratory Standards Institute guidelines.[19] NTSp and *S. pseudopneumoniae* in the present study showed similar non-susceptibility rates to penicillin, amoxicillin/clavulanic acid, ceftriaxone, and cefotaxime as *S. pneumoniae* in our previous study.[39]

There were also some limitations in our study. First, the strains were not collected continuously over time, and there may be differences in molecular epidemiological characteristics and antibiotic susceptibility patterns of strains from different ages. Second, some clinical information on these strains is missing. However, this study is the first to explore the molecular epidemiology and antibiotic susceptibility characteristics of *Streptococcus pneumoniae* strains with negative Quellung reaction test results from children in China. In future study, we will continuously collect more strains and include clinical isolation information of strains as much as possible, so as to fully understand the characteristics of this group of strains.

5 | CONCLUSION

Commonly used methods, including MALDI-TOF MS, could not always accurately differentiate *S. pneumoniae* from *S. pseudopneumoniae*. Most NESp isolates had heterologous genetics as encapsulated *S. pneumoniae*, and the NCC1 and NCC2 types of NESp were first reported in mainland China in this study. *S. pneumoniae* and *S. pseudopneumoniae* have similar antibiotic susceptibility patterns.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Fang Dong, Qingying Meng, Lin Yuan, and Changhui Chen collected the strains. Ju Jia, Fang Dong, and Qingying Meng performed the identification tests. Wei Shi and Lin yuan performed the antibiotic susceptibility test. Ju Jia, Wei Shi, and Kaihu Yao collected data and drafted the article.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the present study are available from the corresponding author Kaihu Yao (email address: jiuhu2655@sina.com) on reasonable request.

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