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CASE SERIES

First Genome-Phenome Characterization of Two Nonencapsulated *Streptococcus pneumoniae* Isolates from Children with LRTI in China

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Abstract: *Streptococcus pneumoniae (S.pn)* is the predominant bacterial pathogen affecting children under 5 years old. Its polysaccharide capsule is the primary virulence factor, enabling the bacteria to evade the immune system and defining the serotypes that current vaccines target. Recently, nonencapsulated *Streptococcus pneumoniae* (NESp) have emerged as significant causes of conjunctivitis, otitis media, and invasive diseases worldwide. This report provides the first integrated genomic and phenotypic analysis of two NESp strains identified in China. The NESp colonies are characterized by their rough texture and small size, and they exhibit a slower growth rate compared to other serotypes. Whole-genome sequencing has identified these NESps strains as belonging to the ST10236 type. Notably, these strains demonstrate multidrug resistance. In comparison to encapsulated strains, NESps possess fewer coding genes related to cell wall biogenesis and basal metabolism. However, they still retain crucial virulence genes including the *pspK* gene. Our findings highlight the clinical significance of NESps and emphasize the need for ongoing surveillance of these "capsule-free" clones in the post-PCV era.

Keywords: nonencapsulated *Streptococcus pneumoniae*, lower respiratory tract infection, multidrug resistance, virulence factors

Introduction

Streptococcus pneumoniae (*S.pn*) has been identified as a Gram-positive opportunistic pathogen that colonizes the nasopharynx and respiratory tract and causes pneumonia, conjunctivitis, otitis media, bacteremia and meningitis.[1] Data from the World Health Organization (WHO) show that pneumonia contributed to 14% of all deaths among children below five years, resulting in 740,180 fatalities in 2019.[2] The incidence and mortality rates of pneumococcal infections in developing countries markedly exceed those in developed ones.[3,4]

The capsular polysaccharide (CPS) can enhance the capacity of *S.pn* to evade host immunity and regulate the colonization and invasion processes.[5] Variations in the composition and structure of CPS form the basis for classifying over 100 distinct serotypes.[6–8] The capsule synthesis is modulated by the capsular polysaccharide synthesis (*cps*) locus, located between the *dexB* and *aliA* genes. The recombination events within the *cps* locus may induce the emergence of NESp strains.[5,9,10] Some of the NESp strains have been reported to be potential causative agents for otitis media, conjunctivitis, pneumoniae, even bacteremia.[11–13]

Due to the absence of a capsular structure, NESp colonies exhibit a "rough" morphology that starkly contrasts with the "smooth" or "mucoid" appearance of encapsulated *S.pn*. Additionally, NESp colonies tend to be smaller than their encapsulated counterparts, complicating their identification. Furthermore, the 23-valent polysaccharide and pneumococcal conjugate vaccines (PCVs), which are designed to target specific polysaccharides that encapsulate the pneumococcus, provide no protection against NESp. This situation presents a significant pathogenic threat to susceptible populations.[13]

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This report presents two pediatric cases of lower respiratory tract disease attributed to NESp infection. Additionally, we analyze the biological characteristics and genomic features of the isolated NESp strains. The aim of this study is to enhance awareness among pediatricians regarding NESp infections and to establish a basis for improved clinical diagnosis and treatment strategies.

Case Presentation

Case 1

A 1-year-old girl was admitted with a primary complaint of cough lasting for 2 days, accompanied by wheezing and shortness of breath that had developed over the past half day. Upon physical examination, bilateral coarse breath sounds were noted, along with widespread moist rales and expiratory wheezing. The major laboratory findings are as follows: a white blood cell (WBC) count of 15.8×10^9/L (with 63.9% neutrophils), a high-sensitivity C-reactive protein (hs-CRP) level of 7.6 mg/L, and a procalcitonin level of 0.19 ng/mL. The nucleic acid tests for *Mycoplasma pneumoniae, Chlamydia* and viruses all returned negative results. Sputum culture revealed predominant growth of NESp. The primary diagnosis of the patient was acute asthmatic bronchitis (ICD code:J21.901). Ceftriaxone was administered clinically for its antibacterial properties, alongside corticosteroids and bronchodilators to alleviate airway spasms. After 5 days of treatment, the patient showed significant improvement and was subsequently discharged from the hospital.

Case 2

A 2-year-old boy, a premature infant born at 28.5 weeks of gestation, was admitted with the chief complaint of recurrent cough accompanied by fever for 2 weeks. On admission, physical examination revealed a body temperature of 38.5°C, coarse breath sounds bilaterally, and a small amount of moist rales in the right lung. Chest X-ray found pneumonia. Chest computed tomography (CT) revealed multiple segmental areas of atelectasis and consolidation in both lungs, suggestive of bronchopulmonary dysplasia complicated by infection. The WBC count was 20.13×10^9/L, with a neutrophil proportion of 86.6% and a nuclear left shift. The hs-CRP level elevated to 10.2 mg/L. Sputum culture revealed predominant growth of NESp. The nucleic acid test for parainfluenza virus returned positive results. The patient was primarily diagnosed with acute bronchopneumonia (ICD code:J18.000). Initially, treatment consisted of ceftriaxone and bronchodilators. Given the patient's close contact with his twin brother, who was infected with influenza, oseltamivir was introduced later during the hospitalization. After 7 days of treatment, the patient was cured and discharged from the hospital.

Materials and Methods

Bacterial Strains

The NESp01 and NESp02 were isolated from two pediatric patients with lower respiratory tract infections in Shenzhen Children's Hospital. The 23F was also a clinical isolate from an inpatient with invasive pneumococcal disease in our previous project.[14] Serotype 23F was selected as a control for the following reasons: first, it is a common, vaccine-type encapsulated serotype known for its high virulence and invasive potential; second, its well-defined clinical background makes it a representative example of invasive *S. pneumoniae*. This comparison allows for an effective highlight of the distinct biological, genomic, and virulence characteristics of the NESp strains.

Sputum Collection

The sputum samples in this study were obtained via phlegm suctioning. In prior to the procedure, patients were instructed to gargle thoroughly with sterile water to avoid oral bacteria. A qualified nurse then introduced a sterile catheter into patient's lower respiratory tract and extracted phlegm using phlegm suctioning device. To evaluate whether sputum meets the validity criteria for bacteriological analysis, the sputum samples were stained by Gram and their containing cells were counted under a microscope. The qualified sputum samples were defined as those containing less than 10 squamous epithelial cells as well as more than 25 polymorphonuclear leukocytes in low-power field. The sputum samples in this study were qualified.

NESp Culture and Identification

Sputum samples were cultured on Columbia blood agar plates and incubated at 35±2°C in a 5% CO_2 atmosphere for 18–24 hours. The solubility test of suspected NESp colonies exhibited positive and the optochin susceptibility test showed sensitive. They were identified as *Streptococcus pneumoniae* by using MALDI-TOF (BioMérieux VITEK MS). Their PCR result by using primers targeting a conserved region within pneumococcal *cps* locus was negative.[15] The serotype of the suspected NESp were NCC1_pspK_non_encapsulated by seroBA.[16] They were named as NESp01 and NESp02, respectively.

In vitro Antimicrobial Susceptibility Testing

Susceptibility to erythromycin, clindamycin, tetracycline, levofloxacin and trimethoprim-sulfamethoxazole was determined by KB disk diffusion method. The MICs of penicillin and ceftriaxone were determined by E-test method. The culture medium were MHA containing 5% sheep blood. The incubation condition was 35±2°C with 5% CO_2 for 20–24 hours. The diameters of the inhibition zones and the MIC values were measured and recorded. All results were interpreted based on the non-meningitis *S. pneumoniae* breakpoints according to CLSI M100 33th. The antibiotic disks are from Oxoid (UK). The E-test strips and MHA are from Autobio (Zhengzhou, China). All the reagents have been tested to be qualified by quality control experiments using *S. pneumoniae* standard strain ATCC49619. The susceptibility tests were performed in triplicate.

Growth Curve Analysis and Statistical Methods

An inoculum for each strain, standardized to a 0.5 McFarland turbidity, was diluted in Todd-Hewitt Broth (THB) and incubated at 37°C with 5% CO_2. At hourly intervals for 16 hours, aliquots were serially diluted and plated on blood agar to determine viable cell counts (CFU/mL). After overnight incubation, colonies were counted, and the data were used to generate growth curves. This entire growth experiment was performed in triplicate. The growth differences were analyzed by comparing peak counts between different serotypes. The statistical method is one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism 9.0.0). The P-value < 0.05 was considered statistically significant.

Whole-Genome Sequencing and Bioinformatic Analysis

Whole-genome sequencing was performed by BGI (Shenzhen, China), using the strategy that combined the DNBSEQ short-read and Oxford Nanopore long-read platforms. Raw data were filtered by SOAPnuke (v1.5.6) and porechop (v0.2.4), respectively. The assembly pipeline involved bacterial de novo assembly of Nanopore long reads by Canu (v1.5), which was subsequently polished and corrected for single-base errors by GATK (v3.4–0). Gene prediction and annotation were performed with tools including Glimmer (v3.02), tRNAscan-SE (v1.3.1), and RNAmmer (v1.2). The functional annotation databases were KEGG, COG, VFDB and AMR. The circular genome maps were illustrated by Proksee.

For comparative genomics, pan-genome analysis was performed with CD-HIT (v4.6.6).

Results

Morphology and Growth Dynamics

Compared to encapsulated *S. pn* serotype 23F (Figure 1A), neither of NESp01 and NESp02 showed capsule structure after Gram staining (Figure 1B and C). The growth dynamics of NESp01, NESp02, and four other *S. pn* serotypes (23F, 19F, 14, and 6B) were monitored to generate growth curves (Figure 2A). The growth curve of the NES serotype exhibits a flatter trend compared to other serotypes. At approximately 11 to 12 hours, the bacterial colonies across all serotypes achieve their peak counts. Notably, the peak count for NESp was significantly lower than that of the other serotypes, specifically 23F, 14, and 6B ($P<0.05$) (Figure 2B).

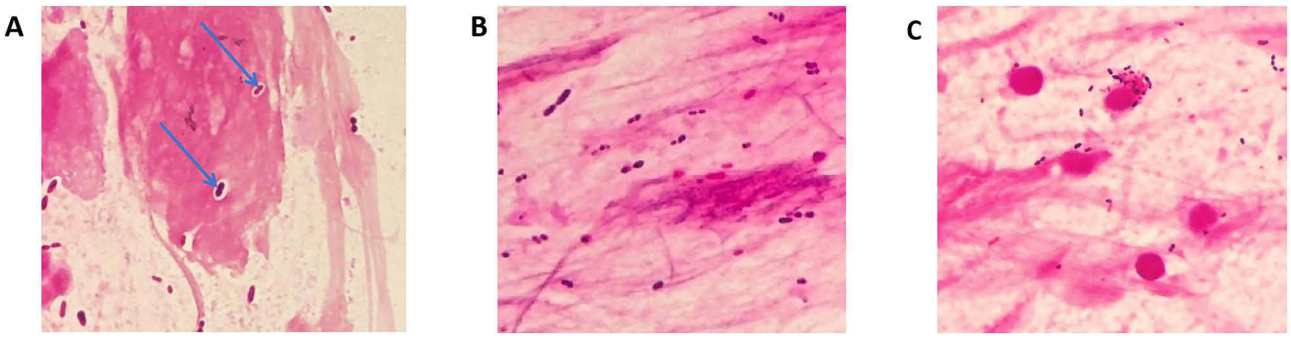

Figure 1 Gram stain morphology of *S.pn* (1000×oil immersion). (**A**) 23F serotype showing unstained capsule (indicated by blue arrow). (**B**) NESp01. (**C**) NESp02.

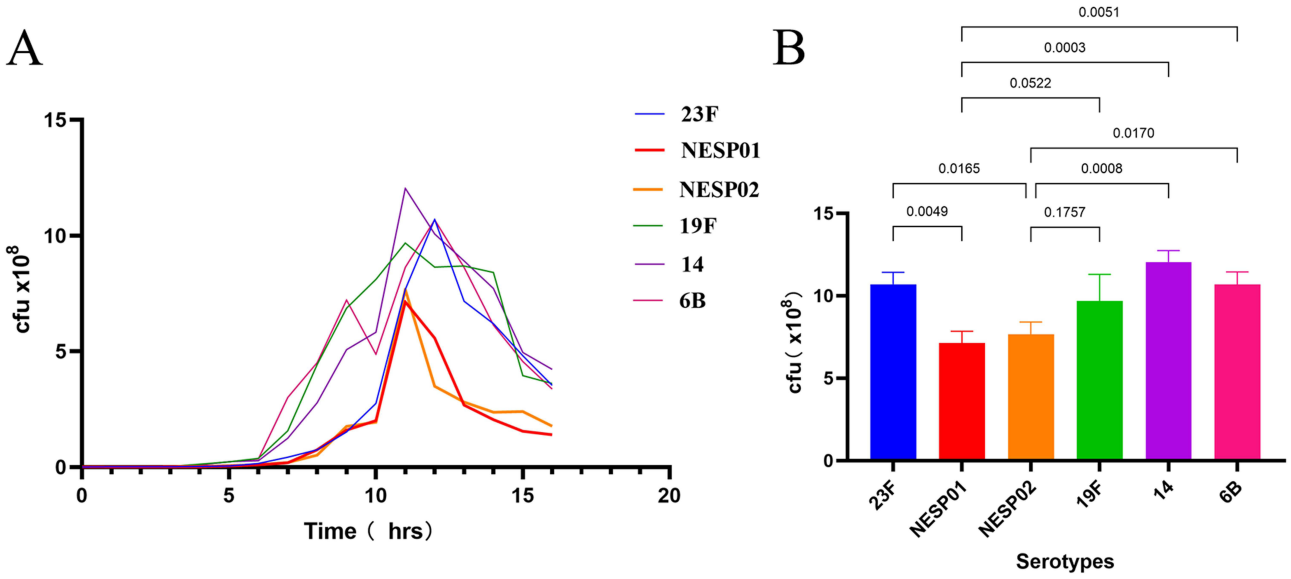

Figure 2 (**A**) In vitro growth curves of NESps and other serotypes. Each serotype is represented by a different color. (**B**) Comparison of growth peaks of different serotypes. The statistical method is one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism 9.0.0). The P-value of one-way ANOVA was 0.0002. The P-value of multiple comparisons test are displayed in bar graph. P< 0.05 is considered statistically significant.

Genomic Features and MLST

The genome of NESp01 is a single chromosome with a total length of 2,131,001 bp, containing 2184 coding sequences (CDS), 12 rRNAs, 59 tRNAs, and 40 sRNAs. The G+C content is 39.70% (Figure 3A). The genome of NESp02 is with a total length of 2,127,172 bp, containing 2194 CDS, 12 rRNAs, 59 tRNAs, and 39 sRNAs. Its genomic G+C content was 39.64% (Figure 3B). Multilocus sequence typing (MLST) revealed that both NESp strains belong to sequence type ST10236. For comparison, a 23F strain (serotype 23F, ST242) was also analyzed. The strain was previously isolated from an inpatient suffering from invasive pneumococcal disease. Its genome comprises a chromosome with 2,192,693 bp, containing 2227 CDS, 12 rRNAs, 58 tRNAs, and 37 sRNAs, with a G+C content of 40.55% (Figure 3C). The number of genes annotated to Clusters of Orthologous Groups (COG) was 1663, 1620, and 1625 for 23F, NESp01, and NESp02, respectively. The COG database categorizes proteins based on their evolutionary relationships across bacteria, algae, and eukaryotes. Each protein sequence is assigned to a specific COG category, consisting of homologous sequences that help infer protein functions. This database is divided into 25 functional categories. Notably, the NESp strains have fewer genes (n=99) associated with the Cell wall/membrane/envelope biogenesis (M) category compared to the 23F strain (n=117) (Figure 3D).

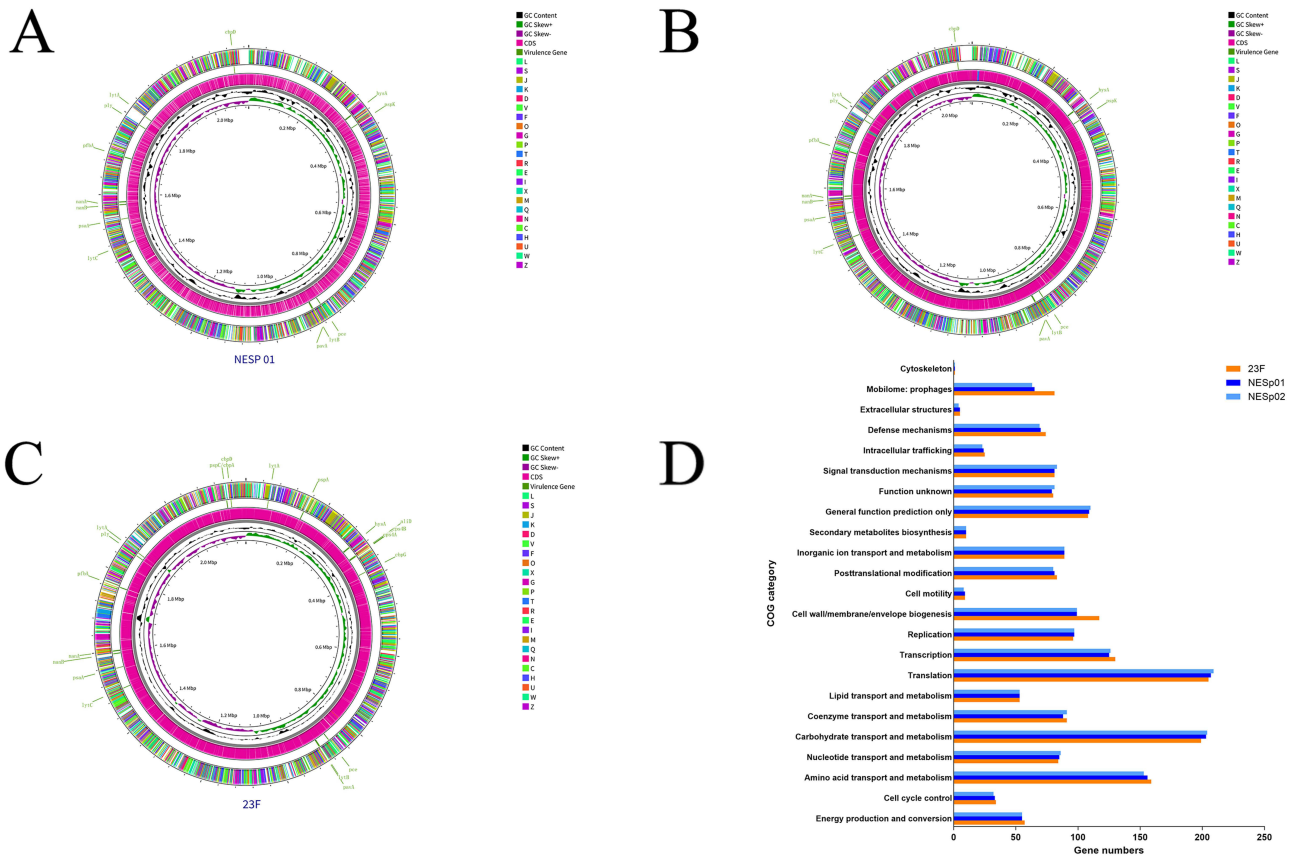

Figure 3 The whole genome circular maps with annotation of CDS, COG clustering and virulence genes. The individual capital letters in the diagram represent COG classes. COG functional categories are grouped into four major groups and subdivided into 25 classes. Information Storage and Processing includes: [J] Translation, ribosomal structure and biogenesis; [A] RNA processing and modification; [K] Transcription; [L] Replication, recombination and repair; and [B] Chromatin structure and dynamics. Cellular Processes and Signaling includes: [D] Cell cycle control, cell division, chromosome partitioning; [M] Cell wall/membrane/envelope biogenesis; [N] Cell motility; [O] Posttranslational modification, protein turnover, chaperones; [T] Signal transduction mechanisms; [U] Intracellular trafficking, secretion, and vesicular transport; [V] Defense mechanisms; [W] Extracellular structures; [Y] Nuclear structure; and [Z] Cytoskeleton. Metabolism includes: [C] Energy production and conversion; [G] Carbohydrate transport and metabolism; [E] Amino acid transport and metabolism; [F] Nucleotide transport and metabolism; [H] Coenzyme transport and metabolism; [I] Lipid transport and metabolism; [P] Inorganic ion transport and metabolism; and [Q] Secondary metabolites biosynthesis, transport and catabolism. The Poorly Characterized categories are: [R] General function prediction only; and [S] Function unknown. (**A**) NESp01 whole genome circular map. (**B**) NESp02 whole genome circular map. (**C**) 23F whole genome circular map. (**D**) COG clustering of encoded genes for the three strains.The description of the matched COG class is on the left side of Y axis.

Virulence Factors

Data presented in Table 1 indicate that the NESp01 and NESp02 possess 12 common virulence genes with 23F including *cbpD, lytA, lytB, lytC, hysA, nanA, nanB, pfbA, pavA, pce, ply* and *psaA*. Notably, the NESp strains obtained specific virulence gene *pspK*. The Pspk protein serves as an adhesin to upper and lower respiratory epithelium and assists NESp to develop invasive diseases.[17,18] Notably, both NESp strains lacked genes that encode major surface proteins, including those for capsular polysaccharide biosynthesis (*cps4A, cps4B*), choline-binding protein G (*cbpG*), pneumococcal surface protein A (*pspA*), and choline-binding protein A (*pspC/cbpA*).

Table 1 Virulence Genes and Virulence Factors of NESp01, NESp02 and 23F

Gene	Virulence Factor	NESp01	NESp02	23F
hysA	Hyaluronidase	+	+	+
nanA	Neuraminidase A	+	+	+
nanB	Neuraminidase B	+	+	+

(*Continued*)

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Table 1 (Continued).

Gene	Virulence Factor	NESp01	NESp02	23F
lytA	Autolysin A	+	+	+
lytB	Autolysin B	+	+	+
lytC	Autolysin C	+	+	+
pce	Phosphocholine Esterase	+	+	+
ply	Pneumolysin	+	+	+
pavA	Pneumococcal Adherence and Virulence factor A	+	+	+
psaA	Pneumococcal Surface Adhesin A	+	+	+
pfbA	Plasmin- and Fibronectin-binding Protein A	+	+	+
cbpD	Choline-Binding Protein D	+	+	+
pspA	Pneumococcal Surface Protein A	-	-	+
pspC/cbpA	Pneumococcal Surface Protein C	-	-	+
pspK	Pneumococcal Surface Protein K	+	+	-
cbpG	Choline-Binding Protein G	-	-	+
cps4A	Capsular Polysaccharide Synthesis protein 4A	-	-	+
cps4B	Capsular Polysaccharide Synthesis protein 4B	-	-	+
aliD	Oligopeptide-binding Lipoprotein D	-	-	+
aliC	Oligopeptide-binding Lipoprotein C	-	-	-

Antimicrobial Susceptibility Test and Antimicrobial Gene Analysis

In vitro antimicrobial test revealed that both NESp strains showed multidrug resistance to erythromycin, clindamycin, tetracycline and Trimethoprim/Sulfamethoxazole. Correspondingly, they both harbored antimicrobial resistance genes as ermB, tetM, and folP_aa_insert_57-70. Both NESp strains exhibit susceptibility to penicillin and ceftriaxone. Li et al developed a classification system that categorizes pneumococcal isolates into distinct "PBP types." This classification is based on the sequence signatures found in the transpeptidase domains (TPDs) of three essential penicillin-binding proteins (PBPs), PBP1a, PBP2b, and PBP2x.[19] Using this PBP typing method, the inferred phenotype of NESp strains is also sensitive to penicillin and cefriaxone. Results from the antimicrobial susceptibility tests and antimicrobial gene analysis are presented in Table 2.

Table 2 Antimicrobial Susceptibility Test and Antimicrobial Gene/PBP Analysis of NESp01, NESp02 and 23F

Strains / Antibiotics	NESp01		NESp02		23F	
	KB Test Result	Predicted Gene	KB Test Result	Predicted Gene	KB Test Result	Predicted Gene
Erythromycin	6/R	ermB	6/R	ermB	10/R	ermB
Clindamycin	6/R	ermB	6/R	ermB	6/R	ermB,mefA-10
Tetracycline	13/R	tetM-12	12/R	tetM-12	18/R	tetM-2

(*Continued*)

Table 2 (Continued).

Levofloxacin	27/S	—	28/S	—	26/S	—
Trimethoprim/ Sulfamethoxazole	6/R	folP_aa_insert_57-70	6/R	folP_aa_insert_57-70, folA_I100L	6/R	folP_aa_insert_57-70, folA_I100L
	E-Test Result	**Predicted MIC**	**E-test Result**	**Predicted MIC**	**E-Test result**	**Predicted MIC**
Penicillin	0.38/S	2/S	0.75/S	2/S	1.5/S	2/S
Ceftriaxone	0.75/S	1/S	0.75/S	1/S	0.75/S	1/S

Notes: Symbol "—" indicates no predicted drug-resistant genes.
Abbreviations: R, antibiotic resistance; S, antibiotic sensitivity.

Comparative Genomics Analysis

Pan-genome analysis of NESp01, NESp02, and 23F identified a core genome consisting of 1763 genes, a pan-genome of 2234 genes, and a dispensable genome comprising 184 genes (Figure 4). To find genomic explanations for phenotypic difference, the specific genes of each strain were clustered according to COG separately. The number of specific genes present in NESp01 and NESp02 is significantly lower than that of 23F across all matched functional classes. This finding suggests that the NES strains exhibit a reduced capacity compared to 23F, particularly in areas such as the bacterial life cycle, cell wall biogenesis, and metabolism (Figure 5).

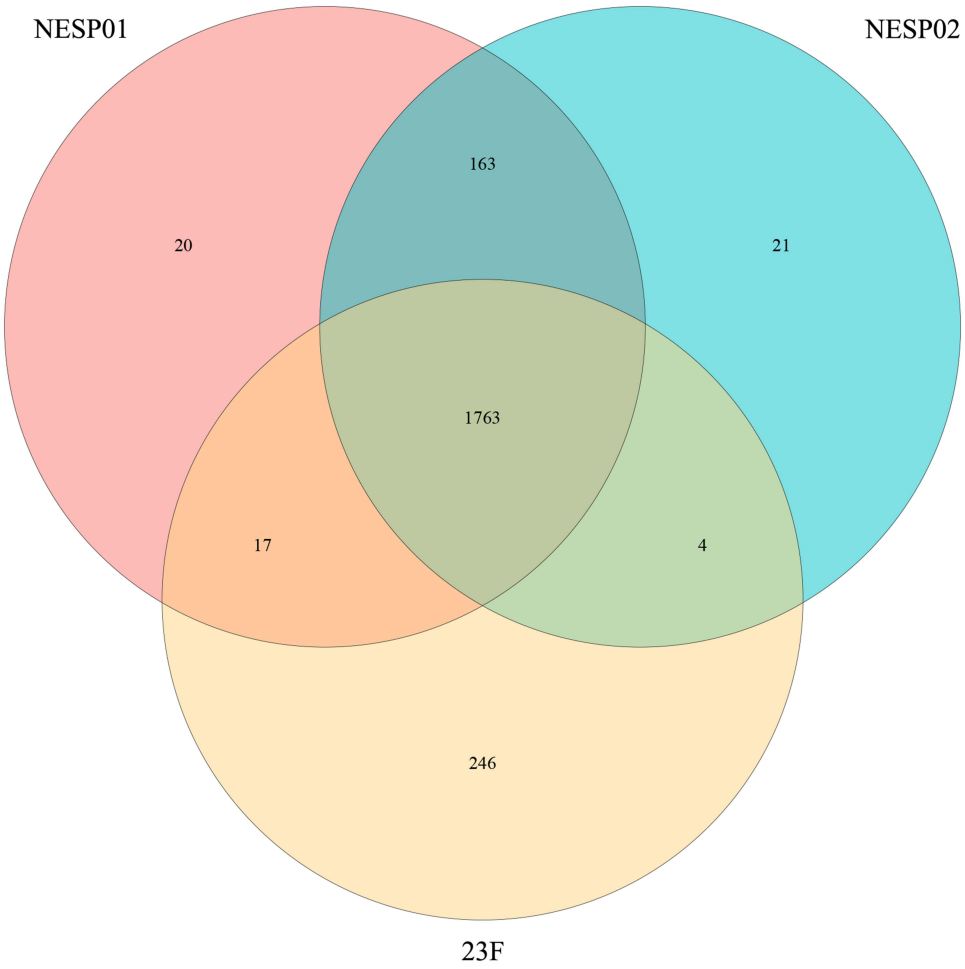

Figure 4 Pan-genome analysis of NESp01, NESp02 and 23F.

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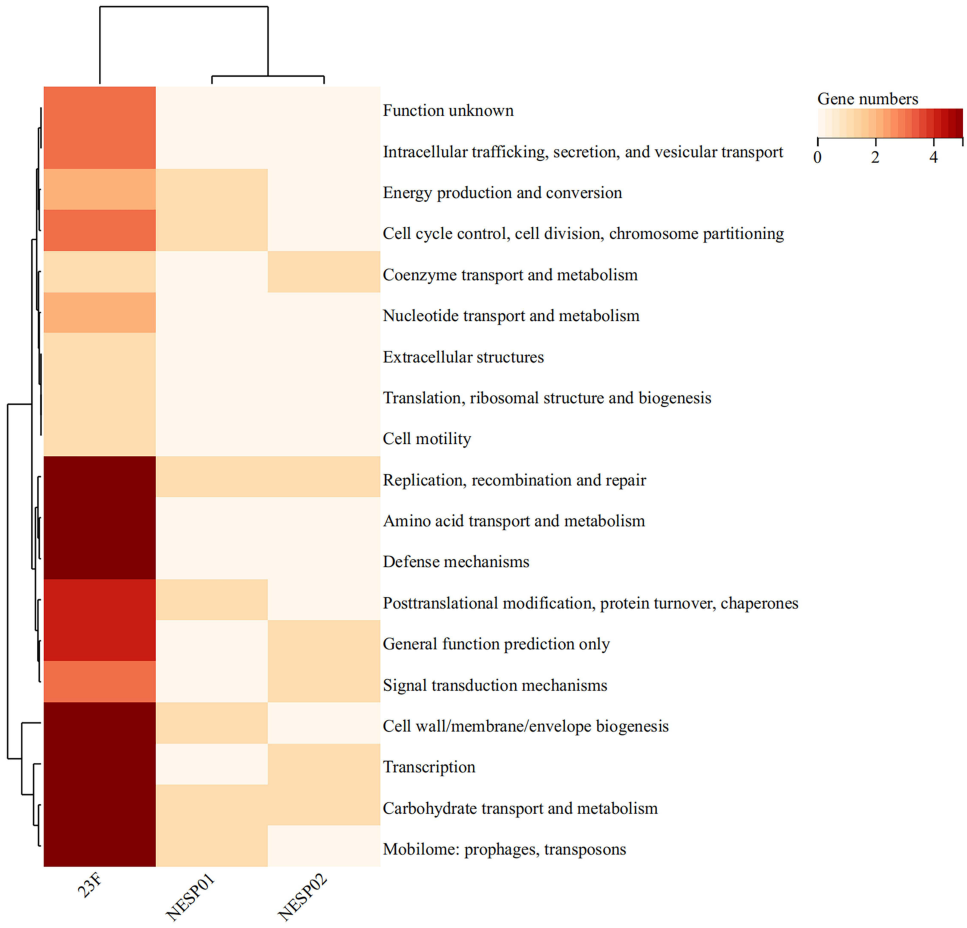

Figure 5 COG clustering of specific genes for NESp01, NESp02 and 23F. Colored blocks represent the number of genes. The description of the matched COG class is on the right side of each column.

Discussion

NESp can be classified into either group I or group II isolates.[13] Group I NESp isolates possess *cps* genes, but they cannot form a capsule due to the presence of genic mutations or deletions. Group II NESp isolates carry a different set of genes as opposed to the *cps* genes and can be subdivided into so-called null capsule clades (NCCs) based on which genes are located within the *cps* locus.[20] The replacing genes are *pspK, aliC* and *aliD*. NCC1 strains are *pspK*-positive, *aliC*-negative, and *aliD*-negative strains. NCC2 strains are *pspK*-negative, *aliC*-positive, and *aliD*-positive strains. To our knowledge, we provide the first genomic-phenomic mechanism for the NCC1 NESp of ST12306.

The cases presented here are linked to the NESp-associated LRTI. This is supported by the following lines of evidence: First, NESp-associated pneumonia has been reported in several epidemiological studies.[11–13] Second, NCC1 NESp can indeed cause lethal pneumonia and bacteremia in intratracheally infected mouse model.[18] Third, Case 1 indicates that NESp02 was the main pathogen. Based on previous studies of coinfection cases,[21,22] NESP02 is probably co-pathogen with parainfluenza virus in Case 2.

Although some surface proteins and CPS proteins were lacking, NESp01 and NESp02 retained genes that encode toxins and virulence-related enzymes (Table 1), Pneumolysin and Pneumococcal Surface Adhesin A are recognized as the key virulence factors of *S. pn*.[23] Pneumolysin can damage host cells and activate inflammatory responses. Pneumococcal Surface Adhesin A contributes to oxidative stress resistance by regulating Mn^2transport. NESp 01 and NESp02 harbored a novel pneumococcal surface protein K, which is a critical for developing invasive diseases.[18]

The analysis showed that the in vitro growth rate of NESp01 and NESp02 was slower relative to that of the encapsulated strains tested. Moreover, the comparative genomics analysis showed that NESp had fewer genes in the

pathway of bacterial life cycle and energy metabolism. The missing genes may explain the observed limited proliferation. However, it is possible that other factors could influence the growth.

According to CDC's survey among children 0–15 months in 9 provinces of China, the urban vaccination coverage of PCV13 in cities is appropriately 50% in 2021.[24] Nonvaccine serotypes and NESp prevalence have increased since the introduction of pneumococcal vaccines.[14,20] Due to their unusual morphology and limited growth, NESps may be overlooked in clinical laboratories, leading to underreporting. This study presents a comprehensive genomic and phenotypic characterization of two clinical NESp strains, which provides essential insights for enhancing diagnostic accuracy. It is crucial for pediatric hospitals to recognize the potential harm posed by NESps and to improve their detection rates by integrating morphological assessment, mass spectrometry identification, and molecular assay.

This report has some limitations including the small sample size, single center scale, and the absence of functional studies. We aim for this study to inspire multicenter surveillance of NESp in China, which should encompass genomic analysis and an assessment of its clinical burden.

Data Sharing Statement

The whole-genome sequencing data generated in this study have been deposited in the NCBI databases under BioProject accession number PRJNA1338348. The assembled genome sequences for *Streptococcus pneumoniae* strains NESP01, NESP02, and 23F are available in GenBank under the accession numbers JBRRGX000000000, JBRRGW000000000, and JBRRGV000000000, respectively. The corresponding raw sequencing reads have been deposited in the Sequence Read Archive (SRA) under the accession numbers SRR35754186 (NESP01), SRR35754185 (NESP02), and SRR35754184 (23F). The BioSample metadata are available under accession numbers SAMN52405016 (NESP01), SAMN52405017 (NESP02), and SAMN52405018 (23F).

Ethical Approval

All procedures performed in studies involving human participants were approved by the ethics committee of Shenzhen Children's hospital (Approval No.: 202209202) and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The informed consent for participation was obtained from parents of participants included in the study. The consent for the publication of the case(s) was also obtained from parents of all participants. The publication of the cases was approved by Shenzhen Children's hospital. Data confidentiality was strictly maintained, and all personal information was anonymized to ensure privacy. The study adhered to rigorous ethical guidelines throughout its duration.

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Disclosure

The authors report no conflicts of interest in this work.

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