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Original Article

Pathogen distribution of severe pneumonia in children and characteristics of Streptococcus pneumoniae biofilm

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Abstract: Objective: To investigate pathogen spectrum in children with severe community-acquired pneumonia (CAP) and the biofilm-formation characteristics of *Streptococcus pneumoniae*. Methods: A retrospective case-control study was performed at the Affiliated Haimen Hospital of Xinglin College, Nantong University. Fifty children with severe CAP (case group) and 50 children with mild CAP (control group) were enrolled. Sputum and blood samples were subjected to bacterial culture and enzyme-linked immunosorbent assay (ELISA). Biofilm optical density at 570 nm ($OD_{570\ nm}$) was detected by microplate reader, and biofilm structure was observed using confocal laser scanning microscopy (CLSM). Fisher's exact test and the Wilcoxon rank-sum test were used for statistical analysis, and cross-tabulation analysis was performed to assess the association between season of onset and pathogen distribution. Results: The pathogen-positive rate was 86.0% in the case group compared with 62.0% in controls ($P < 0.05$). Single bacterial infection predominated among severe cases (42.0%), with *Klebsiella pneumoniae* being the most frequently detected bacterium, whereas respiratory syncytial virus (RSV) was the predominant virus. Severe CAP occurred most frequently in winter (32.0%) and spring (30.0%), with no significant seasonal differences in pathogen distribution ($P > 0.05$). All isolated *S. pneumoniae* strains formed biofilms. Isolates from the case group exhibited markedly higher OD_{570} values than those from the control group (1.31 ± 0.03 vs 0.68 ± 0.10, $P < 0.05$) and showed stronger biofilm-forming capacity. Conclusion: Bacterial infections predominate in severe childhood CAP, with *K. pneumoniae* and RSV being the leading bacterial and viral pathogens, respectively. Severe CAP occurs more frequently in winter and spring. Enhanced biofilm-forming capacity of *S. pneumoniae* correlates with greater disease severity, providing evidence for the clinical diagnosis, treatment, and prevention of severe pediatric CAP.

Keywords: Children, severe pneumonia, pathogenic bacteria, Streptococcus pneumoniae, biofilm

Introduction

Pneumonia is an inflammatory disease of the lung parenchyma and/or interstitium caused by various pathogenic infections. The disease may progress rapidly and manifest as polypnea and dyspnea, sometimes accompanied by altered consciousness, lethargy, dehydration, and loss of appetite [1]. Gram-negative bacilli, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Influenza bacillus*, can cause pneumonia [2]. When host immunity is compromised, individuals become more susceptible to pathogenic infections. For example, oropharyngeal fungi and anaerobic bacteria may cause pneumonia under certain conditions. Moreover, the extensive use of antibiotics has contributed to the emergence of antimicrobial resistance among pathogenic microorganisms [3]. Pneumonia is a common disease in childhood and remains a major cause of death among children under 5 years of age. In developing countries, more than 25% of children are diagnosed with pneumonia every year, and the associated mortality remains high, imposing a substantial burden on families and society [4]. In China, childhood pneumonia is more common in winter and spring. It is mainly caused by pathogen infections, inhalation of amniotic fluid and oily substances, or allergic reactions. The main symptoms include fever, cough, polypnea, and dyspnea. Common bacterial pathogens include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphyloco-*

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ccus aureus, and *Pseudomonas aeruginosa*, whereas viral pathogens include adenovirus, influenza virus, respiratory syncytial virus, and the measles virus. *Mycoplasma pneumonia* and *Chlamydial pneumonia* are also important causes of respiratory tract infections [5]. Severe pneumonia (SP) is an important condition threatening the health of children in China and has attracted considerable attention. Therefore, accurate diagnosis and effective treatment of SP in children remain important priorities in pediatric practice.

According to the setting in which the infection is acquired, pneumonia can generally be classified as hospital-acquired pneumonia (HAP) or community-acquired pneumonia (CAP) [6]. CAP refers to pneumonia acquired outside the hospital or diagnosed within 48 hours after admission. In contrast, HAP develops during hospitalization and may be associated with healthcare-related risk factors and invasive procedures, such as tracheal intubation and central venous catheterization. Appropriate infection-control measures, including isolation when indicated and strict adherence to aseptic techniques, are essential for preventing nosocomial transmission [7]. SP manifests with respiratory symptoms and signs, including dyspnea, tachypnea, cough, pulmonary rales, and gastrointestinal manifestations, such as nausea, vomiting, and loss of appetite, or involvement of other organs. SP is associated with a high risk of mortality and substantial medical costs, imposing a great burden on patients and their families [8]. The pathogen spectrum of SP varies across geographic regions and seasons. With continuous advances in diagnostic and therapeutic technologies and the widespread use of antibiotics, antimicrobial resistance among pathogenic bacteria has increased, potentially altering the pathogen spectrum of the disease. Previous studies have indicated that the pathogens responsible for SP in children vary according to the region, age, and season of onset [9]. *Streptococcus pneumoniae* is one of the major bacterial pathogens causing CAP. Previous studies investigating the mechanisms underlying resistance in *S. pneumoniae* have suggested that biofilm formation is closely related to antimicrobial resistance and recurrent *S. pneumoniae*.

Based on the above considerations, this study aimed to investigate the pathogen spectrum of SP in children and the biofilm-forming characteristics of *S. pneumoniae*. Sputum and blood samples from the enrolled children were cultured and tested using ELISA (Enzyme-linked immunosorbent assay). Meanwhile, confocal laser scanning microscopy (CLSM) was used to evaluate biofilm formation and structure. The findings of this study may provide a reference for the diagnosis and treatment of SP in children.

Materials and methods

Sample size calculation

This sample size was calculated using the formula for case-control studies. The significance level was set at $\alpha = 0.05$, and the statistical power was set at $1-\beta = 0.80$. Based on previous literature on pathogens associated with severe pneumonia in children [10], the estimated pathogen detection rates were 60% in children with mild pneumonia and 85% in children with severe pneumonia, with a between-group difference of 25%. A one-sided test indicated that a minimum of 46 participants per group was required. To account for potential sample loss due to missing data, the target sample size was increased to 50 cases per group, resulting in a total of 100 pediatric patients, which satisfied the requirements for statistical analysis.

Research subjects

This retrospective case-control study included children with CAP who were treated at the Affiliated Haimen Hospital of Xinglin College, Nantong University between 2023 and 2025. Patients in the case group were hospitalized for diagnosis and treatment. They were aged 1-9 years, with a mean age of 3.4 years. The case group included 28 females (56%) and 22 males (44%), corresponding to a male-to-female ratio of 11:14. The control group comprised 50 children with mild CAP treated at the same hospital during the same period. They were aged 1-9 years, with a mean age of 3.2 years, including 26 males and 24 females. This study was approved by the ethics committee of the Affiliated Haimen Hospital of Xinglin College, Nantong University. The graphic abstract of this article is shown in **Figure 1**.

Inclusion criteria: ① Diagnosis of CAP; ② Age range of 28 days to 9 years (infants aged 28 days to 12 months, and preschool- and school-

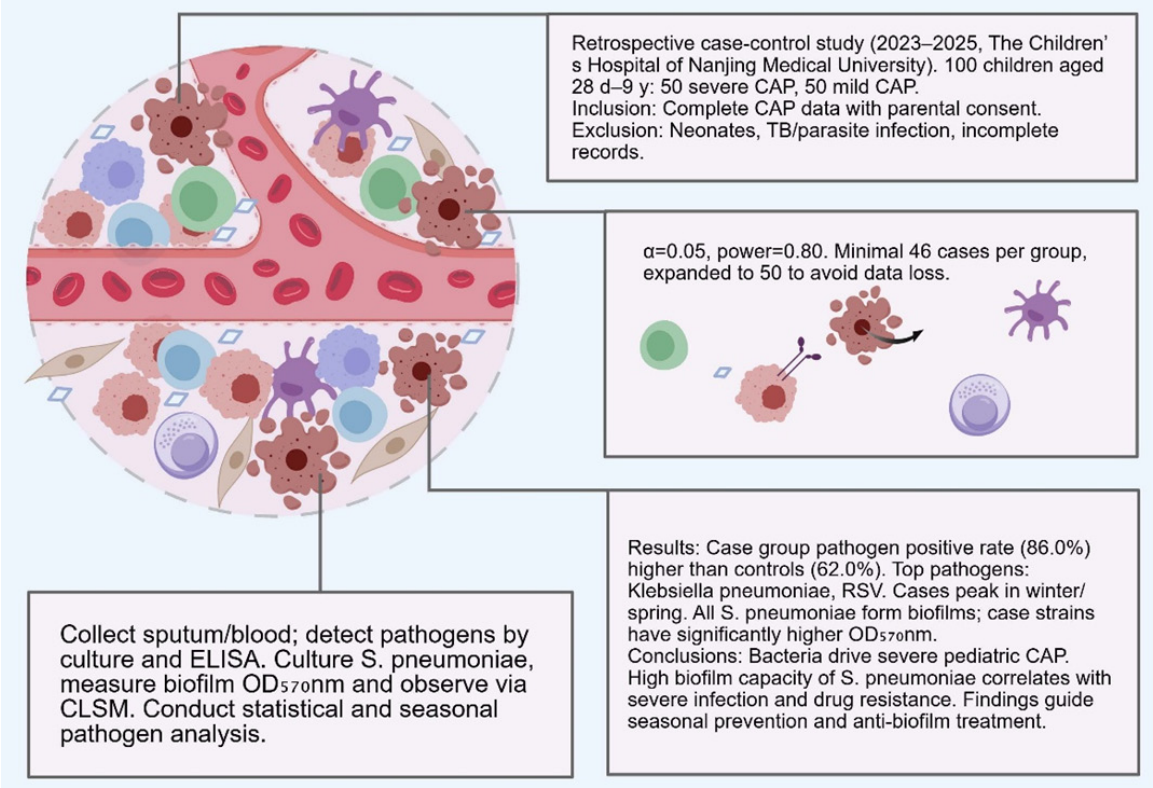


Figure 1. Graphic abstract.

aged children aged 1-9 years); ③ Fulfillment of the diagnostic criteria for severe pediatric pneumonia for the case group or mild pediatric pneumonia for the control group; ④ Availability of complete and traceable medical records, laboratory test results, and imaging data.

Exclusion criteria: ① Neonates aged < 28 days; ② Concurrent tuberculosis or parasitic infections; ③ Missing key data, such as medical records, sputum/blood pathogen test results, or biofilm-related test results, precluding statistical analysis.

Diagnostic criteria for severe pneumonia in children

For infants, severe pneumonia was characterized by a respiratory rate ≥ 70 breaths/min at rest, axillary temperature ≥ 38.5°C, inspiratory chest indrawing, nasal flaring, and cyanosis of the lips. For older children, the diagnostic criteria were similar to those for infants, with a respiratory rate ≥ 50 breaths/min at rest and varying degrees of dehydration.

Data on previous hospitalization were collected, including a history of respiratory-related hospitalization within 1 year before admission and possible exposure to nosocomial infection before the current episode. Previous hospitalization and related healthcare exposure were considered potential confounding factors in the subsequent analyses.

Sample collection

Blood sample collection: Blood samples were collected on the first day of admission before the administration of antibiotics.

Sputum sample collection: Respiratory secretions were collected using a disposable sterile suction catheter and transferred into sterile sputum collection containers. The samples were subjected to microbiological examination within 1 h of collection.

Pathogenic detection: For bacterial culture, the collected samples were inoculated onto appropriate culture media, including chocolate agar,

potato dextrose agar, eosin methylene blue agar, and blood agar, and incubated at 35°C.

Serum virus antibody detection: ELISA was used to detect serum IgM antibodies against respiratory syncytial virus (RSV), influenza B virus (IFB), parainfluenza virus (PIV), and adenovirus (ADV).

Biofilm culture and detection

Colonies were transferred from blood agar plate into sterile tubes containing Todd-Hewitt broth (THB) using a sterile inoculating loop, and the bacterial suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. Then, 7 strains were inoculated on a 96-well plate, with sterile THB without bacteria serving as the blank control. The plates were incubated at 35°C for 24 hours in an atmosphere containing 5% CO_2. After incubation, the wells were washed three times with PBS (phosphate-buffered saline) and dried for 1 hour at 60°C. Subsequently, the biofilms were stained with 1% crystal violet for 15 minutes. After removal of excess crystal violet, the wells were washed, and the bound crystal violet was solubilized with 95% ethanol. Finally, the OD (Optical density) was measured at 570 nm using a microplate reader. Each measurement was repeated 3 times, and the mean value was used for subsequent analysis.

CLSM

In this experiment, FITC-ConA and PI double staining was used. FITC-ConA binds to polysaccharides in the biofilm matrix and emits green fluorescence, whereas PI binds to bacterial DNA and emits red fluorescence. FITC-ConA was diluted with normal saline to a concentration of 5 ug/mL, and PI was diluted with purified water to a concentration of 100 ug/mL. One strain was selected from each of the four groups for CLSM analysis. The selected strains were inoculated onto Columbia blood agar plates and cultured at 35°C in an atmosphere containing 5% CO_2 for 20 hours. An inoculating loop was used to transfer the colonies on the blood agar plate into sterile tubes containing THB culture medium, and the bacterial suspensions were adjusted to a turbidity equivalent to a 0.5 McFarland standard. Sterile coverslips were placed in 24-well plates, and 2 mL of bacteria suspension in THB was added to each well. The samples were incubated at 35°C in 5% CO_2 for 24 hours. After incubation, the coverslips were gently rinsed with PBS and fixed with 2.5% glutaraldehyde for 1.5 h. After further washing with PBS, the coverslips were sequentially stained with FITC-ConA at 4°C for 30 minutes and PI at 4°C for 15 minutes. Next, the coverslips were rinsed three times with PBS for 5 min each and mounted with 40% glycerol-PBS. Biofilm structure was subsequently observed using CLSM.

Pathogen-season cross-analysis

Based on the pathogen detection results and the season of disease onset, season-pathogen cross-tabulations were constructed. The detection rates and proportions of bacteria, viruses, fungi, and *Mycoplasma* in spring, summer, autumn, and winter were calculated to assess the association between season and pathogen distribution.

Grading and visualization of Streptococcus pneumoniae biofilms

The biofilm-forming capacity of *S. pneumoniae* was graded according to $OD_{570\ nm}$ values as follows: (1) Weak biofilm formation: $OD_{570\ nm} \leq 0.8$; (2) Moderate biofilm formation: $0.8 < OD_{570\ nm} \leq 1.2$; (3) Strong biofilm formation: $OD_{570\ nm} > 1.2$. The number of isolates in each biofilm grade was counted for both groups, and differences in the ordinal distribution of biofilm-formation ability between groups were evaluated using the Wilcoxon rank-sum test.

Statistical analysis

SPSS 26.0 software was used for statistical analyses. Continuous variables are presented as mean ± standard deviation (SD). Normally distributed continuous variables with homogeneous variances were compared using the independent-samples t-test, whereas continuous variables non-normal or uncertain distributions were analyzed using the Mann-Whitney *U* test. Categorical variables are expressed as numbers and percentages. Intergroup comparisons of categorical variables were performed using the Pearson chi-square test or Fisher's exact test as appropriate. A two-sided *P* value < 0.05 was considered statistically significant.

Table 1. Comparison of baseline characteristics between the two groups

Parameters	Case group (n = 50)	Control group (n = 50)	χ^2	*P* value
Age group			0.216	0.832
1-3 years	27 (54.0)	25 (50.0)		
4-6 years	16 (32.0)	17 (34.0)		
7-9 years	7 (14.0)	8 (16.0)		
Underlying disease			0.510	0.774
Congenital heart disease	8 (16.0)	6 (12.0)		
Bronchial asthma	10 (20.0)	9 (18.0)		
Malnutrition	5 (10.0)	4 (8.0)		
Immunocompromised	4 (8.0)	3 (6.0)		
No underlying disease	23 (46.0)	28 (56.0)		
Complication			21.364	< 0.001
Respiratory failure	12 (24.0)	0 (0.0)		
Heart failure	7 (14.0)	0 (0.0)		
Electrolyte imbalance	9 (18.0)	2 (4.0)		
Sepsis	5 (10.0)	0 (0.0)		
No complications	17 (34.0)	48 (96.0)		

Table 2. Comparison of pathogen detection rate between the two groups

Pathogen type	Case group (n = 50) n (%)	Control group (n = 50) n (%)	χ^2	*P* value
Bacterial infection alone	21 (42.0)	13 (26.0)	4.889	0.027
Viral infection alone	18 (36.0)	15 (30.0)	0.513	0.474
Fungal infection alone	1 (2.0)	1 (2.0)	0.000	1.000
Mycoplasma infection alone	2 (4.0)	2 (4.0)	0.000	1.000
Mixed infection	1 (2.0)	0 (0.0)	2.041	0.153
Total positive rate	43 (86.0)	31 (62.0)	7.683	0.006

Results

Baseline characteristics and pathogen distribution in children with severe pneumonia

As shown in **Table 1**, the overall chi-square tests showed no significant differences between groups in age distribution (χ^2 = 0.216, *P* = 0.832) or the distribution of underlying diseases (χ^2 = 0.510, *P* = 0.774). In contrast, the overall distribution of complications differed significantly between the two groups (χ^2 = 21.364, *P* < 0.001).

As shown in **Table 2**, among the 50 children with mild pneumonia in the control group, 31 tested positive for pathogens, with an overall pathogen detection rate of 62.0%. Among these, there were 13 cases (26.0%) of bacterial infection alone, 15 cases (30.0%) of viral infection alone, 1 case (2.0%) of fungal infection alone, and 2 cases (4.0%) of *Mycoplasma* infection alone; no mixed infections were detected. The overall pathogen detection rate in the case group (86.0%) was significantly higher than that in the control group (χ^2 = 7.683, *P* = 0.006).

Bacterial detection results in children with severe pneumonia

Of the 43 pathogen-positive children in the case group, bacterial infection alone was detected in 21 cases (48.8%). A total of 21 bacterial isolates representing five species were identified: *Klebsiella pneumoniae* (6 isolates), *Pseudomonas aeruginosa* (5 isolates), *Streptococcus pneumoniae* (4 isolates), *Escherichia coli* (4 isolates), and Staphylococcus aureus (2 isolates). Of these, 15 isolates were Gram-negative bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*), and the remaining 6 strains were Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*).

In the control group, among the 13 children with bacterial infection alone, *S. pneumoniae* was detected in 5 cases, *K. pneumoniae* was detected in 3 cases, *E. coli* was detected in 2 cases, *S. aureus* in 2 cases, and *P. aeruginosa* in 1 case. Gram-positive bacteria were predominant in the control group. The detection rates of *K. pneumoniae* and *P. aeruginosa* were significantly lower in the control group than in the case group (*P* < 0.05) (**Table 3**).

Virus detection results in children with severe pneumonia

Of the 43 pathogen-positive children in the case group, 18 cases had viral infection alone

Table 3. Comparison of bacterial pathogen distribution between the two groups

Pathogen species	Case group with bacterial infection (n = 21)		Control group with bacterial infection (n = 13)		χ^2 value	*P* value
	Number of cases	Composition ratio (%)	Number of cases	Composition ratio (%)		
Klebsiella pneumoniae	6	28.6	3	23.1	0.121	0.728
Pseudomonas aeruginosa	5	23.8	1	7.7	1.317	0.251
Escherichia coli	4	19	2	15.4	0.061	0.805
Streptococcus pneumoniae	4	19	5	38.5	1.317	0.251
Staphylococcus aureus	2	9.5	2	15.4	0.241	0.623
Total	21	100	13	100	-	-

Table 4. Comparison of viral pathogen distribution between the two groups

Virus type	Case group with viral infection (n = 18)		Control group with viral infection (n = 15)		χ^2 value	*P* value
	Number of cases	Composition ratio (%)	Number of cases	Composition ratio (%)		
Respiratory syncytial virus	7	38.9	6	40.0	0.004	0.951
Adenovirus	6	33.3	5	33.3	0.000	1.000
Influenza B virus	3	16.7	3	20.0	0.059	0.808
Parainfluenza virus	2	11.1	1	6.7	0.171	0.679
Total	18	100	15	100	-	-

Table 5. Comparison of the seasonal distribution of disease onset between the two groups

Season of onset	Case group (n = 50)		Control group (n = 50)		χ^2 value	*P* value
	Number of cases	Proportion (%)	Number of cases	Proportion (%)		
Spring	15	30	13	26	0.204	0.651
Summer	10	20	11	22	0.051	0.821
Autumn	9	18	10	20	0.052	0.819
Winter	16	32	16	32	0.000	1.000
Total	50	100	50	100	-	-

(41.9%). Of these 18 cases, respiratory syncytial virus (RSV) was detected in 7 (38.9%), and adenovirus (ADV) in 6 (33.3%), influenza B virus in 3 (16.7%), and parainfluenza virus (PIV) in 2 (11.1%). Among the 15 children with viral infection alone in the control group, RSV was detected in 6 cases, ADV in 5, IBV in 3, and PIV in 1. There was no significant difference in the distribution of the major viral pathogens between the two groups ($P > 0.05$) (**Table 4**).

Seasonal distribution characteristics of children with severe pneumonia

Of the 50 children with severe pneumonia, disease onset occurred in spring in 15 cases (30.0%), summer in 10 cases (20.0%), autumn in 9 cases (18.0%), and winter in 16 cases (32.0%). Among the 50 children in the control group, 13 cases occurred in spring, 11 in summer, 10 in autumn, and 16 in winter. The two groups showed similar seasonal distribution patterns ($P > 0.05$), with a greater proportion of cases occurring in winter and spring (**Table 5**).

Semi-quantitative assessment of Streptococcus pneumoniae biofilm formation

Four *S. pneumoniae* isolates were obtained from children with SP in the case group, whereas three isolates were obtained from children in the control group. Biofilm-forming ability was

Table 6. Comparison of OD570 values of Streptococcus pneumoniae biofilm among groups

Group	Number of strains	$OD_{570\ nm}$	Comparison with blank control group (U/P)	Comparison with case group (U/P)
Case group	4	1.31 ± 0.03	U = 0, P < 0.001	-
Control group	3	0.68 ± 0.10	U = 0, P < 0.01	U = 0, P < 0.05
Blank control	-	0.12 ± 0.03	-	-

Note: $OD_{570\ nm}$ = Optical density value at 570 nm.

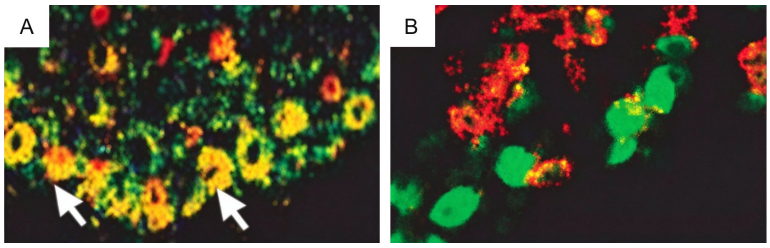


Figure 2. CLSM visualization of *Streptococcus pneumoniae* biofilm formation and polysaccharide matrix. A. *Streptococcus pneumoniae* biofilm; B. Polysaccharide matrix. Note: CLSM, Confocal laser scanning microscopy.

assessed by measuring $OD_{570\ nm}$ values. The mean $OD_{570\ nm}$ value of the 4 isolates from the case group was 1.31 ± 0.03 (individual isolates: 1.32 ± 0.15, 1.28 ± 0.11, 1.35 ± 0.13, 1.29 ± 0.14), and the mean value in the control group was 0.68 ± 0.10 (individual isolates: 0.65 ± 0.09, 0.72 ± 0.11, 0.67 ± 0.08). The OD_{570} value of the blank control containing culture medium without bacteria was 0.12 ± 0.03. The Mann-Whitney *U* testing indicated that OD_{570} values were significantly higher in isolates from the case group than in those from the blank control group (U = 0, $P < 0.001$) as well as the control group (U = 0, $P < 0.05$) (**Table 6**).

CLSM

CLSM observations showed that *S. pneumoniae* had formed an initial biofilm after 24 h of culture, with both polysaccharide matrix and bacterial DNA clearly visualized (**Figure 2**). The polysaccharide matrix emitted green fluorescence following FITC-ConA staining, whereas bacterial DNA exhibited red fluorescence following PI staining. Colocalization between the green and red fluorescence appeared yellow, indicating the spatial overlap of the polysaccharide matrix and bacterial cells within the developing biofilm.

Distribution of pathogen infections according to season of onset

A total of 100 children were included in this study, including 28 cases with disease onset in spring, 21 in summer, 19 in autumn, and 32 in winter. The detection rates of bacterial infection were relatively high in winter and spring, at 53.1% and 50.0%, respectively. The proportion of viral infections was highest in summer, reaching 50.0%. Fisher's exact test showed no statistically significant difference in pathogen distribution across seasons ($P > 0.05$). The detection rates of fungal and *Mycoplasma* infections were relatively higher in winter and spring than in summer and autumn (**Table 7**).

Analysis of the biofilm-forming capacity of Streptococcus pneumoniae

The biofilm-forming capacity of *S. pneumoniae* was graded according to $OD_{570\ nm}$ values. As shown in **Table 8**, all four isolates from the case group were classified as having strong biofilm-forming ability, whereas all three isolates from the control group were classified as having weak biofilm-forming capacity. The Wilcoxon rank-sum test showed a statistically significant difference in biofilm formation grades between the two groups (Z = -2.418, $P = 0.016$).

Discussion

The findings of this study showed that the overall pathogen positivity rate in the severe pneumonia group was 86.0%, and bacterial infection alone accounted for 42.0% of cases; both proportions were significantly higher than those in the mild control group (62.0% and 26.0%, respectively). These findings suggest that bacterial infection may be associated with greater

Table 7. Distribution of pathogen infections according to season of onset

Pathogen Type	Statistical Indicator	Spring (n = 28)	Summer (n = 21)	Autumn (n = 19)	Winter (n = 32)	*P* value
Bacterial infection	Detection rate (%)	50.00 (14/28)	33.30 (7/21)	31.60 (6/19)	53.10 (17/32)	0.792
	Constituent ratio (%)	44	38.90	40	47.200	-
Viral infection	Detection rate (%)	39.30 (11/28)	42.90 (9/21)	36.80 (7/19)	37.50 (12/32)	0.615
	Constituent ratio (%)	34	50.00	46.700	33.300	-
Fungal/Mycoplasma	Detection rate (%)	25.00 (7/28)	9.50 (2/21)	10.50 (2/19)	21.90 (7/32)	0.678
	Constituent ratio (%)	22	11	13	19	-

Note: Detection rate = number of cases detected by single pathogen examination/total number of cases in the current season; Composition ratio = number of detected cases of single pathogen/total number of positive cases of pathogen in the current season.

Table 8. Biofilm-forming capacity of *Streptococcus pneumoniae*

	Isolation counts	Weak biofilm-forming capacity ($OD_{570\ nm} \leq 0.8$)	Medium biofilm-forming capacity ($0.8 < OD_{570\ nm} \leq 1.2$)	Strong biofilm-forming capacity ($OD_{570\ nm} > 1.2$)	Z value	*P* value
Case group	4	0	0	4	-2.418	0.016
Control group	3	3	0	0		

Note: OD_{570} = Optical density value at 570 nm.

disease severity in children with pneumonia. After bacterial invasion, an excessive systemic inflammatory response may occur, causing extensive lung tissue damage, and in severe cases, multiple organ dysfunction [11]. In infants and young children, the airways are relatively narrow, the lung parenchyma is still developing, and cough reflexes and immune function are not yet fully mature, which may increase susceptibility to lower respiratory tract infection. Additionally, children with severe pneumonia may exhibit immune dysfunction, and the sustained release of pro-inflammatory cytokines can further aggravate pulmonary injury, contributing to a vicious cycle of inflammation and tissue damage [12]. Therefore, the relatively high frequency of bacterial infection observed in severe pneumonia may provide useful information for disease severity assessment.

Regarding bacterial pathogen spectrum, differences were observed between the Gram-negative and Gram-positive groups: Gram-negative opportunistic bacteria, particularly *K. pneumoniae* and P. *aeruginosa*, were more frequently detected in children with severe pneumonia, while Gram-positive bacteria like *S. pneumoniae*. was more common in children with mild pneumonia. *K. pneumoniae* and *P. aeruginosa* are opportunistic pathogens that can be encountered in community and hospital environments, possessing multiple virulence mechanisms, including strong adhesion, invasion, toxin production, and immune evasion. Their virulence factors may contribute to alveolar and vascular endothelial injury, inducing pulmonary edema, consolidation, and even necrotizing lung injury [13, 14].

This is a retrospective hospital-based study, and some children had a history of hospitalization for respiratory diseases before admission. Previous healthcare visit may increase the likelihood of exposure to healthcare-associated and antimicrobial-resistant Gram-negative organisms, including *K. pneumoniae* and *P. aeruginosa* [15], therefore contributing, at least in part, to the pathogen distribution observed in children with severe pneumonia. Clinicians should carefully inquire about previous hospitalization during consultation. In patients with relevant risk factors, the possibility of infection with resistant Gram-negative pathogens should be considered when selecting empirical antimicrobial therapy.

Compared with the differences observed in the bacterial pathogen spectrum, the distribution of viral pathogens did not differ significantly between the two groups. RSV accounted for 38.9% of viral infections in the severe group and 40.0% in the mild group, while the proportions of adenovirus, influenza B virus, and para-

influenza virus were also similar between the groups. These findings indicate that the distribution of the major viral pathogens identified in this study was not significantly associated with pneumonia severity. A phase III clinical trial evaluating the durability of protection provided by an RSV vaccine showed that the RSVPreF3 OA vaccine reduced the risk of RSV-associated lower respiratory tract disease in older adults over three consecutive epidemic seasons, although vaccine efficacy declined over time [16]. Although these findings were obtained in an older adult population and cannot be directly extrapolated to children, they highlight the substantial clinical burden of RSV infection and the potential value of effective preventive strategies. In children, RSV infection can damage the respiratory epithelium and impair local host defenses, which may facilitate bacterial adherence, colonization, and subsequent bacterial infection. Subsequent viral-bacterial interactions may therefore contribute to rapid disease deterioration in some children. Therefore, during periods of high viral activity, children with viral pneumonia should be closely monitored for clinical or laboratory evidence of secondary bacterial infection, particularly when symptoms worsen or fail to improve as expected.

To further analyze the association between season and pathogen epidemiological characteristics, a season-pathogen cross-analysis was performed. The results showed that bacterial detection rates were relatively high in winter and spring, reaching 53.1% and 50.0%, respectively, whereas the proportion of viral infections was highest in summer at 50.0%. Fungal and *Mycoplasma* infections were also frequently detected in winter and spring. However, the overall distribution of pathogens did not differ significantly across seasons, suggesting that no clear seasonal variation in the overall pathogen composition was identified in this cohort. Low temperature and dry air in winter and spring may impair respiratory mucosal defense, while indoor crowding and reduced ventilation can facilitate the transmission of respiratory pathogens. In this study, *K. pneumoniae* and RSV were also more frequently detected during these seasons. Another controlled trial specifically investigating cross-immunity against coronaviruses confirmed that full vaccination and booster vaccination against COVID-19 provide virtually no effective cross-protection against common seasonal coronaviruses [17]. Combined with the seasonal epidemiological data from this study, this result indicates that there is no cross-immunity barrier among different respiratory viruses; during winter and spring, when multiple viruses circulate alternately, children are prone to co-infections with multiple viruses, further compromising the integrity of the respiratory mucosal barrier and simultaneously increasing the risk of secondary bacterial infection. This also provides an immunological explanation for the high incidence of severe pediatric pneumonia in winter and spring. In addition, seasonal variation in host susceptibility and exacerbation of underlying conditions, such as congenital heart disease and asthma, may also contribute to the clustering of severe pediatric pneumonia during winter and spring. Clinically, seasonal epidemiological patterns may help inform pathogen surveillance and preventive strategies. During winter and spring, greater attention should be paid to bacterial infections and rational antimicrobial use, whereas during periods of increased viral activity, timely viral testing and supportive management should be emphasized, together with close monitoring for possible secondary bacterial infection [18].

One of the key findings of this study was the difference in the biofilm-forming capacity of *S. pneumoniae* isolates between the two groups. The mean OD_{570} value of isolates from the severe group was 1.31 ± 0.03, and all isolates were classified as strong biofilm producers. In contrast, the mean OD_{570} value of isolates from the mild group was 0.68 ± 0.10, and all were classified as weak biofilm-forming strains. The Wilcoxon rank-sum test showed a statistically significant difference in biofilm formation grades between the two groups ($Z = -2.418$, $P = 0.016$).

S. pneumoniae forms biofilm structures by secreting a polysaccharide matrix that envelops the bacterial cells, and the luxS quorum-sensing gene and the CiaRH two-component regulatory system are the core molecular pathways regulating biofilm synthesis: luxS mediates the synthesis of quorum-sensing signal molecules, upregulating the expression of polysaccharide matrix-related encoding genes and promoting the formation of dense biofilm structures; ciaR

enhances bacterial adhesion and colonization capacity by regulating bacterial membrane transport proteins and the release of virulence factors. On one hand, the dense biofilm structure physically blocks the penetration of antibiotic molecules, greatly reducing the bactericidal efficiency of conventional antibiotics; on the other hand, it shields bacterial antigens, helping bacteria evade macrophage recognition and phagocytosis, ultimately enabling long-term respiratory colonization and inducing recurrent infections [19]. The high-biofilm-activity strains isolated from children with SP can persistently colonize the airways, continuously release inflammatory toxins, progressively damage lung tissue, prolong the disease course, and worsen clinical symptoms. Thus, the biofilm-forming ability of *S. pneumoniae* can serve as a virulence marker directly associated with pneumonia severity and is also an important laboratory indicator for assessing disease prognosis and guiding anti-infective regimen selection. If a highly biofilm-active *S. pneumoniae* strain is isolated clinically, priority should be given to antimicrobial agents capable of penetrating the biofilm matrix to optimize overall anti-infective therapy.

In summary, severe pediatric pneumonia likely results from the combined effects of multiple factors, including pathogen characteristics, host immune status, previous healthcare exposure, and seasonal environmental conditions [20]. In the present study, *K. pneumoniae* was more frequently detected among children with severe pneumonia, whereas *S. pneumoniae* isolates from the severe group exhibited greater biofilm-forming capacity than those from the mild group. In addition, severe pneumonia cases were more frequently observed in winter and spring. In clinical practice, pediatricians should consider the local pathogen spectrum, previous healthcare exposure, individual risk factors, and antimicrobial susceptibility data when selecting empirical anti-infective therapy. During winter and spring, enhanced respiratory infection prevention and early clinical assessment may help reduce the risk of disease progression.

This study has several limitations. First, it was a single-center retrospective study with a relatively limited overall sample size. Only seven *S. pneumoniae* isolates were available for biofilm-related experiments, limiting the representativeness and statistical robustness of the biofilm findings. Second, biofilm formation was evaluated only in *S. pneumoniae*. Biofilm-forming capacity and related resistance or virulence mechanisms were not investigated in other important pathogens identified in severe cases, such as *K. pneumoniae* and *P. aeruginosa*. In addition, biofilm-associated regulatory genes in S. pneumoniae were not directly examined. Future multicenter, large-sample cohort studies could be conducted to systematically dissect the biofilm regulatory pathways of multiple pathogens, thereby providing a more comprehensive theoretical basis for the prevention and precise anti-infective treatment of severe pediatric pneumonia.

Conclusion

This study investigated the pathogen distribution and biofilm-forming characteristics of *S. pneumoniae* in children with severe pneumonia. Bacterial infections were more frequently detected in severe cases, with *K. pneumoniae* and *P. aeruginosa* among the commonly identified bacterial pathogens, whereas RSV was the most frequently detected viral pathogen. Severe pneumonia cases were more frequently observed in winter and spring. All *S. pneumoniae* isolates evaluated were capable of forming biofilms, and isolates from children with severe pneumonia exhibited stronger biofilm-forming capacity than those from children with mild pneumonia. However, because of the small number of isolates analyzed, this association should be interpreted cautiously. Future multicenter, large-sample studies are needed to further elucidate the epidemiological patterns and pathogen resistance characteristics of severe pediatric pneumonia, so as to provide a more robust evidence base for clinical prevention and treatment.

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Disclosure of conflict of interest

None.

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