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Clinical Features and Value of Tracheal Aspirate Metagenomic Next-Generation Sequencing for Severe Pneumonia in Children in Pediatric Intensive Care Unit

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Abstract

Pneumonia is a leading cause of mortality in children. While metagenomic next-generation sequencing (mNGS) has the potential to detect all the microorganisms in pneumonia patients, the relationship between these microorganisms and the patients' clinical characteristics remains to be established. Fifty-five children, diagnosed with severe pneumonia and undergoing tracheal aspirate (TA) mNGS for pathogen detection at The Heilongjiang Hospital of Beijing Children's Hospital between July 2021 and November 2022, were included in this study. The clinical characteristics, pathogen distribution, and microbiome features of these children were analyzed. Results showed that the rate of mixed infections was notably high (80%, 44/55), with bacterial-viral infections being the most common. *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* (MP), *Candida albicans*, and Respiratory syncytial virus (RSV) were the most common pathogens in this cohort. Furthermore, RSV and *S. pneumoniae* were the most prevalent pathogens in children younger than 12 months (infants), while MP and *Haemophilus influenzae* were more commonly identified in children between 12 and 144 months. Increased richness and diversity of the microbiota were observed in the TA of the older children. Linear discriminant analysis (LDA) effect size (LEfSe) analysis identified that RSV and *Streptococcus mitis* were the specific species associated with infants. In contrast, *Human bocaparvovirus 1* and *Prevotella histicola* were significantly enriched in the older children. In addition, the top 20 most abundant species exhibited correlations with neutrophil count and C-reactive protein. This study emphasizes the significance of employing mNGS to understand better the clinical characteristics and microbial diversity in pediatric patients with severe pneumonia.

Keywords: metagenomic next-generation sequencing, severe pneumonia, children, microbiome, age

Introduction

Pneumonia is a significant cause of morbidity and mortality in children (Liu et al. 2015). Severe pneumonia accounts for 7–13% of these cases (Rudan et al. 2008; Black et al. 2010; Rudan et al. 2013). Due to its rapid onset, severe symptoms, rapid progression, and high incidence of complications, accurate diagnosis and treatment of severe pneumonia are crucial for reducing mortality rates and improving cure rates. However, identifying the pathogen that causes pneumonia can be challenging due to the diverse clinical symptoms that vary depending on children's age, gender, immune status, and underlying diseases (Shi et al. 2020; Anteneh et al. 2023).

The distribution of pathogens in severe pneumonia cases among children of different age groups was studied. Respiratory syncytial virus (RSV) was detected more frequently in younger children, while parainfluenza, adenovirus, and influenza were identified more commonly in older children (Ning et al. 2017; Chen et al. 2018; Shin et al. 2018; Zhao et al. 2019; Chen et al.

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2023). Older children were more likely to be infected with multiple pathogens, with mycoplasma and viruses commonly found in their pathogenic spectrum (Chen et al. 2023). Nonetheless, there remains a lack of research on the changes in respiratory system microbial components among children of different ages.

Pathogens, the lung microbiome, and the host response are the three core elements of respiratory tract infections (Zhang et al. 2021). Altering the respiratory microflora early in life may lead to the development of respiratory diseases. *Streptococcus*, *Moraxella*, or *Haemophilus* were the dominant taxa for most children aged 6 months to 5 years, and *Streptococcus*, *Prevotella*, or *Pasteurellaceae* were the dominant taxa for children aged 5 to 18 years (Pettigrew et al. 2016). Additionally, specific taxa in the lung microbiota are linked to the severity of community-acquired pneumonia in children (Pettigrew et al. 2016). No further comparative analysis between the age groups was carried out in the previous study.

Tracheal aspirate (TA) sampling is less invasive than mini-bronchoalveolar lavage (mBAL). Nevertheless, TA was previously considered a suboptimal sample due to contamination by oral microbes. Previous studies showed that the lung microbiome composition is similar between TA and mBAL in patients with LRTI (Kalantar et al. 2019). TA samples can also better reflect the lung microbiome of children with LRTI (Mick et al. 2023). The Infectious Diseases Society of America (IDSA) and American Thoracic Society (ATS) currently recommend non-invasive TA sampling with semiquantitative cultures over other investigative techniques, including BAL, in the diagnosis of ventilator-associated pneumonia (VAP) in adults (Kalil et al. 2016). Katayama et al. (2010) demonstrated that gram staining of TAs is highly effective in guiding antibiotic therapy for VAP in extremely preterm neonates with lifelong hospital admission and mean onset of VAP at > 30 days. These results show that TA can be used for pathogen identification and lung microbiome research. The prospects of studying the lung microbiome include various aspects, such as investigating its potential as a biomarker for diagnosing respiratory diseases, exploring its spatial dynamics, and understanding the mechanisms underlying its interaction with the host (Yi et al. 2022). Metagenomic next-generation sequencing (mNGS) is an emerging method that has shown significant advantages in the unbiased detection of pathogens causing various infectious diseases (Gu et al. 2019; Li et al. 2022b, 2022a; Chen et al. 2024; Wu et al. 2023). Additionally, it can analyze the microbial landscape of the respiratory tract (Langelier et al. 2018; Zhang et al. 2023). mNGS has been widely used to study upper respiratory tract samples in children (Ogunbayo et al. 2023). However, its application in less invasive lower respiratory tract samples, such as tracheal aspirates, remains in the preliminary exploration stage. Systematic research on its clinical utility in diagnosing pediatric lung infections is still lacking. Thus, in-depth exploration of metagenomics in this sample type could elucidate the pathogen profile and microbiome composition in childhood pneumonia and provide new insights for optimizing clinical diagnosis and treatment strategies.

The goal of etiologic diagnosis and lung microbiome research is to achieve precision medicine. The purpose of this study was to investigate the relationship between clinical features, specific pathogen distribution, and microbiome characteristics in children of different ages with severe pneumonia. The findings have significant implications for the clinical management of infectious diseases and future research directions.

Experimental

Materials and Methods

Patient population and study design. A total of 63 children who were suspected of having a pulmonary infection and were admitted to Heilongjiang Hospital of Beijing Children's Hospital between July 2021 and November 2022 were enrolled in this study. The inclusion criteria for participants were as follows: i) diagnosis of severe pneumonia according to the criteria set by the World Health Organization (Bradley et al. 2011; Chisti et al. 2015; Williams et al. 2016); ii) parental consent for TA collection for mNGS analysis; iii) use of at least one of the following samples for conventional microbiological testing (CMT): TA, blood, or urine. Samples were collected according to standard procedures. Children undergoing mNGS testing are selected at the clinician's discretion. mNGS testing is primarily used for children with severe disease, atypical clinical symptoms, or those whose cause cannot be determined through conventional microbiological testing. Due to the high cost of mNGS testing, informed consent from the child's family is required before performing the test. Approximately 60–70% of children with severe pneumonia who were admitted to the intensive care unit and met the criteria for metagenomic testing were enrolled in this cohort. Participants were excluded from the study if they were not diagnosed with severe pneu-

monia. According to WHO standards, when a child's breathing becomes faster and there is inspiratory retraction of the chest wall or wheezing is heard in the lungs, severe pneumonia should be considered (Bradley et al. 2011). Children's demographic data, clinical data, serum inflammatory markers, imaging information, and medication use were collected. The final clinical diagnoses were based on several factors, including clinical manifestations, laboratory test results, imaging results, pathogens detected by mNGS and CMT, the effects of antibiotic use, and epidemiology.

Samples were immediately subjected to several tests after collection. The CMT methods included culture, fungal D-glucose testing, antibody testing for seven respiratory viruses [Influenza A virus (IAV), Influenza B virus (IBV), RSV, Adenovirus (ADV), Parainfluenza viruses (PIVs) 1, 2, and 3], or antibody testing for eight respiratory pathogens (ADV, *Legionella pneumophila* (LP), *Chlamydia pneumoniae* (CP), RSV, IFA, IFB, *Mycoplasma pneumoniae* (MP), PIVs 1, 2, and 3]. In addition, a test for mycoplasma antibodies was conducted. Off the samples, 80% (44/55) had culture results, and 80% underwent the MP test.

To determine the causative pathogens, final comprehensive clinical diagnoses were independently confirmed by two to three clinical adjudicators, according to mNGS results, complete laboratory examinations, the patients' treatment response, and clinical experiences.

Processes of mNGS sequencing and pathogen identification. DNA was extracted from the TA (collected by routine endotracheal suctioning) samples using the PathoXtract® Basic Pathogen Nucleic Acid Kit (WYXM03211S, WillingMed Corp, China), and RNA was extracted using PathoXtract® Virus DNA/RNA Isolation Kit (WYXM03009S, WillingMed Corp, China), following the manufacturer's protocol. After combining DNA and RNA, the RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript™ Double-Stranded cDNA Synthesis Kit (11917020, Invitrogen™, Thermo Fisher Scientific Inc., USA USA). For cfDNA libraries, the KAPA DNA HyperPrep Kit (KK8504, Kapa Biosystems, USA) was used, and their quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). High-quality libraries were sequenced using the NextSeq™ 550Dx sequencer (Illumina, Inc., USA) with a 75-bp single-end method. No-template control (NTC) containing nuclease-free water was included in each sequencing run to control the effect of contaminating DNA. Trimmomatic v0.40 (Bolger et al. 2014) was employed to process the obtained sequence data in FASTQ format to filter out low-quality sequences, contaminated adapters, duplicated reads, and reads shorter than 36 bp. The resulting sequences were aligned against the human reference genome GRCh37 (hg19) using Bowtie2 v2.4.3 to remove human sequences (Langmead and Salzberg 2012). For the classification and identification of microbial reads, we used Kraken2 v2.1.0 software and the non-redundant nucleotide sequences database of the National Center for Biotechnology Information (NCBI) (Wood et al. 2019). To report the positive pathogens, we used the following criteria for reads per ten million (RPTM): bacteria and fungi with RPTM ≥ 20, viruses with RPTM ≥ 3, and special pathogens (including *Cryptococcus*, *Mycobacterium*, *Mycoplasma*, *Chlamydia*, and parasites) with RPTM ≥ 1 (Langelier et al. 2018; Chen et al. 2022).

Clinical adjudication of mNGS results. To confirm the composite clinical diagnosis, two experienced pediatric ICU specialists independently reviewed each patient's medical records and mNGS results. Based on laboratory findings and clinical information, the clinician panel categorized the likelihood of mNGS results being a causative agent of severe pneumonia into four categories: definite, probable, possible, and unlikely. This classification is based on a previous report by Blauwkamp et al. (2019). Our study classified definite, probable, and possible microorganisms as clinically relevant pathogens, while unlikely microorganisms were considered colonization or contamination.

Statistical analysis. Continuous variables are presented as mean ± standard deviation and were compared using an unpaired *t*-test. Skewed continuous variables are expressed as quartiles and were compared using the Mann–Whitney *U* test. The chi-square test was used for categorical variables. Statistical calculations were performed using GraphPad Prism v9.0 (GraphPad Software, USA, www.graphpad.com). Statistical significance was determined by *p*-values less than 0.05. For evaluating compositional similarity among samples within different groups, the Bray–Curtis measure of β diversity was utilized to compare the detected species. Analysis of similarity (ANOSIM) was conducted to identify the factors that differentiated the microbial community. The analysis was conducted using the vegan package in R, and the resulting plot was created with the ggplot2 package (Wickham 2016). A barplot was generated to display the top 10 species with high relative abundance. To identify the distinc-

tive species between the groups, linear discriminant analysis (LDA) effect size (LEfSe) was performed using the lefse package of R.

Results

General characteristics of the severe pneumonia children. Sixty-three children who were hospitalized in Heilongjiang Hospital of Beijing Children's Hospital and underwent TA mNGS were included in this study (Fig. 1). After excluding eight cases without severe pneumonia, 55 children diagnosed with severe pneumonia were included in this study. The patients' characteristics and baselines were presented in Table I. Their median age was 5.97 months, with 43.64% being male. Upon admission, over 60% of the patients presented symptoms such as coughing, respiratory abnormalities, and mental decline. The mean serum C-reactive protein (CRP) concentration was 21.06 ± 43.04 mg/l, and procalcitonin (PCT) concentration was 2.61 ± 6.85 ng/ml. The mean length of hospital stay (LOHS) was 16.49 ± 8.83 days, with a mean length of stay in the intensive care unit (ICU) being 15.24 ± 8.87 days. Furthermore, 94.55% (52/55) of children had respiratory failure, and 23.64% (13/55) had sepsis. 43.64% of cases had their antibiotic usage adjusted based on the mNGS results. Only two patients died upon discharge from the hospital. Children aged 12–44 months (n = 22) had significantly lower platelet counts (PLT) and percentage of lymphocytes (LY%) compared to those younger than 12 months (n = 33), but their neutrophil count (NEUT), and CRP and PCT levels were significantly elevated ($p < 0.05$). Thirty patients underwent intubation and mechanical ventilation. The Sequential Organ Failure Assessment score (SOFA score) and Acute Physiology and Chronic Health Evaluation II score (APACHE II score) between the two groups showed no significant difference ($p > 0.05$). Indicators related to arterial gas and blood analysis also showed no significant difference ($p > 0.05$), except for Partial pressure of carbon dioxide (PaCO2), which was sig-

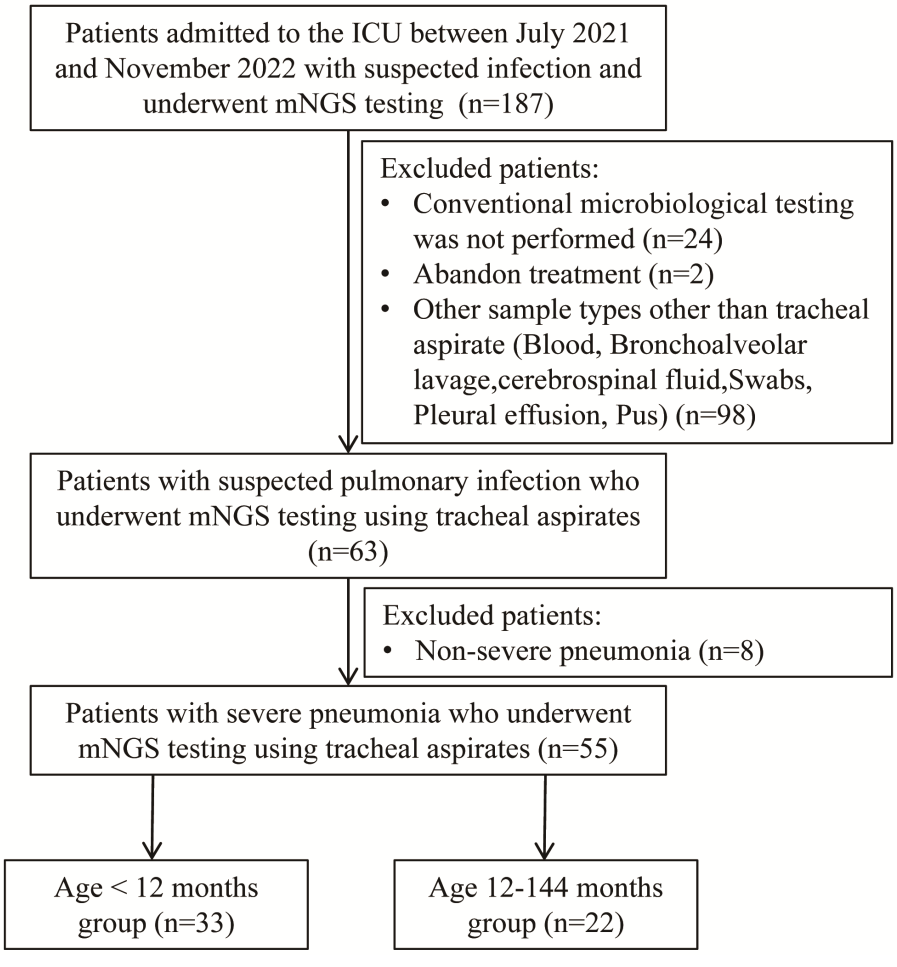

Fig. 1. Flow diagram of patient inclusion and exclusion.

Table I
Demographic and clinical characteristics of patients.

Characteristics	Severe pneumonia (n = 55)	Children less than 12 months (n = 33)	Children aged 12~144 months (n = 22)	*p*-value[a]
Age, month (M, IQR)	5.97 (3.28–30.50)	3.70 (2.80–4.63)	40.50 (24.75–51.75)	< 0.0001
< 12	33	/	/	/
12–144	22	/	/	/
Gender, male, n (%)	24 (43.64%)	18 (54.55%)	6 (27.27%)	0.0457
Symptoms, n (%)				
Fever	24 (43.64%)	9 (27.27%)	15 (68.18%)	0.0027
Cough	41 (74.55%)	26 (78.79%)	15 (68.18%)	0.3764
Abnormal breathing	51 (92.73%)	33 (100%)	18 (72%)	0.0012
Unconsciousness	6 (10.91%)	3 (9.09%)	3 (13.64%)	0.5963
Apathetic	43 (78.18%)	23 (69.70%)	20 (90.91%)	0.0620
Fidgety	10 (18.18%)	2 (6.06%)	8 (36.36%)	0.0043
Laboratory examination (mean ± SD)				
Pulse (times per minute)	106.9 ± 28.95	172.88 ± 22.49	143 ± 28.69	<0.0001
Respiratory rate (times per minute)	42.31 ± 11.39	44.55 ± 9.95	38.96 ± 12.77	0.0742
WBC	10.42 ± 4.79	11.03 ± 4.52	9.49 ± 5.14	0.2465
Hb	105.8 ± 18.72	104.82 ± 14.19	107.27 ± 24.30	0.6382
PLT	368.13 ± 183.67	441.64 ± 187.03	257.86 ± 110.54	0.0001
NEUT%	12.38 ± 23.53	9.60 ± 17.41	16.56 ± 30.53	0.2866
LY%	12.36 ± 23.38	17.66 ± 28.21	4.42 ± 9.15	0.0386
NEUT	5.02 ± 3.80	3.82 ± 2.49	6.80 ± 4.71	0.0035
HCT	31.38 ± 7.91	31.38 ± 6.79	31.38 ± 9.52	0.9990
CRP	21.06 ± 43.04	7.35 ± 19.80	41.63 ± 58.54	0.0029
PCT	2.61 ± 6.85	0.91 ± 2.84	5.09 ± 9.78	0.0260
BUN	4.93 ± 4.61	4.67 ± 2.95	5.33 ± 6.44	0.6157
Arterial blood pH	7.38 ± 0.08	7.37 ± 0.08	7.40 ± 0.08	0.2710
PaO_2	58.29 ± 17.37	57.67 ± 14.03	59.23 ± 21.77	0.7474
$PaCO_2$	43.78 ± 12.70	47.33 ± 11.44	38.45 ± 12.88	0.0097
HCO_3^-	26.32 ± 8.26	26.93 ± 5.14	25.40 ± 11.55	0.5058
PaO_2/FiO_2	213.13 ± 64.00	221.33 ± 48.89	200.82 ± 81.41	0.2478
Premature, n	7 (12.73%)	6 (18.18%)	1 (4.55%)	0.1371
Post operation, n	3 (5.45%)	1 (3.03%)	2 (9.09%)	0.3322
Intubation, n	30 (54.55%)	19 (57.58%)	11 (50%)	0.5804
Mechanical ventilation, n	30 (54.55%)	19 (57.58%)	11 (50%)	0.5804
SOFA score	3.58 ± 2.03	3.48 ± 1.68	3.73 ± 2.51	0.6691
APACHE II score	13.45 ± 4.69	13.58 ± 3.95	13.27 ± 5.72	0.8167
LOHS (mean ± SD), day	16.49 ± 8.83	17.39 ± 9.72	15.14 ± 7.30	0.3579
ICU (mean ± SD), day	15.24 ± 8.87	16.76 ± 9.75	12.96 ± 6.97	0.1203
Outcomes, n				
Cured	53 (96.36%)	32 (96.97%)	21 (95.45%)	0.7687
Died	2 (3.64%)	1 (3.03%)	1 (4.55%)	0.7687

a*p*-Value was the statistically significance result between children aged less than 12 months and 12~144 months. The Student's *t*-test was used to compare differences between groups, and the chi-square test was used for analyzing categorical variables. $p < 0.05$ was considered statistically significant.

M – median; IQR – interquartile range; WBC – white blood cell; Hb – hemoglobin; PLT – blood platelet; NEUT – neutrophil cell count; LY – lymphocyte count; HCT – hematocrit; CRP – C-reactive protein; PCT – procalcitonin; BUN – urea nitrogen; PaO2 – partial pressure of oxygen; PaCO2 – partial pressure of carbon dioxide; PaO2/FiO2 – the ratio of PaO2 to fractional inspired oxygen (FiO2); SOFA score – sequential organ failure assessment score; APACHE II score – acute physiology and chronic health evaluation II score; ICU – intensive care unit; LOHS – length of hospital stay.

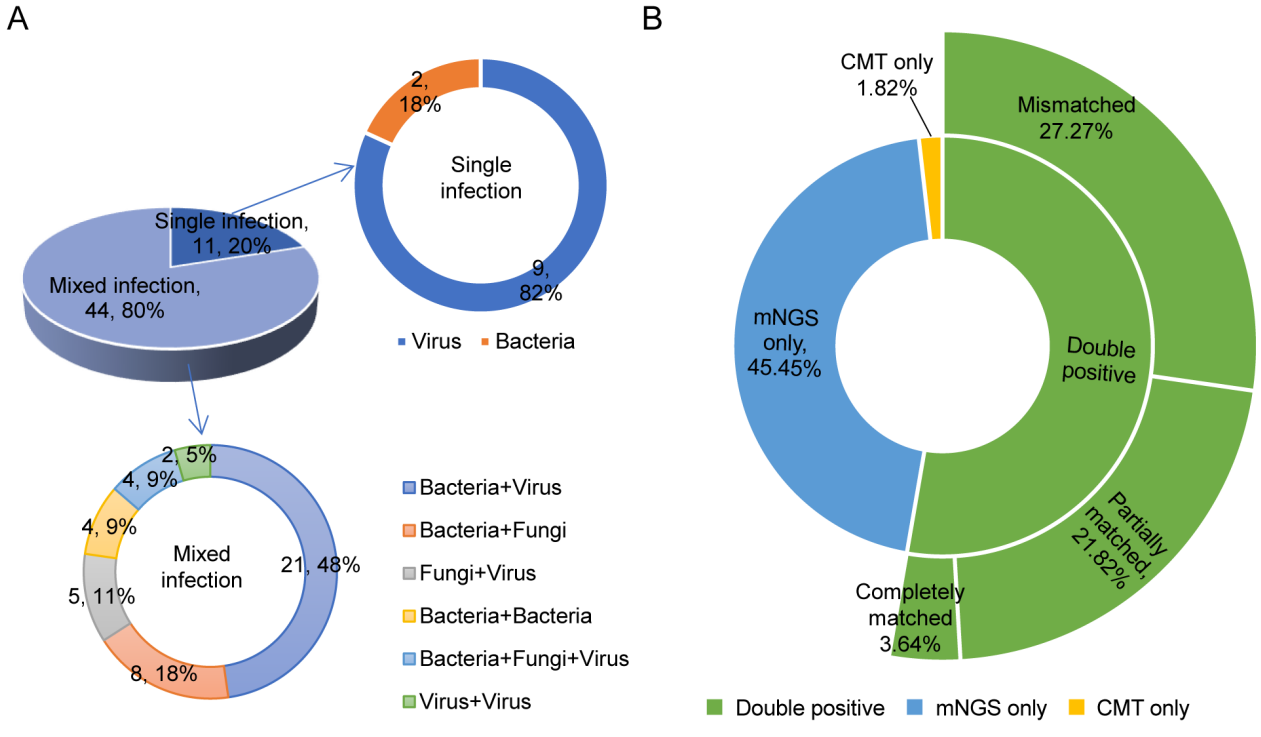

Fig. 2. The pathogen detected results of mNGS and CMT.
A) The types of infection identified in the children with severe pneumonia. The solid pie chart represents the proportion of single and mixed infections, and the other two hollow pie charts represent the specific pathogen types of single and mixed infections.
B) The coincidence between mNGS and CMT for pathogen identification.

nificantly higher in children < 12 months than in children aged 12–144 months (47.33 ± 11.44 vs. 38.45 ± 12.88, $p = 0.0097$).

Comparative analysis of mNGS and CMT results. Among the 55 children with severe pneumonia, 11 children were diagnosed with a single infection while the remaining 44 children were diagnosed with a mixed infection, with bacteria-virus co-infection being the most common, followed by bacteria-fungi, and fungi-virus co-infections (Fig. 2A). Twenty-nine (52.73%) samples were positive by both mNGS and CMT, an additional 45.45% were mNGS positive, and only one was CMT positive. Among the double positive samples, a concordance rate of 26% (at least one microorganism matched) for mNGS and CMT was identified (Fig. 2B).

A total of 29 underlying pathogens were identified in 55 children with severe pneumonia, including bacteria (11), fungi (4), and viruses (14). mNGS detected almost all pathogens, while CMT identified only eight species. *Streptococcus pneumoniae* (17/55, 30.91%), *Staphylococcus aureus* (7/55, 12.73%), MP (7/55, 12.73%), and *Haemophilus influenzae* (5/55, 9.09%) were the most common bacteria. *Candida* represented 91.67% (11/12) of the identified fungi, with *C. albicans* (8/12, 66.67%) being the most common. One case of *Pneumocystis jirovecii* was detected. The most frequently detected viruses were RSV (24/55, 43.64%), followed by cytomegalovirus (CMV, 5/55, 9.09%) (Fig. 3A). Pathogens showed an age-dependent diversity. RSV and *S. pneumoniae* were the most common pathogens in children younger than 12 months, whereas MP and *H. influenzae* were more prevalent in older children (Fig.3B).

Respiratory microbiota in children with severe pneumonia. Next, we analyzed the respiratory microbiota of children with severe pneumonia. At the species level, 160 bacterial species, 5 fungal species, and 23 viral species were identified in these children. The heatmap showing the relative abundance among the children demonstrated increased coexistence of bacteria and viruses in older children (Fig. S1). As children grow older, the coexistence of multiple microorganisms is more commonly detected, while in infants, the detected microorganisms are more homogeneous, with *Rothia mucilaginosa* being the most common bacterium. RSV was the most common virus detected. As patients age, the variety of microorganisms in the respiratory tract increases. In older children, the most common bacteria were *R. mucilaginosa*, *Streptococcus salivarius*, and *S. pneumoniae*, while respiratory virus-

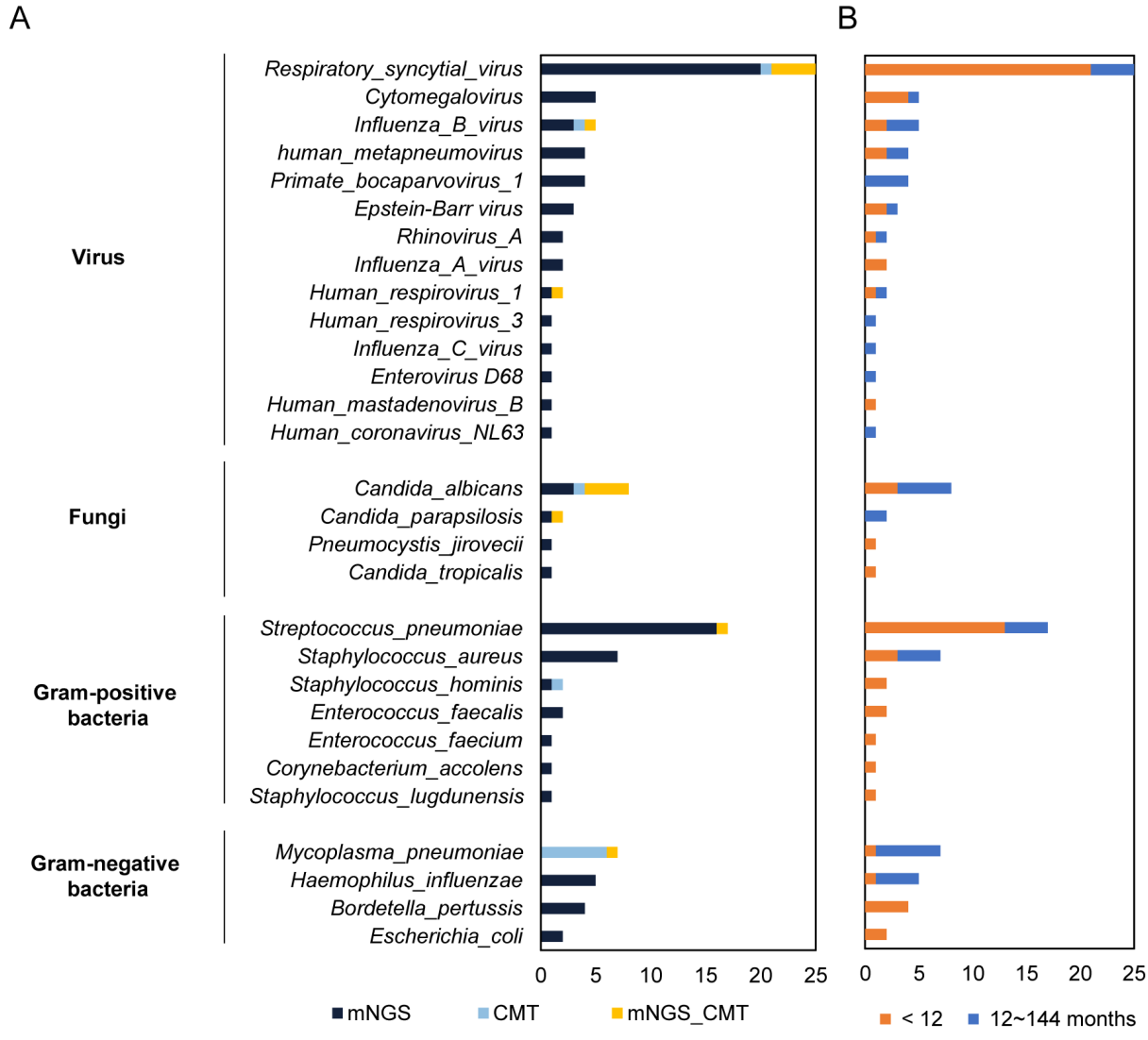

Fig. 3. The distribution of the identified pathogen spectrum.
A) The distribution of pathogens detected by mNGS and CMT.
B) The distribution of pathogens for children in the < 12 months group and the 12~144 months group.

es such as *Primate bocaparvovirus 1* (HBoV-1), *Human metapneumovirus*, and IBV were also frequently detected.

Additionally, the alpha diversity analysis revealed that children aged 12 to 144 months displayed increased richness and diversity of the microbiota, as compared to children younger than 12 months (Fig. 4A). Beta diversity was analyzed using principal coordinate analysis (PCoA) and indicated the creation of two clustering age groups; however, many samples from the two groups were mixed together (Fig. 4B). Moreover, the significant difference between groups was analyzed using ANOSIM. The results revealed that the differences in microbial community structure between groups were significantly greater than the differences within groups ($R > 0$, $p < 0.05$) (Fig. 4C).

The top 20 species with the highest relative abundance were shown. Among them, 6 of the 14 bacteria were species of *Streptococcus*, and the only fungal species identified was *C. albicans*. Five viruses including RSV, HBoV-1, CMV, enterovirus D68 (EV-D68), and influenza C virus (ICV). *S. pneumoniae* accounted for the highest proportion, reaching 19.14%; RSV and *Streptococcus peroris* accounted for 7.19% and 6.50%, respectively (Fig. 5A). The difference in abundance of the top 20 species between children younger than 12 months and 12 to 144 months was also analyzed. RSV, MP, *Corynebacterium accolens*, *R. mucilaginosa*, and *Streptococcus mitis* had increased abundance in infants, while HBoV-1, EV-D68, and ICV had higher abundance in older children (Fig. 5B).

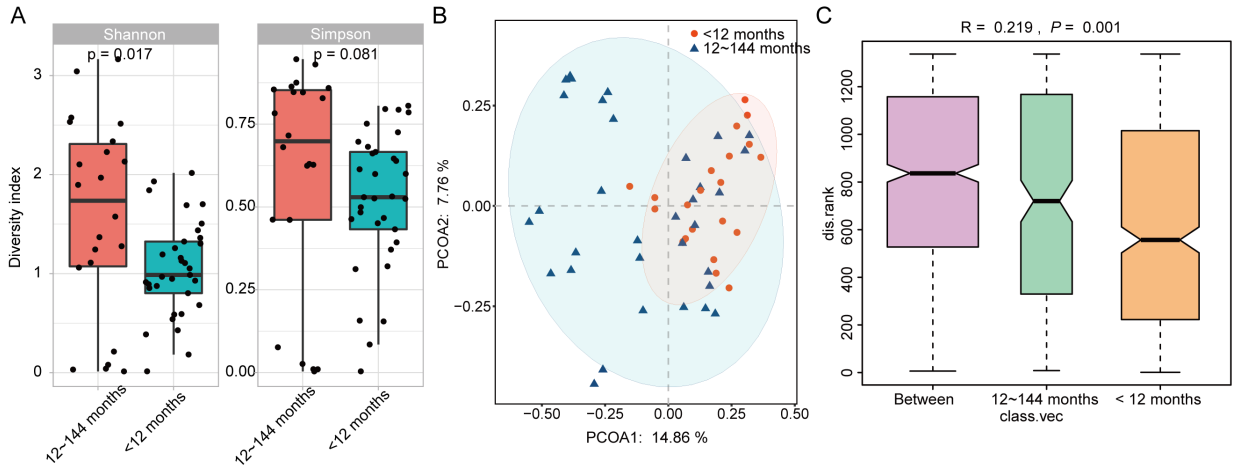

Fig. 4. Analysis of microbiome alpha and beta diversity between patients < 12 months and 12~144 months.
A) Alpha diversity differences of microbiota communities at the species level between groups. Each point represents one sample from each group. B) PCoA plot is based on Bray–Curtis's distance between the children in groups. The x-axis is for grouping information, the y-axis is for distance information, the *R*-value is between (−1, 1) and there are significant differences between groups when the *R*-value is greater than zero and the P value is less than 0.05.

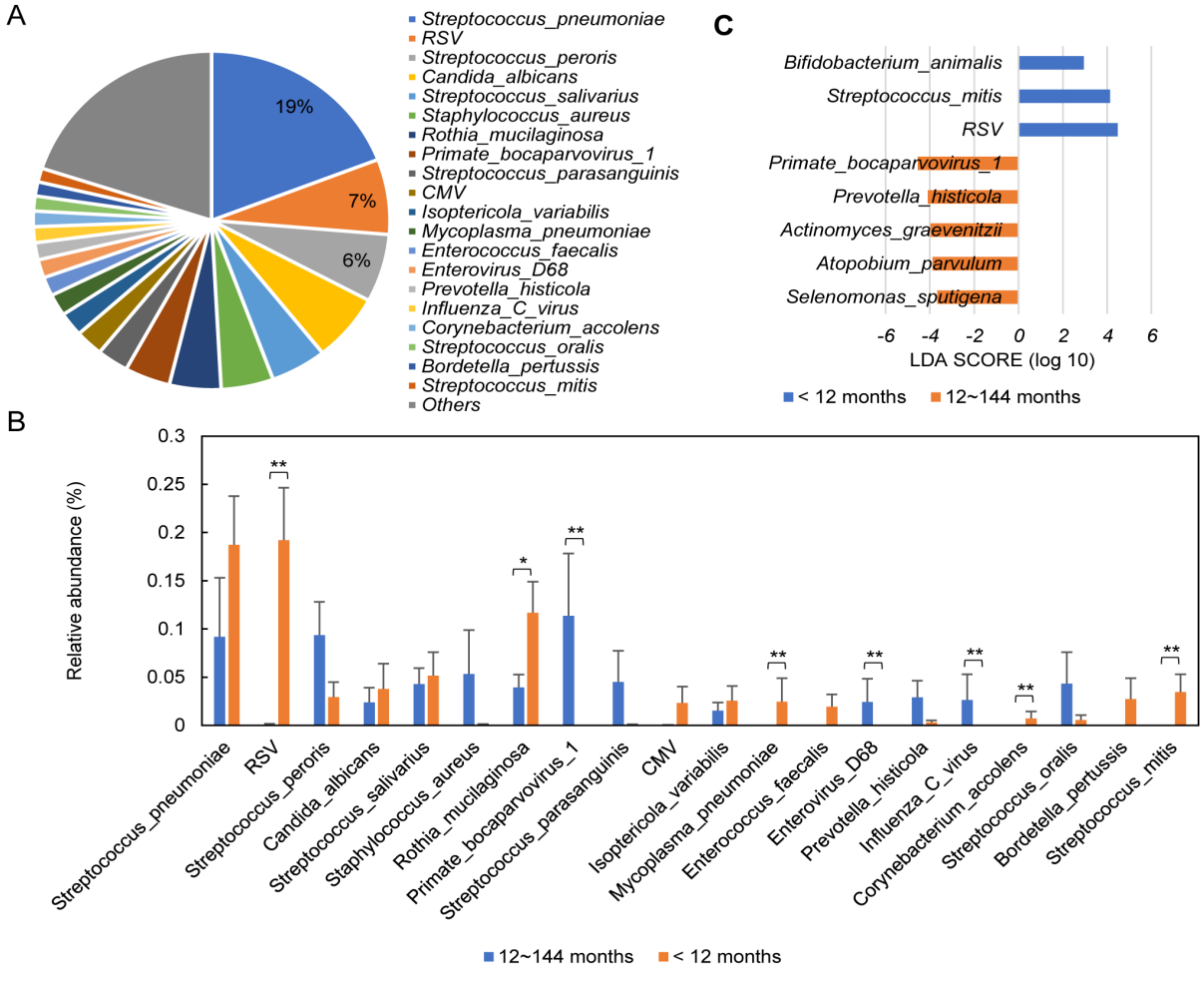

Fig. 5. The top 20 microorganisms and their correlation with patient clinical data.
A) Top 20 most abundant species and their relative abundance. B) Differences between age groups for the top 20 most abundant microbes. * *p*-adjust < 0.05; ** *p*-adjust < 0.01; *** padjust < 0.001. Metastats analysis, *p*-value was adjusted using the Benjamini and Hochberg method. C) LEfSe analysis of respiratory microbiome between patients < 12 months and 12~144 months. Bar chart showing the log-transformed LDA scores of pathogen species identified by LEfSe analysis (the log-transformed LDA score of 4 as the threshold).

Linear discriminant analysis (LDA) effect size (LEfSe) was performed to evaluate the influence of microorganisms on significantly different groups based on LDA scores (cutoff of 4.0). The results showed that the relative abundance of RSV and *S. mitis* was higher in children younger than 12 months compared to children between 12 and 144 months, and the relative abundance of HBoV-1 and *Prevotella histicola* was higher in older children (Fig. 5C).

Correlation between microbial and clinical characteristics in pediatric severe pneumonia patients. Spearman correlation analysis was conducted to investigate the correlation between clinical parameters, including age, routine blood indicators, inflammatory and other indicators, and the top 20 abundant species. The most abundant and prevalent bacterium, *S. pneumoniae*, exhibited a strong negative correlation with PLT, whereas it showed a positive correlation with CRP and PCT. RSV, the most abundant and typical virus in children less than 12 months, positively correlated with LY% and negatively correlated with HCT. HBoV-1, a prevalent pediatric virus, had a positive correlation with NEUT% and a negative correlation with NEUT. Another typical pediatric species, *P. histicola*, showed a strong positive correlation with age, NEUT%, and weight, while negatively correlated with pulse. A special pathogen, MP, was positively correlated with the length of hospital and ICU stay (Fig. 6).

Discussion

Respiratory microbes undergo dramatic changes in the early stages of childhood (Biesbroek et al. 2014). Several studies have suggested that respiratory microbes play a key role in the pathogenesis of respiratory infections (Hurst et al. 2022). From infancy, microbial diversity increases significantly with age (Hurst et al. 2022). The action of external factors has a long-term impact on establishing the microbiota. These effects may be due to the natural environment, diet, seasons, exposure history, drug use, and lifestyle habits (McCauley et al. 2022). With advancing age, the immune system shapes microbiota diversity through regulatory mechanisms such as immune tolerance, selective immune responses, and adaptation (Kloepfer and Kennedy 2023). Therefore, this study aimed to comprehensively evaluate the association between these microorganisms and the clinical characteristics of patients with severe pneumonia in different age groups.

Our study found that mNGS demonstrated good consistency and a broad pathogen profile in children with severe pneumonia when CMT was used as the reference standard, with RSV, *S. pneumoniae*, and *C. albicans* being the most common pathogens. Pathogen distribution and microbiota composition vary with age. In our study, co-infection was common (80%), particularly bacterial-viral co-infection, consistent with previous studies (Li M et al. 2023; Shen et al. 2023). These co-infections correlated with disease severity, and RSV co-infection with *H. influenzae*, *S. aureus*, and rhinovirus significantly increased the incidence of severe pneumonia (Cebey-López et al. 2016; Meskill and O'Bryant 2020; Liu et al. 2023). In this study, one case of co-infection involving *P. jirovecii* and CMV was identified. Previous studies have indicated that co-infection-induced pneumonia can have fatal consequences, particularly in infants and young children, if not promptly diagnosed and treated (Williams et al. 2001; Lyu et al. 2022). Investigating pathogen co-infections provides valuable insights for clinicians, helping them make more accurate diagnoses and treatment decisions. In children with severe pneumonia, mNGS can help doctors identify complex co-infections in a timely manner, enabling them to select targeted antimicrobial drugs for combination therapy and reduce unnecessary drug use.

By analyzing the respiratory microbiota of children with severe pneumonia, we found that bacteria and viruses are more likely to coexist in older children. Over time, older children's immune systems mature, and environmental exposure increases the diversity of microorganisms in the lower respiratory tract (Muhlebach et al. 2018; Zhu et al. 2021). Most children under one year of age have low bacterial densities in the lower respiratory tract due to the immaturity of their immune system (Renz et al. 2018). This increased diversity may also contribute to a higher risk of opportunistic infections by colonizing bacteria. The LEfSe analysis revealed differential species between the two age groups. RSV and *S. mitis* are microbiological markers of severe pneumonia in children under 12 months of age, while HBoV-1 and *P. histicola* are more commonly found in children aged 12 to 144 months. This study highlights RSV as the main causative species, consistent with previous research (Esposito et al. 2013; Khuri-Bulos et al. 2018). Additionally, the uneven distribution of RSV among hospitalized children of different ages supports the idea that younger age is

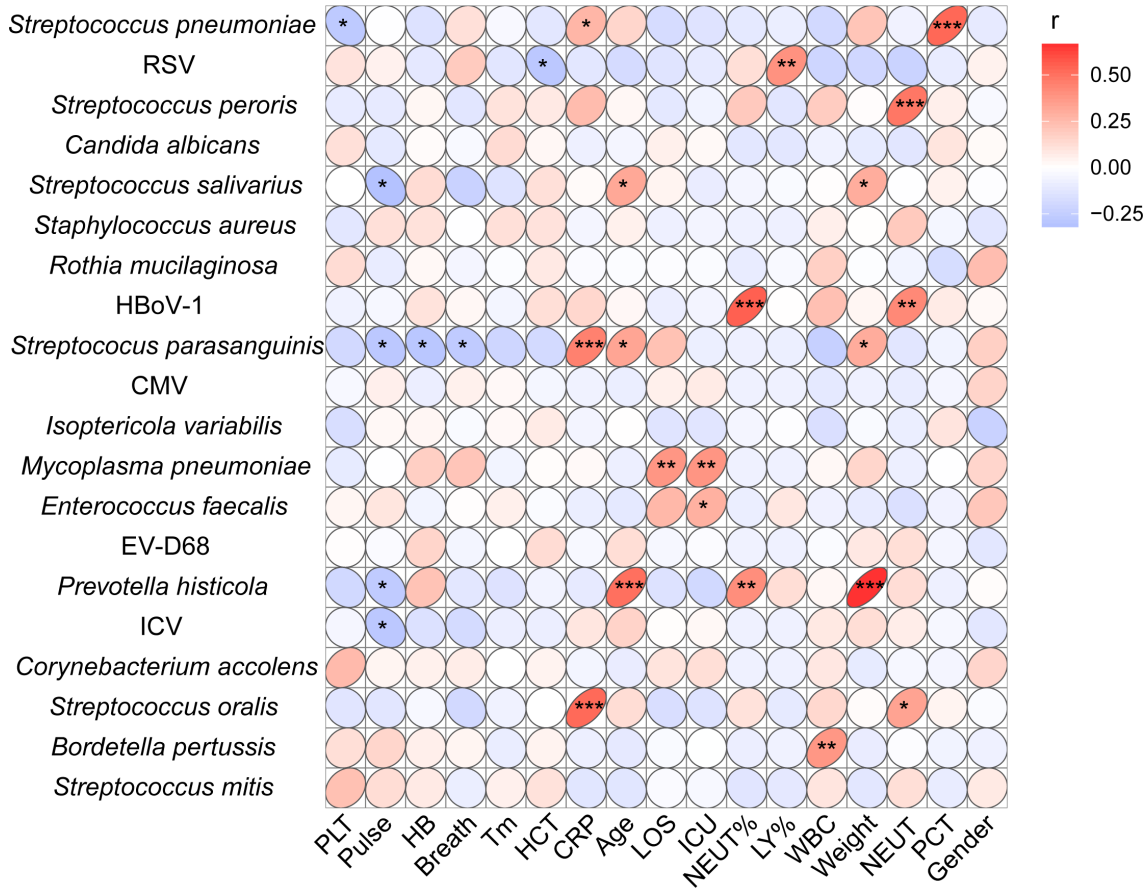

Fig. 6. Spearman correlations between the top 20 most abundant species and clinical data.
* $0.01 < p < 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

closely associated with severe RSV infection, as mentioned by Do et al. (2020) and Gonapaladeniya et al. (2021). The difference is that more *S. mitis* is found in children under 12 months of age. *S. mitis*, a respiratory colonizing bacterium, has been reported to inhibit the growth of various respiratory pathogens (Men et al. 2025). Our study found that *S. mitis* may be involved in developing severe pneumonia as a potential pathogen in infants with incomplete immune function. HBoV-1 was detected only in children aged 12 to 144 months, which differs from previous studies. Several studies have found that severe HBoV pneumonia most commonly occurs in younger children under 2 years of age, particularly in infants, who are more likely to develop HBoV co-infection (Zhang et al. 2018; Li Z et al. 2025). The epidemiological characteristics of different regions may influence these differences. Rapid identification of early species-specific infections is essential for prompt treatment in infants and children. When RSV and *S. mitis* are detected in infants, and HBoV-1 and *P. histicola* are detected in children, clinicians should focus more on controlling disease exacerbations.

The microbiome and host interaction significantly influences human health (Vayssier-Taussat et al. 2014; Kumpitsch et al. 2019). Our study showed that the high abundance of microorganisms in TA samples from children was closely related to inflammatory markers, especially *S. peroris*, HBoV-1, *Staphylococcus oralis*, *S. pneumoniae*, and *Streptococcus parasanguinis*. Previous studies have also found that an increased abundance of colonizing microorganisms may induce more significant epithelial damage and the expression of inflammatory factors, leading to exacerbation of the disease (McCauley et al. 2019; Kloepfer and Kennedy 2023).

However, there are several limitations worth noting. Firstly, this study is retrospective, potentially introducing a certain degree of selection bias. Secondly, the sample size was relatively small, consisting of only 55 patients from a single center, which may limit the statistical power. Thirdly, although TA mNGS is superior to CMT in detecting infections in children with severe pneumonia, the actual clinical situation is that not all patients undergo CMT testing of TA samples.

Therefore, we used CMT results from different sampling sites for comparative analysis. Differences in sample types may impact the interpretation of the results. Lastly, not all viral events detected by mNGS underwent confirmatory PCR testing.

Additionally, our study is retrospective, and a single sampling site cannot thoroughly analyze the dynamics of microorganisms throughout the disease course. Previous studies have conducted longitudinal research on adult lung microbes to explore the relationship between microbes, disease, and disease progression after treatment. In future studies, longitudinal research on the microbial communities of children with severe pneumonia using mNGS detection technology can better clarify the disease's potential mechanisms and therapeutic effects.

In conclusion, we performed a comprehensive analysis to elucidate the characteristics of pediatric patients suffering from severe pneumonia, specifically focusing on variations in clinical symptoms, characteristic indices, pathogens, and the microbiome among various age groups. RSV, *S. pneumoniae*, and *C. albicans* were the dominant pathogens, and co-infections were the primary cause of severe pneumonia. Older children showed increased microbial richness and diversity compared to infants, with representative microorganisms identified in both groups. These results provide a solid basis for enhancing our understanding of the relationship between severe pneumonia and pulmonary microorganisms in children.

Availability of data and material

The data presented in the study are deposited in the SRA (https://www.ncbi.nlm.nih.gov/sra/) repository, accession number PRJNA1058125.

Ethical statement

The studies involving humans were approved by the Ethics Committee of The Heilongjiang Hospital of Beijing Children's Hospital, Capital Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Due to the retrospective nature of the study, the need for informed consent was waived by the Ethics Committee of The Heilongjiang Hospital of Beijing Children's Hospital, Capital Medical University. The patients were anonymized, and their information was nonidentifiable.

Author contributions

XYY: methodology, investigation, formal analysis, writing – original draft, writing – review, and editing, funding. JCL and RY: provided the resources and collected samples. WG and YFZ: methodology, formal analysis, writing – original draft, writing – review, and editing. All authors critically revised and provided the final approval for this manuscript.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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