

Detection of *Pneumocystis jirovecii* Pneumonia in Infants with Non-Human Immunodeficiency Virus Admitted to Pediatric Intensive Care Using Metagenomics Next-Generation Sequencing

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Objective: This study aimed to investigate the characteristics of the non-human immunodeficiency virus (HIV) pneumocystis pneumonia (PCP) via the microbial composition of *Pneumocystis jirovecii* pneumonia in the lower respiratory tract in infants with severe pneumonia who were hospitalized in the study's pediatric intensive care unit (PICU).

Methods: The clinical characteristics of 16 infants with non-HIV PCP (the PCP group) and 33 infants with severe pneumonia (the control group) who were hospitalized at the same time in the PICU were analyzed retrospectively. Using metagenomic next-generation sequencing (mNGS), the bronchoalveolar lavage fluid (BALF) of the two groups was analyzed, and the microbial results and clinical data were compared.

Results: Compared with the control group, the infants in the PCP group had a lower incidence of cough (25% vs 78.8%; $P < 0.05$), a greater history of surgery (50.0% vs 39.1%; $P < 0.05$), and a more significant decrease in C3, C4, and CD4/CD8 ratios (all $P < 0.05$). The pathogenic bacteria in the BALF included *P. jirovecii*, respiratory syncytial virus, cytomegalovirus (CMV), and *Staphylococcus aureus*. The predominance of viral infection in the PCP group was significantly higher than in the control group ($P < 0.05$), especially CMV (43.5% vs 15.2%; $P < 0.05$). The top five symbiotic microorganisms detected in the BALF of the 49 infants were *Streptococcus*, *Propionibacterium*, *Rothia*, *Staphylococcus*, and *Moraxella*. There was no significant difference in the relative abundance of common symbiotic microorganisms between the two groups (all $P > 0.05$).

Conclusion: Non-HIV PCP has a higher incidence in PICU infants with severe pneumonia, especially those with underlying diseases or who are immunocompromised, which are clinically difficult to treat. A BALF analysis using mNGS is helpful for early and clear diagnoses. It also helps to clarify the distribution of pathogenic and lower respiratory tract colonizing bacteria in infants with severe pneumonia.

Keywords: non-HIV *Pneumocystis jirovecii* pneumonia, metagenomic next-generation sequencing, pathogen detection, lower respiratory tract microorganisms, severe pneumonia, infants

Introduction

Pneumocystis pneumonia (PCP) is a highly fatal opportunistic lung disease caused by infection with *Pneumocystis jirovecii*; it is commonly known as PCP-after-HIV (human immunodeficiency virus) infection.¹ The main symptoms in patients with PCP are fever, cough, and dyspnea, and respiratory failure may occur in severe cases.² The increase in the number of patients with tumors, organ transplants, and autoimmune diseases in conjunction with improvements in bronchoscopes and microbiological detection technology over recent years have significantly increased the incidence and mortality of non-HIV PCP.^{1,3} However, few studies have been conducted involving infants. Therefore, this article retrospectively analyzed the clinical characteristics of non-HIV PCP diagnosed using metagenomic next-generation

sequencing (mNGS), the pathogens of severe infant pneumonia in the study's pediatric intensive care unit (PICU), and the distribution of lower respiratory tract microorganisms.

Materials and Methods

Subjects

A total of 49 infants (27 males and 22 females) with severe pneumonia who were admitted to the PICU of the Guangdong Women and Children's Hospital between September 1, 2020, and March 31, 2021, were enrolled in the study. The diagnostic criteria were severe pneumonia with two or more of the following:⁴ 1. respiratory rate >70 rpm for infants <12 months of age, 2. Apnea, 3. increased breathing effort, including retractions, dyspnea, nasal flaring, and grunting, 4. partial pressure of oxygen in arterial blood to a fraction of inspired oxygen ratio <250 mmHg, 5. multilobar infiltrates, 6. altered mental status, 7. Hypotension, 8. pleural effusion, 9. comorbid condition, 10. unexplained metabolic acidosis, and 11. a pediatric early warning score of >6.⁵

The inclusion criteria were 1. aged <6 months, 2. bronchoalveolar lavage fluid (BALF) mNGS was performed, and 3. complete clinical data. The single exclusion criterion was a positive HIV test.

Research Methods

The clinical data were collected and retrospectively analyzed.

Patient Grouping

The patients were split into two groups: the PCP (n = 16; 8 males and 8 females) and control groups (n = 33; 19 males and 14 females). The diagnostic criteria for PCP were as follows: (1) *P. jirovecii* was detected in the BALF samples, (2) patients presented with the clinical characteristics of a PCP infection, such as fever, cough, tachypnea, and dyspnea, (3) patients showed interstitial pneumonia changes in lung imaging,⁶ and (4) empirical treatment was effective. The control group consisted of infants <6 months of age with severe pneumonia who were hospitalized during the same period with mNGS results from their BALF samples that were negative for *P. jirovecii* DNA and RNA.

Bronchoalveolar Lavage Fluid Collection and Inspection

Standardization was achieved by strict adherence to the operating procedures, quality controls, and guidelines for qualified BALF standards.^{7,8} Specifically, all operations were performed by an experienced and skilled respiratory intervention physician using a curved bronchoscope (outer diameter = 2.8 mm). The qualification requirements for BALF specimens were ① a recovery rate ≥40%, ② not mixed with blood (red blood cells <10% and epithelial cells <5%), ③ diseased lobe segment lavage was collected according to recent chest Computed Tomography results; for cases with diffuse lung disease, the right middle lobe or left lung lingual segment lavage was collected, ④ volume of lavage: 1 mL/kg/time, ≤20 mL/time, and total ≤5–10 mL/kg. The BALF samples (10–20 mL) were collected in a sterile container, kept at room temperature, and sent for analysis within 2 h. Specimens that could not be sent for analysis within this timescale were stored in a refrigerator at 4°C. All specimens were analyzed within 24 h.

Metagenomic Next-Generation Sequencing Analysis

① Nucleic acid extraction: Nucleic acid extraction was performed according to the instructions of the TIANamp Micro DNA Kit (Tiangen Biology, Beijing), and 0.5–2-mL BALF was extracted and mixed with glass beads. Nucleic acids were quantified and quality assessed using a Qubit 4.0 (Thermo Fisher Scientific, USA) fluorometer. ② Library construction and sequencing: Targeted amplification of the 16-srDNA gene segment was performed using Ion 16S Metagenomics Kit targeting, and the library was constructed. The DNA library concentrations were quality controlled and analyzed using a Qubit dsDNA HS Assay Kit and Agilent 2100 QC library inserts, and they were sequenced using an Ion PGM. Then, 50–1000 ng of RNA was mixed with ribosomal RNA (rRNA) depletion probes for rRNA depletion. Fragmentation, the synthesis of the first and second strands, end repairment, and adaptor ligation were conducted for library construction using an RNA library construction kit (Vision Medicals, Guangzhou, China). After pooling the amplified libraries, sequencing was completed on an Illumina NextSeq sequencer 550 (Vision Medicals), and a minimum of 5M 75 bp single-end reads per sample were obtained for metagenomic analysis. ③ Bioinformatics analysis: A data

analysis was performed using the IDseq™ (Vision Medicals) commercial bioinformatic pipeline. The low-quality short (35 bp) reader and adapter were removed before mapping to the human genome for host background depletion. The remaining reads were examined using a curated microbial database consisting of viruses, bacteria, fungi, and parasites and were taxonomically classified and annotated after removing reads with low complexity and those belonging to plasmids. Pathogens were defined based on the *ABX Guide: Diagnosis and Treatment of Infectious Diseases*.⁹

Statistical Analysis

Analyses were carried out using the SPSS version 19.0 platform. Normally distributed variables were expressed as mean $\pm$ standard deviation and were compared using a Student's *t*-test. Nonparametric continuous variables were presented as median (interquartile range) values and compared using a Mann–Whitney *U*-test. Categorical variables were expressed as percentages and compared using an χ^2 test. A value of $P < 0.05$ was considered statistically significant.

Results

Clinical Features and Bronchoscopic Findings in the Pneumocystis Pneumonia Group

During the study period, 16 infants with severe pneumonia (8 males, 8 females) were diagnosed with PCP by mNGS examination of their BALF samples. The average age of these infants was 3.69 ± 1.25 months, ranging from 1 month + 9 days to 5 months + 17 days. In total, 79 cases of severe pneumonia were hospitalized with mNGS detection during the study period, of which 49 were less than 6 months old. The detection rate of *P. jirovecii* among all 79 cases of severe pneumonia was 20.3% (16/79), while in infants less than six months old with severe pneumonia, the detection rate was 32.7% (16/49).

In the PCP group, seven cases (43.8%, 7/16) were malnourished, four (25%, 4/16) were born prematurely (among which one was super premature at 27 ± 1 weeks with a birth weight of 880 g), four (25%, 4/16) had community-acquired pneumonia, and 12 (75%, 12/16) had basic diseases, including three cases of congenital diaphragmatic hernia, two cases of congenital heart disease, four cases of airway malformation, two cases of esophageal atresia, two cases of metabolic disease, one case of bronchopulmonary dysplasia, and one case of cholestatic hepatitis. Eight cases had a history of surgery (50%, 8/16); of these, three had diaphragmatic surgery, two had cardiac surgery, two had esophagus surgery, one had airway surgery, one had biliary surgery, and one had both congenital heart disease and a diaphragmatic hernia. Both operations were performed. None of the infants had undergone organ transplantation or had tumors (including solid tumors and hematologic malignancies). Nine infants received mechanical ventilation treatment (56.3%, 9/16). All the infants in the PCP group were treated with broad-spectrum antibiotics between 1 and 90 days before mNGS detection. Five cases (31.3%, 5/16) received a combination of three or more antibiotics, while four (25%, 4/16) received carbapenems and/or vancomycin/linezolid.

The clinical manifestations in the PCP group were fever (43.8%, 7/16), cough (25%, 4/16), wheezing (25%, 4/16), and pulmonary rales (81.3%, 13/16). Chest radiographs showed typical ground-glass changes in two cases (12.5%, 2/16). Other chest radiographs were varied due to primary disease and mixed infection; they revealed extensive exudation of both lungs in three cases, extensive exudation with pulmonary consolidation and atelectasis in four cases, and extensive exudation with emphysema in one case.

In the auxiliary examination, routine blood tests and C-reactive protein, procalcitonin, and other infection indices were almost the same as if a bacterial infection were present. The alanine aminotransferase (ALT) elevation of liver function was observed in four cases (25%, 4/16), including mild ALT elevation in two cases (>40 U/L) and more than double the ALT elevation in two cases (>80 U/L). Aspartate aminotransferase increased in seven cases (43.8%, 7/16), of which four had a mild increase (>40 U/L) and two had more than double the increase (>80 U/L). Myocardial creatine kinase increased in three cases (18.8%, 3/16), and creatine kinase-MB increased in six cases (37.5%, 6/16), all of which were only slightly elevated. Renal function (creatinine and urea nitrogen) was normal in all cases.

Immune function tests were completed in seven cases. The results showed that humoral immunity was not significantly abnormal: immunoglobulin G was not decreased in one case, and immunoglobulin A was decreased in only one case (<0.08 g/L). The main manifestations of the immune function test were decreases in C3 (<0.79 g/L) in five

cases (71.4%, 5/6), in C4 (<0.16 g/L) in five cases (71.4%, 5/7), in NK cells ($<9.9\%$) in six cases (85.7%, 6/7), in CD4 T cells ($<28.4\%$) in four cases (57.1%, 4/7), and in CD4/CD8 (<1.02) in four cases (57.1%, 4/7) (see Table 1).

On genetic examination, one of the infants in the PCP group was confirmed to have X-linked severe combined immunodeficiency (see Table 1). Regarding bronchoscopic changes, two infants in the PCP group showed typical ground-glass changes on their chest radiographs, with small amounts of white sticky secretions revealed under a bronchoscope. Other bronchoscopy findings due to primary disease and mixed infection showed large amounts of gray–white and yellowish–white viscous secretions accompanied by the congestion of some bronchial segments, resulting in local ventilation obstruction.

The patients in the PCP group were treated with oral sulfamethoxazole and trimethoprim (SMZ/TMP) in combination with human immunoglobulin and anti-infective therapies according to the patients' other combined pathogens. Two of the infants died (12.5%, 2/16), of which one died when the support was withdrawn. All the other cases were controlled. By the end of the study, 10 cases had improved and were discharged from the hospital.

Comparison of the Two Groups

Comparison of the PCP and control groups showed that the age of the PCP group was significantly higher ($P < 0.05$), but there were no significant differences in weight, preterm infants, infants who were smaller than their gestational age, and malnutrition ($P > 0.05$). The incidence of cough was significantly lower in the PCP group ($P < 0.05$), but there were no significant differences between the two groups in the incidence of fever, wheezing, and pulmonary signs (all $P > 0.05$). The number of infants with a history of surgery in the PCP group was significantly higher than in the control group ($P < 0.05$).

There were no significant differences between the two groups in the combination of basic diseases, medication before diagnosis (such as antibiotics and glucocorticoids), imaging changes, biochemical tests (such as liver/kidney function and myocardial enzymes), and auxiliary tests (such as complete blood count, including the elevation of eosinophils) ($P > 0.05$). In terms of immune function, the decreases of the C3, C4, and CD4/CD8 ratios in the PCP group were significantly greater than in the control group (all $P < 0.05$). Although the decrease of CD4 was greater in the PCP group than in the control group, the difference was not statistically significant ($P > 0.05$) (see Table 1).

Due to missing data, no further multifactor analysis was conducted. Furthermore, due to a large number of covariables and the small number of cases with PCP in the sample, it was not appropriate to conduct multivariate logistic regression analysis to explore the risk factors.

Metagenomic Next-Generation Sequencing Analysis

The mNGS analysis of BALF samples obtained via bronchoscopy can accurately and quickly detect the presence of pathogenic bacteria in infants with pneumonia and identify the bacterial species and flora changes of suspected human symbiotic microorganisms in the lower respiratory tract. Metagenomic next-generation sequencing is a process that includes specimen collection, sample processing, library construction, gene sequencing, biological information analysis, and report interpretation. During the process, nucleic acids are extracted and sequenced, the human-derived hosts are removed, species identification is performed with reference to the database, and reports are interpreted based on the clinical features. According to the number of sequence alignments, genome size, and genome conservation, the species can be accurately identified by weighting and scoring to determine the credibility of the microorganism in the sample and ascertain the presence of a pathogen. According to the classification of viruses, bacteria, fungi, and parasites as well as the size of the genome, the relative proportion of the pathogen in the corresponding classification can be calculated to determine the quantity of the pathogen.

Analysis of Pathogenic Microorganisms

In the 49 infants with severe pneumonia who were enrolled in this study, the pathogenic microorganisms identified were *P. jirovecii* ($n = 16$), respiratory syncytial virus (RSV) ($n = 16$), cytomegalovirus (CMV) ($n = 13$), *Staphylococcus aureus* ($n = 9$), *Escherichia coli* ($n = 7$), enterovirus (EV) ($n = 6$), human respiratory virus type 3 ($n = 6$), *Klebsiella pneumoniae* ($n = 6$), torque teno virus ($n = 5$), and others ($n = 1-4$, see Figure 1). In the PCP

Table I Comparison of Clinical Data and Pathogenic Microorganisms Between the Two Groups

Item	PCP Group (n=16 Cases)	Control Group (33 Cases)	T/z/ χ^2	p
Gender				
Age (month)	3.69±1.25	2.30±1.16	3.741	0.001
Weight (kg)	4.75±1.43	4.35±1.04	0.939	0.359
Malnutrition	7(43.8%)	6(18.2%)	3.614	0.086
Preterm infants	4(25.0%)	10(30.3%)	0.148	0.926
SGA	2(12.5%)	1(3.0%)	1.681	0.245
Fever	7(43.8%)	17(51.5%)	0.260	0.762
Cough	4(25%)	26(78.8%)	13.132	0.000
Wheezing	4(25%)	8(24.2%)	0.003	1.000
Lung rales	13(81.3%)	29(87.9%)	0.387	0.668
Operation History	8(50.0%)	3(9.1%)	10.358	0.003
Background disease	11(68.8%)	26(78.8%)	0.587	0.491
Breathing machine	9(56.3%)	9(27.3%)	3.893	0.048
Ground glass opacity in chest films	2(12.5%)	7(21.2%)	0.545	0.698
Elevation of ALT	4(25.0%)	9(27.3%)	0.029	1.000
Elevation of AST	7(43.8%)	11(33.3%)	0.503	0.537
Elevation of CK-MB	6(37.5%)	10(30.3%)	0.254	0.748
Elevation of Cr or/and BUN	0	3(9.0%)	/	/
Time of antibiotic use before examination	4(3, 17)	4(3, 10)	0.187	0.851
Use of triple antibiotics	5(31.1%)	4(12.1%)	2.630	0.13
Use of glucocorticoids throughout the body	5(31.3%)	10(30.3%)	0.005	1.000
White blood cells	13.31±7.26	10.04±3.99	1.954	0.057
Neutrophils	47.99±15.80	46.47±16.52	0.295	0.770
Lymphocytes	41.59±15.58	39.83±15.67	0.350	0.728
Increase of E counts	3(18.8%)	8(24.2%)	0.187	1
Increases of E ratio	3(18.8%)	6(18.2%)	0.002	1
High IgG	3(3/7, 42.9%)	5(5/12, 41.7%)	0.003	1
Low IgA	2(2/7, 28.6%)	2(2/12, 16.7%)	0.377	0.603
High C3	5(5/7, 71.4%)	3(3/12, 25%)	3.909	0.048
Low C4	5(71.4%)	1(1/12, 8.3%)	8.146	0.01
Decrease of total T lymphocytes	3(3/7, 42.9%)	1(1/12, 8.3%)	3.170	1.117
Increase of total B lymphocytes	4(4/7, 57.1%)	6(6/12, 50.0%)	0.090	1.000
Decrease of NK	5(5/7, 71.4%)	4(4/12, 33.3%)	2.574	0.17
Decrease of CD4 ⁺ T	3(3/7, 42.9%)	2(2/12, 16.7%)	1.564	0.305
Decrease of CD4 ⁺ /CD8 ⁺ T	5(5/7, 71.4%)	2(2/12, 16.7%)	5.698	0.045
CMV	7(43.8%)	5(15.2%)	4.766	0.04
RSV	4(25%)	12(36.4%)	0.663	0.526
EV	4(25%)	2(6.1%)	3.597	0.08
Staphylococcus aureus	4(25)	5(15.2%)	0.697	0.449
Klebsiella pneumoniae	0	6(18.2%)	/	/
Escherichia coli	1(6.3%)	6(18.2%)	1.253	0.402
Tiny Ureaplasma	2(12.5%)	2(6.1%)	0.596	0.588
Ureaplasma Urealyticum	2(12.5%)	0	/	/
Candida albicans	2(12.5%)	2(6.1%)	0.596	0.588
Virus dominated	10(62.5%)	14(42.2%)	4.332	0.037
Bacteria dominated	3(18.8%)	12(36.4%)	1.574	0.324
Multiple infection	6(37.5%)	6(18.2%)	2.175	0.169

Note: /: There is no need to compare the two groups because one of them is "0".

Abbreviations: SGA, small for gestational age; CMV, cytomegalovirus; RSV, respiratory syncytial virus; EV, rhinovirus; ALT, glutamic pyruvic transaminase; AS, glutamic oxalacetic transaminase; CK-MB, creatine kinase -MB; Cr, creatinine; BUN, blood urea nitrogen; E, eosinophilic granulocyte; NK, natural killer cell.

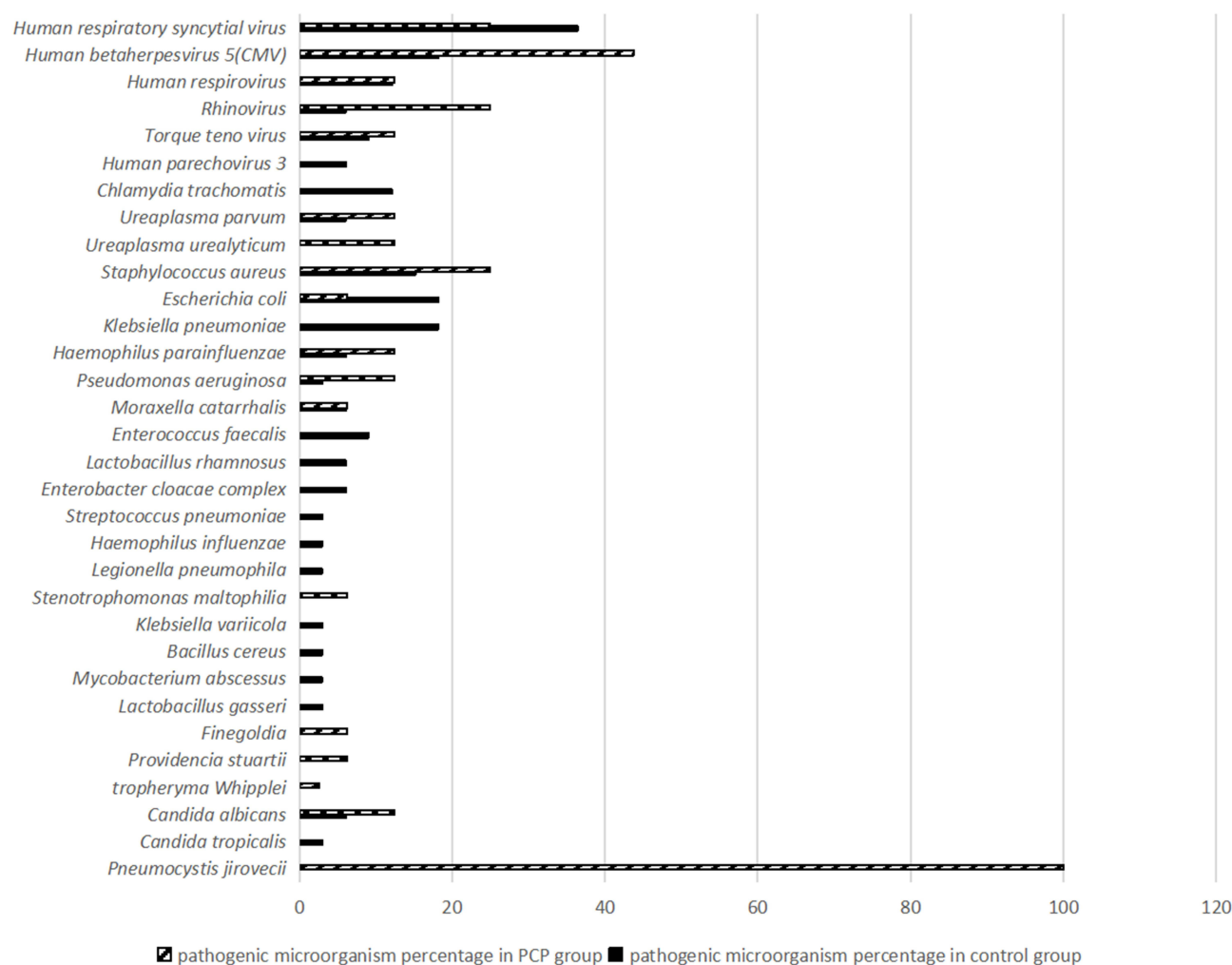


Figure I Distribution of pathogens detected by mNGS in BALF of two groups pathogens.

group, the relative abundance of *P. jirovecii* DNA was 71.3% (0.85–98.7%), with sequence number 13.0 (0.5–2229.0), and the relative abundance of RNA was 43.7% (8.0–96.9%), with sequence number 58.5 (8.5–1,484.25). Except for Case 9, none of the cases were simple sporozoite infections and were often complicated by viruses ($n = 12$), bacteria ($n = 8$), mycoplasma ($n = 4$), fungi ($n = 2$), and other pathogens. Six cases were complicated by multiple infections, ie, a combination of three or more pathogens.

According to the relative abundance and sequence number of pathogenic bacteria associated with PCP, viral infection was dominant in 10 cases in the PCP group (62.5%, 10/16), bacterial infection dominated in three cases (18.8%), and *Ureaplasma urealyticum* dominated in one case (6.2%). Cytomegalovirus had the highest detection rate (43.8%, 7/16), and the second-most-common viruses were RSV and EV (both 25%, 4/16). Four cases of *Ureaplasma* were detected, including two cases of *U. parvum* and two of *U. urealyticum*. Of those co-infected bacteria, *S. aureus* was the most common (25%, 4/16), followed by *Haemophilus parainfluenzae* and *Pseudomonas aeruginosa* (both 12.5%, 2/16) and *Moraxella catarrhalis*, *E. coli*, *Stenotrophomonas maltophilia*, *Providencia stuartii*, *Fascioloides magna*, and *Tropheryma whipplei* (all 6.2%, 1/16). The two cases with combined fungal infections were both *Candida albicans* (see Table 2).

In addition to *P. jirovecii*, the four most common pathogens in the PCP group were CMV, EV, RSV, and *S. aureus*. However, the four most common pathogens in the control group were RSV, CMV, *E. coli*, and *K. pneumoniae*. Compared with the control group, the CMV detection rate in the PCP group was significantly higher ($P < 0.05$). *K. pneumoniae* was not detected in the PCP group, and there was no statistical difference in the remaining detection rates between the two

Table 2 Detection of Pneumocystis and Other Pathogenic Microorganisms in 16 Children with PCP

Cases	Pneumocystis				Virus					Bacteria					Others	
	Relative Abundance of DNA (%)	DNA Sequence Number	Relative Abundance of RNA (%)	RNA Sequence Number	CMV	RSV	EV	Torque Teno Virus	Human Respiratory Virus Type 3	<i>Pseudomonas aeruginosa</i>	Parainfluenza Virus	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Tropheryma whipplei</i>	<i>Candida albicans</i>	<i>Ureaplasma urealyticum</i>
1	97.4	49	83	210	+											
2	99	14,285	100	144,369	+		+								+	
3	53.1	13	97.9	1539		+		+								+
4	0	0	41.9	16		+	+									
5	0	0	21.6	13		+	+									
6	22.2	2	0	0				+								
7	0	0	7.2	19			+				+					+
8	0	0	36.2	42									+			+
9	3.4	6	0	0												
10	89.5	13	45.4	75	+				+		+					
11	99.9	2921	99.5	17,932	+				+							
12	97.6	86	92.5	816						+		+				
13	95.5	153	93.7	1320								+				+
14	99.8	23,518	0	0	+									+		
15	38.1	9	10.4	7	+	+						+	+			
16	99.8	8680	100	200,402	+										+	

Note: “+”Indicates other pathogenic microorganisms in addition to pneumocystis in this case.

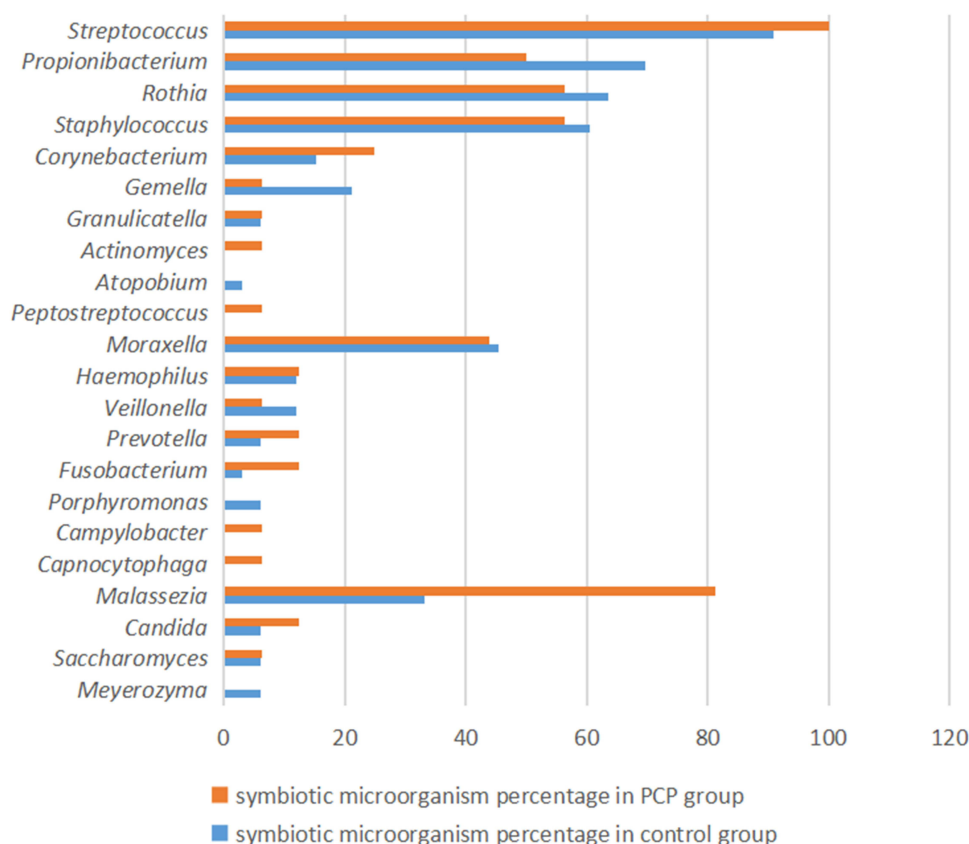


Figure 2 Distribution of human symbiotic microorganism detected by MNGs in BALF of the two groups.

groups ($P > 0.05$). In summary, the predominance of viral infection in the PCP group was significantly higher than in the control group ($P < 0.05$, see Figure 1 and Table 1).

Analysis of Symbiotic Microorganisms

In the 49 infants enrolled in the study, a total of 22 species of suspected human symbiotic microorganisms were detected. Of these, 19 were detected in the PCP group and 18 in the control group. The five most commonly detected species were *Streptococcus* ($n = 46$; relative abundance of DNA 49.15% [17.65–66.45%] and RNA 3.95% [1.775–31.22%]), *Propionibacterium* ($n = 31$; relative abundance of DNA 1.3% [0.2–2.3%] and RNA 2.7% [1.55–7.5%]), *Rothia* ($n = 30$; relative abundance of DNA 4.1% [1.58–10.2%] and RNA 0.6% [0.25–1.2%]), *Staphylococcus* ($n = 29$; relative abundance of DNA 1.9% [0.2–4.5%] and RNA 2.05% [1.02–5.68%]), and *Moraxella* ($n = 22$; relative abundance of DNA 1.6% [0.5–2.6%] and RNA 1.1% [0.35–2.05%]).

A comparison between the two groups showed that the five most common symbiotic microorganisms in the PCP group were *Streptococcus* ($n = 16$), *Staphylococcus* ($n = 9$), *Rothia* ($n = 9$), *Propionibacterium* ($n = 8$), and *Moraxella* ($n = 7$), while the control group had *Streptococcus* ($n = 30$), *Propionibacterium* ($n = 23$), *Rothia* ($n = 21$), *Staphylococcus* ($n = 20$), and *Moraxella* ($n = 15$). Although the sequence of the detection rate of symbiotic microorganisms was slightly different between the two groups, there were no significant differences in the percentages or their relative abundance ($P > 0.05$). In the PCP group, six cases (37.5%, 6/16) had a high relative abundance of *Streptococcus* ($>50\%$), but there was no significant difference between this and the 17 cases in the control group (51.5%; 17/33; $\chi^2 = 0.850$; $P = 0.382$). The main symbiotic microorganisms in infants with severe pneumonia were found to be *Streptococcus mitis*, including *S. pseudopneumoniae*, *S. parasanguinis*, and *S. salivarius*, all of which are oral colonization bacteria. *Staphylococcus*, including *S. hominis* and *S. epidermidis*, colonizes the skin and mucous membranes of the human body (see Figure 2 and Table 3).

Table 3 Distribution of Symbiotic Microorganisms in the Two Groups

Category	Genus of Symbiotic Bacteria	Species of Symbiotic Bacteria	PCP Group (n=16)	Control Group (n=33)	Total (n=49)
G ⁺ bacteria	Streptococcus	<i>S. peroris</i>	16(100%)	30(90.9%)	46(93.9%)
		<i>S. mitis</i>	1	2	3
		<i>S. vestibularis</i>	9	26	35
		<i>S. pseudopneumoniae</i>	1	3	4
		<i>S. parasanguinis</i>	7	12	19
		<i>S. salivarius</i>	3	11	14
		<i>S. dysgalactiae</i>	3	6	9
		<i>S. sanguinis</i>	0	1	1
		<i>S. sanguinis</i>	0	1	1
	Propionibacterium		8(50.0%)	23(69.7%)	31(63.3%)
		<i>P. acnes</i>	4	20	24
	Rothia		9(56.3%)	21(63.6%)	30(61.2%)
		<i>R. mucilaginosa</i>	8	22	30
		<i>R. aeria</i>	1	1	2
	Staphylococcus		9(56.3%)	20(60.6%)	29(59.2%)
		<i>S. epidermidis</i>	5	16	21
		<i>S. hominis</i>	3	5	8
		<i>S. capitis</i>	2	4	6
		<i>S. cohnii</i>	1	3	4
		<i>S. pettenkoferi</i>	0	1	1
		<i>S. pettenkoferi</i>	0	1	1
	Corynebacterium		4(25.0%)	5(15.2%)	9(18.4%)
		<i>C. aurimucosum</i>	0	2	2
		<i>C. propinquum</i>	4	1	5
		<i>C. pseudodiphtheriticum</i>	0	1	1
		<i>C. jeikeium</i>	1	1	2
		<i>C. striatum</i>	0	2	2
		<i>C. accolens</i>	0	1	1
	Gemella		1(6.3%)	7(21.2%)	8(16.3%)
		<i>G. haemolysans</i>	1	7	8
	Granulicatella		1(6.3%)	2(6.1%)	3(6.1%)
		<i>G. elegans</i>	1	1	2
		<i>G. adiacens</i>	0	1	1
	Actinomyces		1(6.3%)	0(0.0%)	1(2.0%)
		<i>A. massiliensis</i>	1	0	1
	Atopobium		0	1	1
		<i>A. parvulum</i>	0	1	1
	Peptostreptococcus		1(6.3%)	0(0.0%)	1(2.0%)
		<i>P. stomatis</i>	1	0	1
G ⁻ bacteria	Moraxella		7(43.8)	15(45.5%)	22(44.9%)
		<i>Moraxella osloensis</i>	7	13	21
	Haemophilus		2(12.5%)	4(12.1%)	6(12.2%)
		<i>H. haemolyticus</i>	2	5	7
	Veillonella		1(6.3%)	4(12.1%)	5(10.2%)
		<i>V. dispar</i>	0	1	1
		<i>V. atypica</i>	1	2	3
		<i>V. parvula</i>	0	1	1
	Prevotella		2(12.5%)	2(6.1%)	4(8.2%)
		<i>P. melaninogenica</i>	1	2	3

(Continued)

Table 3 (Continued).

Category	Genus of Symbiotic Bacteria	Species of Symbiotic Bacteria	PCP Group (n=16)	Control Group (n=33)	Total (n=49)
Fungi	<i>Fusobacterium</i>	<i>P. nigrescens</i>	1	0	1
		<i>P. bivia</i>	0	1	1
			2(12.5%)	1(3.0%)	3(6.1%)
		<i>F. nucleatum</i>	1	1	2
		<i>F. hwasookii</i>	1	0	1
	<i>Porphyromonas</i>	<i>F. periodonticum</i>	1	0	1
			0(0.0%)	2(6.1%)	2(4.1%)
		<i>P. catoniae</i>	0	1	1
		<i>P. somerae</i>	0	2	2
			1(6.3%)	0(0.0%)	1(2.0%)
	<i>Campylobacter</i>		1	0	1
	<i>Capnocytophaga</i>	<i>C. concisus</i>	1	0	1
			1(6.3%)	0(0.0%)	1(2.0%)
	<i>Malassezia</i>	<i>C. sputigena</i>	1	0	1
			5(3.1%)	11(33.3%)	16(32.7%)
		<i>M. restricta</i>	1	6	7
		<i>M. globosa</i>	1	4	5
		<i>M. japonica</i>	1	5	6
	<i>Candida</i>	<i>M. furfur</i>	4	0	4
			2(12.5%)	2(6.1%)	4(8.2%)
		<i>C. parapsilosis</i>	2	1	3
		<i>C. metapsilosis</i>	1	0	1
			1(6.3%)	2(6.1%)	3(6.1%)
	<i>Saccharomyces</i>		1	0	1
	<i>Meyerozyma</i>	<i>S. cerevisiae</i>	1	0	1
			0(0.0%)	1(3.0%)	1(2.0%)
		<i>M. guilliermondii</i>	0	1	1

Abbreviations: G⁺ bacteria, Gram-positive bacteria; G⁻ bacteria, Gram-negative bacteria.

Discussion

The pathogen of PCP is *P. jirovecii*; this was believed to be a protozoan in the past but is now considered a fungus based on the analysis of its ultrastructure and its development rRNA germline.⁶ Infant PCP was seen in malnourished (43.8%, 7/16) and premature infants (25%, 4/16) aged <6 months (3.69 ± 1.25 months), which is consistent with the description by Hu et al⁶ of the subacute course of HIV PCP. Non-HIV PCP presents with an acute course and a high incidence of respiratory failure and mortality.¹⁰ However, in the present study, due to the implementation of mNGS, 16 cases of non-HIV PCP were detected in the six months between September 1, 2020, and March 31, 2021, and the mortality rate was only 12.5% thanks to early diagnosis and treatment. The Beijing Children's Hospital reported 60 cases of non-HIV PCP in the last 10 years, with a mortality rate of 41.7% (25/60).¹¹ Therefore, although non-HIV PCP is a serious threat to infants' life and health, early diagnosis and targeted medication can greatly improve their prognosis.

Non-HIV PCP primarily occurs in patients with underlying diseases. Burghi et al¹² reported 321 cases of non-HIV PCP in which the underlying diseases included chronic lymphocytic leukemia (n = 19), organ transplantation (n = 94), solid malignant tumors (n = 39), allogeneic hematopoietic stem-cell transplantation (n = 14), other hematological malignancies (n = 75), and other immunosuppressive therapies (n = 57). The meta-analysis conducted by Liu et al¹⁰ included 867 patients with non-HIV PCP from 13 studies; the main underlying diseases identified were hematologic malignancies (29.1%), autoimmune diseases (20.1%), organ or bone marrow transplantation (14.0%), and solid tumors (6.0%). The majority of the underlying causes were tumors or immunosuppression after transplantation. However, both reports only analyzed adult cases. Ling et al¹¹ reported that for infants, the underlying diseases in patients with non-HIV PCP were connective tissue disease (n = 23; 38.3%), hematological system diseases (n = 14; 23.3%), kidney disease (n = 8; 13.3%), and congenital immune deficiency (n = 10; 16.7%).

In the present study, infants aged <6 months accounted for 62.0% (49/79) of the total pediatric patients with severe pneumonia who were admitted in the six-month study period, of which 16 had non-HIV PCP (32.7%, 16/49); this indicates that infants under six months old, especially those with underlying diseases, are more prone to severe pneumonia and susceptible to pneumocystis. In the present study, the number of cases of congenital developmental deformities that underwent surgery in the PCP group was significantly higher than in the control group ($P < 0.05$), indicating that it is vital for clinicians to be aware of the possibility of *P. jirovecii* infection in infants younger than six months who do not have HIV or tumors and who have not undergone organ transplantation, especially those with severe pneumonia after surgery for congenital developmental abnormalities.

In terms of clinical manifestations, the PCP group had significantly fewer cough symptoms than the control group ($P < 0.05$), but there were no significant differences between the two groups in other symptoms and signs, routine laboratory examinations, or imaging changes ($P > 0.05$). However, it should be noted that typical ground-glass changes in the lungs are not easily identified, and eosinophilia lacks specificity, so this could be related to the fact that infants with PCP are often complicated with other pathogenic bacterial infections, resulting in characteristics being easily masked. However, in the PCP group, significantly more patients needed mechanical ventilation than in the control group ($P < 0.05$), suggesting that the disease is aggravated easily, and the incidence of respiratory failure is high. Two cases in the PCP group with typical ground-glass chest imaging changes showed small amounts of white viscous secretions under the bronchoscope. Therefore, it is suggested that infants with PCP alone may be characterized by fewer bronchoscopic changes that do not correspond to the severity of a patient's clinical symptoms.

A decrease in immune function is one of the primary causes of PCP. Ling et al¹¹ reported that the area under the receiver operating characteristic curve of the CD4/CD8 T-cell ratio in the diagnosis of non-HIV PCP was 0.902 (95% confidence interval: 0.849–0.955), and the diagnostic threshold was 0.715 (sensitivity 89.2%, specificity 80.4%). In the present study, the genetic examination found that one case in the PCP group had X-linked severe combined immunodeficiency. The decrease in complements C3 and C4 and the CD4/CD8 ratio in the PCP group were significantly greater than in the control group ($P < 0.05$), and although the proportions of NK cells and CD4+ T cells were reduced in the PCP group, the difference between the two groups was not statistically significant.

In terms of humoral immunity, there was no significant abnormality in either group. This, combined with the fact that most of the infants with PCP in the present study were complicated with viral infections (62.5%, 10/16), it can be speculated that the decrease in cellular immunity after viral infection may be one of the reasons for the susceptibility to complicated sporozoite infection. Furthermore, the detection rate of CMV in the PCP group was significantly higher than in the control group (43.8%, $P < 0.05$). Further study is required to determine whether other mechanisms exist in addition to the effect on the immune function.

The present study found that the incidence of multiple infections was significantly higher in the PCP group than in the control group ($P < 0.05$), which is most likely caused by damaged immune functions. These multiple infections can further damage immune function, resulting in mutual causality. Six patients were tested for blood (1,3)- β -D-glucan, and four tested positive. In two of these cases, *C. albicans* was detected. Due to the small number of cases examined, it is impossible to determine whether PCP caused the elevation in blood (1,3)- β -D-glucan levels. However, Lahmer et al¹³ confirmed that detecting the (1,3)- β -D glucan levels in BALF is helpful for diagnosing PCP.

The results of the present study indicate that PCP in infants is characterized by atypical symptoms, rapid and severe disease development, and often mixed infection with multiple pathogens, which is consistent with prior studies.^{14–16} Difficulty in diagnosis delays the implementation of targeted anti-infective treatment, leading to higher mortality rates. Therefore, some researchers have proposed preventive therapy for high-risk patients who have undergone transplantation, receive long-term high-dose immunosuppressants, have severe primary immunodeficiency, and have CD4 T cells of $<200/\mu\text{L}$.^{17,18} However, a recent meta-analysis¹⁰ showed that preventive treatment did not significantly improve the prognosis of patients with non-HIV PCP. Therefore, the early detection of *P. jirovecii* is a more effective method for guiding treatment and improving prognosis.

The mNGS technology developed in recent years requires no preset, culture, or bias and can directly extract DNA and RNA from clinical samples for high-throughput sequencing. Special pathogen database comparison and bioinformatics analysis can complete the detection of pathogens, such as bacteria, fungi, viruses, and parasites. Its advantage in the field

of infectious disease diagnosis lies in its ability to detect pathogens that cannot be detected by other traditional means, so it has become an important means of infection detection, especially in critically ill patients or those with complicated diseases.¹⁹ Bronchoalveolar lavage fluid samples can be obtained easily during bronchoscopy in patients with respiratory diseases. They can be used as high-quality clinical test specimens for pathogen assessment in patients with respiratory infections. The technique's effectiveness is due to the fact that BALF can contain a higher concentration of nucleic acid genetic information, which makes it more sensitive when applied to metagenomic sequencing, especially in the detection of viruses and fungi.^{20,21} The present study used the mNGS of BALF samples to diagnose early-stage PCP and observe the microorganism distribution in the lower respiratory tract of infants with severe pneumonia. The three most common pathogens were *P. jirovecii*, RSV, and CMV. This shows that *P. jirovecii* has a high detection rate, although it is easily neglected in clinical practice at present.

However, these results also suggest that viral infection, especially via CMV, is more common in infants and may promote the occurrence of PCP. The most common bacterial infections of severe pneumonia in infants were found to be *S. aureus*, *E. coli*, and *K. pneumoniae*. *Streptococcus pneumoniae*, which is common in domestic community-acquired pneumonia,²² was only detected in one case, which could be related to the fact that the patients enrolled in this study were treated mainly for severe pneumonia after being transferred from other hospitals. Therefore, they had been treated with various antibiotics, and many had undergone surgery. These results indicate that using mNGS in each treatment center can guide treatment.

The term "microbiome" refers to the total number of microbes in a system, ecosystem, or specific area. The coexistence of these microorganisms with their human hosts means that microbial dysbiosis can lead to disease, and the growth of opportunistic pathogens can threaten the health and life of the hosts. Therefore, there is a global effort to characterize the microbiome in various parts of the body to understand the relationship between the microbiome and its host.^{23,24} The development of mNGS technology provides a favorable means for studying human microbiomes. The present study attempted to investigate whether there were characteristic changes in the microflora of the lower respiratory tract in infants with PCP. The BALF analysis revealed that a variety of oral and skin symbiotic microorganisms were colonizing the lower respiratory tract of the infants, with the most common bacteria being *Streptococcus*, *Propionibacterium*, *Rothia*, *Staphylococcus*, *Moraxella*, and *Malassezia* (fungus). No statistical difference was found in the microecological distribution of the main symbiotic microorganisms between the PCP and control groups ($P > 0.05$). However, due to the small sample size, an association between the flora distribution and *P. jirovecii* infection cannot be excluded.

Symbiotic microorganisms can also be opportunistic pathogens: a high relative abundance (>50%) of *Streptococcus* was detected in 23 infants (47.0%, 23/49), and there were four cases (8.2%) with a high relative abundance of *Staphylococcus* (>50%). These colonizing bacteria multiply when the body's immune system is weak, so they need to be considered pathogenic bacteria. Based on a comprehensive consideration of the relative abundance of pathogenic and colonizing bacteria, a clinical center can select the most appropriate antibiotic(s) for treatment. For example, in Case 9 in the PCP group (see Table 2), the DNA sequence number of *Pityrosporum ovale* was only 6, and the relative abundance was only 3.4%. No other pathogenic microorganisms were detected, but the DNA sequence number of human skin symbiotic *S. epidermidis* was 91,892, and the relative abundance was 70.7%. This patient was a premature infant with bronchopulmonary dysplasia who was ventilator assisted and treated with various antibiotics in the early stage. Based on the results, *S. epidermidis* was considered a pathogenic bacterium, and anti-infective treatment with SMZ/TMP combined with vancomycin was implemented.

The present study has several limitations. It was a single-center retrospective cohort study with a relatively small sample size. The epidemiology, risk factors, and relationship between non-HIV PCP and other microorganisms in infants require further investigation.

Conclusion

The morbidity rate of infant non-HIV PCP is high, and it is difficult to distinguish based on clinical features because of its frequent combination with other infections. Clinicians should be vigilant regarding PCP in infants under the age of six months with a history of surgery, rapid disease progression, and need for ventilator support. Using mNGS examination is

an effective means of gaining more accurate feedback on the infection status and lower respiratory tract microbial colonization of infants and is conducive to clear diagnosis and precise treatment.

Ethics Approval and Consent to Participate

This study was conducted with approval from the Ethics Committee of Peking University Shenzhen Hospital (2021-056). This study was conducted in accordance with the declaration of Helsinki and patient data were kept confidential. Due to the retrospective nature of the study, informed consent was waived.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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