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ORIGINAL ARTICLE

Detection of *Pneumocystis Jirovecii* in Immunocompetent Children with Lower Respiratory Tract Infection

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SUMMARY

Background: **Few studies have been conducted on *Pneumocystis jirovecii* infection in immunocompetent children with respiratory symptoms, and co-infection patterns are limited. This study aimed to describe the detection of *Pneumocystis jirovecii* by targeted next-generation sequencing (tNGS) in immunocompetent children with lower respiratory tract infection (LRTI) and compare co-infection patterns.**
Methods: **From March through July 2023, 117 immunocompetent children with LRTI underwent bronchoalveolar lavage fluid (BALF) testing via tNGS at the Maternal and Child Health Hospital in Meizhou (Guangdong, China). The prevalence of *Pneumocystis jirovecii* and co-infection with other pathogens were analyzed.**
Results: **Respiratory pathogens were identified in 114 children (97.4%), and *Pneumocystis jirovecii* was detected in 18 (15.4%). The median age of the *Pneumocystis jirovecii* positive group was significantly younger than that of the negative group (0.8 vs. 3.9 years, p = 0.008). Co-infections with *Pneumocystis jiroveci* were common, but only cytomegalovirus and human respiratory syncytial virus showed a positive correlation and *Mycoplasmoides pneumoniae* showed a negative correlation. Two out of three children treated with trimethoprim-sulfamethoxazole and fifteen children with no specific therapy recovered well from clinical and radiological signs. Literature review showed that *Pneumocystis jirovecii* was relatively common in immunocompetent children with respiratory symptoms (0.8% to 32.4%).**
Conclusions: **The clinical significance of detection of *Pneumocystis jiroveci* in immunocompetent children with LRTI should be further investigated, and specific therapy could be considered individually based on clinical symptoms, radiological features, and co-infection patterns.**
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KEYWORDS

Pneumocystis jirovecii, children, immunocompetence, respiratory co-infection, epidemiology

INTRODUCTION

In 2022, WHO published a report highlighting the first-ever list of fungal "priority pathogens" [1]. In the medium priority group, *Pneumocystis jirovecii* is a globally distributed, opportunistic pathogenic fungus. *Pneumocystis jirovecii* pneumonia (PJP) is a life-threatening disease in immunocompromised individuals and a serious public health problem. Recent global estimates suggest that ~500,000 cases of PJP occur annually [2].

Pneumocystis jirovecii infection without leading to PJP (defined as colonization) is also common in immunocompetent individuals, particularly children [3]. Colonization has been detected by PCR in nasopharyngeal aspirates, sputum, bronchoalveolar lavage fluid (BALF), and lung tissue, and the prevalence can be as high as 82% [4]. The clinical significance of colonization is not yet fully understood, but it may have several important clinical implications. Colonization is the first step in the progression to PJP and is the potential reservoir for transmission [5]. Although data are limited, researchers have documented the possible association of colonization with bronchiolitis, self-limiting upper respiratory tract infection, respiratory distress syndrome, and abnormal lung development in immunocompetent children [6-9].

PCR-based assays have long been the preferred method for the diagnosis of *Pneumocystis jirovecii* infection, with higher sensitivity than traditional tests [10,11]. Several studies have also shown that quantitative PCR (qPCR) can help differentiate colonization from PCP with acceptable sensitivity and specificity based on lab-developed cutoff values [12-14]. However, the lack of standardization between qPCR assays limits its application. In recent years, next-generation sequencing (NGS), including metagenomic NGS (mNGS) and targeted NGS (tNGS), has become more widely used for pathogen detection in various indications. A meta-analysis demonstrated that mNGS had a pooled sensitivity of 0.974 and specificity of 0.943 for the diagnosis of PJP [15]. A major advantage of NGS-based assays over PCR is the ability to identify co-infections in the diagnosis of *Pneumocystis jirovecii* infection, which has been widely described but has only been limited to several pathogens identified by PCR, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [16-18].

The aim of this retrospective study was to describe the detection of *Pneumocystis jirovecii* by tNGS in a cohort of immunocompetent children with lower respiratory tract infection (LRTI). The frequency of other respiratory pathogens was compared between the *Pneumocystis jirovecii*-positive and -negative groups. A brief literature review was also performed to summarize the prevalence of *Pneumocystis jirovecii* in immunocompetent children with respiratory symptoms.

MATERIALS AND METHODS

Patient enrollment and data collection

From March 2023 through July 2023, pediatric patients admitted to the Maternal and Child Health Hospital in Meizhou (Guangdong, China) that fulfilled clinical and radiological criteria for LRTI were enrolled in the study. Inclusion criteria were the absence of immunocompromising conditions, no underlying diseases, and fulfilling the indications for bronchoscopy. BALF was collected for pathogen detection, including conventional microbiology and tNGS testing. Demographic and clinical data were collected by bulk retrieval from the hospital's electronic medical record system.

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of Maternal and Child Health Hospital in Meizhou. Informed consent was waived due to the retrospective nature of this study.

Targeted NGS for detection of respiratory pathogens

The respiratory pathogens detected by tNGS are shown in Table 1, including 46 bacteria, 9 fungi, and 43 viruses. The tNGS test was performed by Guangzhou Da An Clinical Laboratory Center. DNA and RNA were simultaneously isolated from BALF followed by reverse transcription. The library was constructed using the Respiratory Pathogen Detection Kit (Daan Gene, China). Briefly, reverse transcription products were used for multiplex PCR amplification, and AMPure XP beads (Beckman Coulter, US) were used for size selection. Sequencing linkers and barcodes for sample identification were added to obtain sequencing libraries. Libraries were quantified by Qubit 4.0 fluorometer (Thermo Fisher Scientific, US) and then diluted and mixed. High-throughput sequencing was performed on the Illumina MiniSeq platform (Illumina, US) with single-end sequencing. Raw data were analyzed, and pathogens were identified using a laboratory-developed bioinformatic analysis pipeline.

Results for opportunistic bacteria were interpreted semi-quantitatively using genomic copies of nucleic acid per milliliter of specimen (copies/mL) to estimate relative abundance within a sample. Atypical bacteria, mycobacteria, viruses, and fungi were interpreted qualitatively.

Definitions

According to the revised EORTC/MSGERC criteria, the diagnosis of proven PJP is based on clinical and radiological criteria, plus identification of *Pneumocystis jirovecii* by conventional or immunofluorescence staining in tissue or respiratory tract specimens [10]. And probable PJP is defined by the presence of host factors and clinical and radiological criteria, plus detection of *Pneumocystis jirovecii* by qPCR in respiratory tract specimens and/or 1,3-β-dglucan (BDG) in serum. These criteria are not applicable to immunocompetent children, and conventional or immunofluorescence staining for *Pneumocystis jirovecii* is not routinely used in our hospital. Therefore, in the present study, we used "putative PJP" to guide diagnosis and treatment, which was based on a composite clinical standard consisting of clinical symptoms and signs, radiographic features, and co-infection patterns. Otherwise, the detection of *Pneumocystis jirovecii* was classified as colonization.

Table 1. List of the pathogens detected by the targeted NGS.

Category	Pathogen		
Bacteria (46)	*Acinetobacter baumannii*	*Bacillus anthracis*	*Bacillus cereus*
	Bordetella parapertussis	*Bordetella pertussis*	*Brucella*
	Burkholderia cenocepacia	*Burkholderia cepacia*	*Chlamydia pneumoniae*
	Chlamydia psittaci	*Corynebacterium diphtheriae*	*Coxiella burnetii*
	Enterobacter cloacae	*Enterococcus faecalis*	*Enterococcus faecium*
	Escherichia coli	*Haemophilus influenzae*	*Klebsiella pneumoniae*
	Legionella pneumophila	*Leptospira interrogans*	*Listeria monocytogenes*
	Moraxella catarrhalis	*Mycobacterium avium complex*	*Mycobacterium intracellulare*
	Mycobacterium kansasii	*Mycobacterium marinum*	*Mycobacterium massiliense*
	Mycobacterium tuberculosis	*Mycobacteroides chelonae*	*Mycolicibacterium fortuitum*
	Mycoplasmoides pneumoniae	*Nocardia abscessus*	*Nocardia asteroides*
	Nocardia brasiliensis	*Nocardia farcinica*	*Orientia tsutsugamushi*
	Proteus vulgaris	*Pseudomonas aeruginosa*	*Serratia marcescens*
	Staphylococcus aureus	*Stenotrophomonas maltophilia*	*Streptococcus agalactiae*
	Streptococcus pneumoniae	*Streptococcus pyogenes*	*Tropheryma whipplei*
	Ureaplasma urealyticum		
Fungus (9)	*Aspergillus*	*Aspergillus flavus*	*Aspergillus fumigatus*
	Aspergillus terreus	*Candida albicans*	*Candida tropicalis*
	Cryptococcus neoformans	*Pneumocystis jirovecii*	*Talaromyces marneffei*
Virus (43)	Cytomegalovirus	Dengue virus	Enterovirus A
	Enterovirus A71	Enterovirus B	Enterovirus C
	Enterovirus D	Enterovirus D68	Epstein-Barr virus
	Hantavirus	Herpesvirus type 6	Herpesvirus type 7
	Human adenovirus B	Human adenovirus C	Human adenovirus E
	Human bocavirus 1	Human coronavirus 229E	Human coronavirus HKU1
	Human coronavirus NL63	Human coronavirus OC43	Human herpesvirus type 1
	Human herpesvirus type 2	Human metapneumovirus	Human parainfluenza 1 virus
	Human parainfluenza 2 virus	Human parainfluenza 3 virus	Human parainfluenza 4 virus
	Human respiratory syncytial virus A	Human respiratory syncytial virus	Influenza A virus H1N1
	Influenza A virus H3N2	Influenza A virus H5N1	Influenza A virus H7N9
	Influenza B virus	Measles virus	Mumps virus
	Rhinovirus A	Rhinovirus B	Rhinovirus C
	Rubella virus	SARS-CoV-2	SFTS bunyavirus
	Varicella-zoster virus		

Statistical analysis

SPSS 25.0 software was used for statistical analysis. Continuous variables are presented as medians and in terquartile ranges (IQRs) and categorical variables as counts and percentages. Comparisons were performed using Mann-Whitney, chi-squared or Fisher´s exact tests, as appropriate. A two-tailed p-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics and pathogen spectrum identified by tNGS

A total of 117 pediatric patients were ultimately included in this study. The median age was 3.6 years (range 0.2 - 13.6, IQR 1.7 - 5.6), and there were 70 males (59.8%) and 47 females (40.2%). Respiratory pathogens were identified by tNGS in 114 patients

Table 2. Comparison between the *Pneumocystis jirovecii*-positive and -negative groups.

	Positive group (n = 18)		Negative group (n = 99)		p
Age, median years (IQR)	**0.8 (0.5 - 2.2)**		**3.9 (2.1 - 6.3)**		**0.008**
Gender					
Male	**13**	**72.2%**	**57**	**57.6%**	**0.244**
Female	**5**	**27.8%**	**42**	**42.4%**	**0.244**
Virus					
Cytomegalovirus	**10**	**55.5%**	**20**	**20.2%**	**0.004**
Human respiratory syncytial virus	**10**	**55.5%**	**24**	**24.2%**	**0.007**
Human parainfluenza virus	**4**	**22.2%**	**10**	**10.1%**	**0.288**
Rhinovirus	**4**	**22.2%**	**24**	**24.2%**	**1.000**
Epstein-Barr virus	**3**	**16.7%**	**23**	**23.2%**	**0.758**
Human adenovirus	**2**	**11.1%**	**8**	**8.1%**	**1.000**
Human coronavirus OC43	**1**	**5.5%**	**9**	**9.1%**	**0.972**
Enterovirus	**0**	**0**	**2**	**2.0%**	**1.000**
Human bocavirus 1	**0**	**0**	**2**	**2.0%**	**1.000**
Human metapneumovirus	**0**	**0**	**2**	**2.0%**	**1.000**
Influenza A virus	**0**	**0**	**2**	**2.0%**	**1.000**
Herpesvirus type 6	**0**	**0**	**1**	**1.0%**	**1.000**
Bacteria					
Streptococcus pneumoniae	**4**	**22.2%**	**16**	**16.2%**	**0.773**
Haemophilus influenzae	**3**	**16.7%**	**10**	**10.1%**	**0.684**
Moraxella catarrhalis	**3**	**16.7%**	**11**	**11.1%**	**0.785**
Tropheryma whipplei	**2**	**11.1%**	**4**	**4.0%**	**0.503**
Mycoplasmoides pneumoniae	**0**	**0**	**23**	**23.2%**	**0.022**
Bordetella pertussis	**0**	**0**	**7**	**7.1%**	**0.593**
Staphylococcus aureus	**0**	**0**	**4**	**4.0%**	**1.000**
Mycobacterium tuberculosis	**0**	**0**	**2**	**2.0%**	**1.000**
Pseudomonas aeruginosa	**0**	**0**	**2**	**2.0**	**1.000**
Legionella pneumophila	**0**	**0**	**1**	**1.0**	**1.000**
Stenotrophomonas maltophilia	**0**	**0**	**1**	**1.0**	**1.000**
Fungus					
Aspergillus fumigatus	**1**	**5.5**	**0**	**0**	**0.154**

(97.4%), 83 of whom were co-infected. The pathogens species consisted of 11 bacteria, 12 viruses, and 2 fungi. As shown in Figure 1, the five most common bacteria were *Mycoplasmoides pneumoniae* (23, 19.6%), *Streptococcus pneumoniae* (20, 17.1%), *Moraxella catarrhalis* (14, 12.0%), *Haemophilus influenzae* (13, 11.1%), and *Bordetella pertussis* (7, 6.0%). Six patients were positive for *Tropheryma whipplei*. *Mycobacterium tuberculosis* and *Legionella pneumophila* were detected in 2 patients and 1 patient, respectively.

Human respiratory syncytial virus (RSV) was the most common virus (34, 29.0%), followed by CMV (30, 25.6%) and rhinovirus (28, 23.9%). Other relatively common viruses were EBV (26, 22.2%), human parainfluenza virus (14, 12.0%), human adenovirus (10, 8.5%), and human coronavirus OC43 (10, 8.5%).

Pneumocystis jirovecii was detected in 18 patients (15.4%), one of whom was also positive for *Aspergillus fumigatus*.

Pneumocystis jirovecii prevalence in different age groups

The 117 children were divided into 7 age groups. As shown in Figure 2, *Pneumocystis jirovecii* was detected in 9 out of 18 (50%) patients aged < 1 year. The prevalence decreased to 23.5% (4/17), 11.1% (2/18), 7.1%

Table 3. Summary of the prevalence of *Pneumocystis jirovecii* in immunocompetent children with respiratory symptoms.

Study	Country	Clinical characteristic	Sample type	Method	Median age	*Pneumocystis jirovecii* prevalence
Vargas et al. 2001	Chile	URTI or LRTI	NPAs	Nested PCR	1 month	24/74 (32.4%)
Nevez et al. 2001	Fance	LRTI	NPAs	Nested PCR	5.4 months	45/178 (25.3%)
Larsen et al. 2007	Denmark	URTI or LRTI	NPAs	qPCR	3.7 months	67/422 (15.9%)
Monroy-Vaca et al. 2014	Cuban	Whooping cough	NPS	qPCR	8 months	48/163 (29.4%)
Nevez et al. 2020	France	URTI or LRTI	NPAs	qPCR	2 months	26/109 (23.8%)
Guo et al. 2022	China	LRTI	BALF	mNGS	5 years	1/121 (0.8%)
Harizanov et al. 2023	Bulgaria	LRTI	Multiple *	qPCR	- **	4/83 (4.8%)
Xu et al. 2023	China	LRTI	BALF	mNGS	5 years	4/172 (2.3%)
Li et al. 2023	China	LRTI	BALF	mNGS	5.5 months	6/173 (3.5%)
This study	China	LRTI	BALF	tNGS	3.6 years	18/117 (15.4%)

NPAs nasopharyngeal aspirates, NPS nasopharyngeal swab, BALF bronchoalveolar lavage fluid, mNGS metagenomic next-generation sequencing, tNGS targeted next-generation sequencing, URTI upper respiratory tract infection, LRTI lower respiratory tract infection.
*** Throat secretion, induced sputum, tracheal aspirate, and BALF.**
**** 23 were 0 - 12 months, 34 were 1 - 9 years, and 26 were 10 - 18 years.**

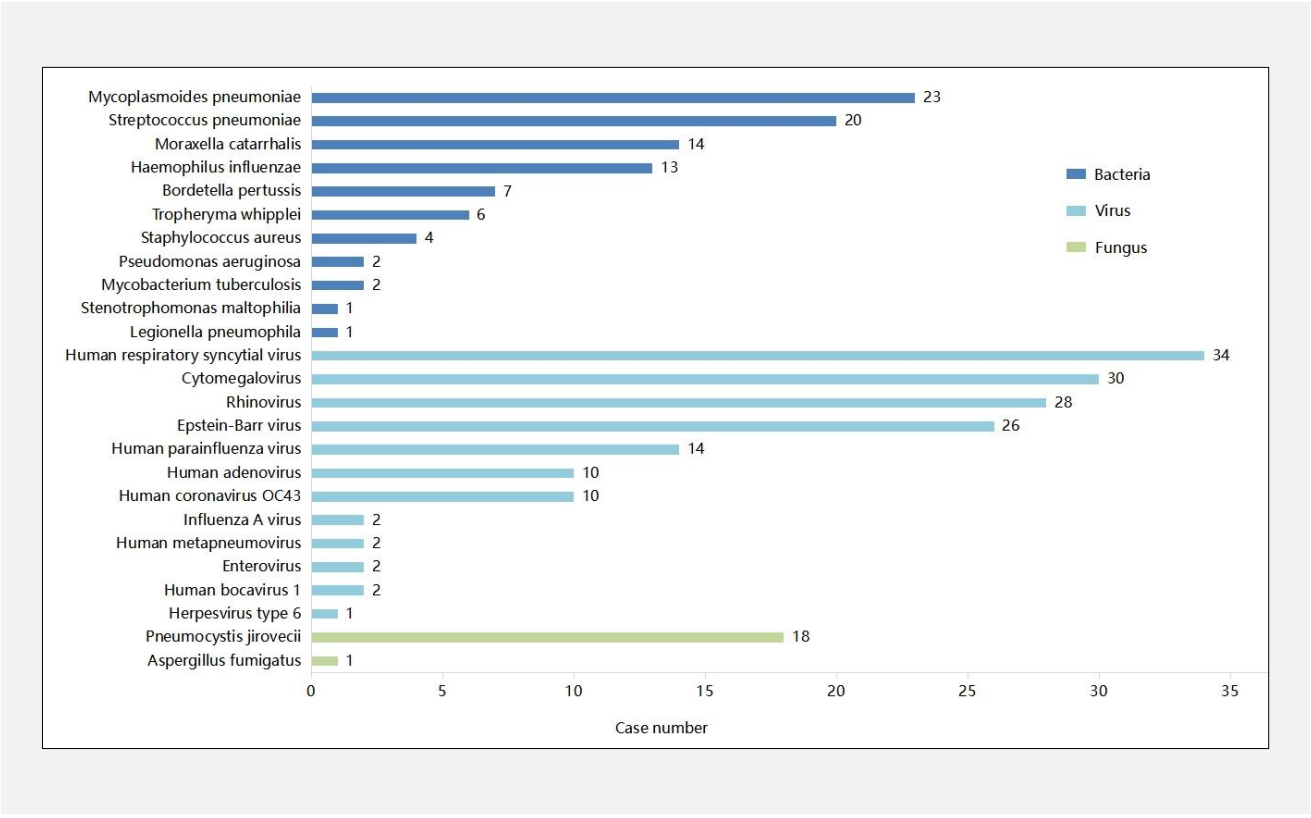

Figure 1. Pathogens identified in 117 immunocompetent children with lower respiratory tract infection by targeted next-generation sequencing.

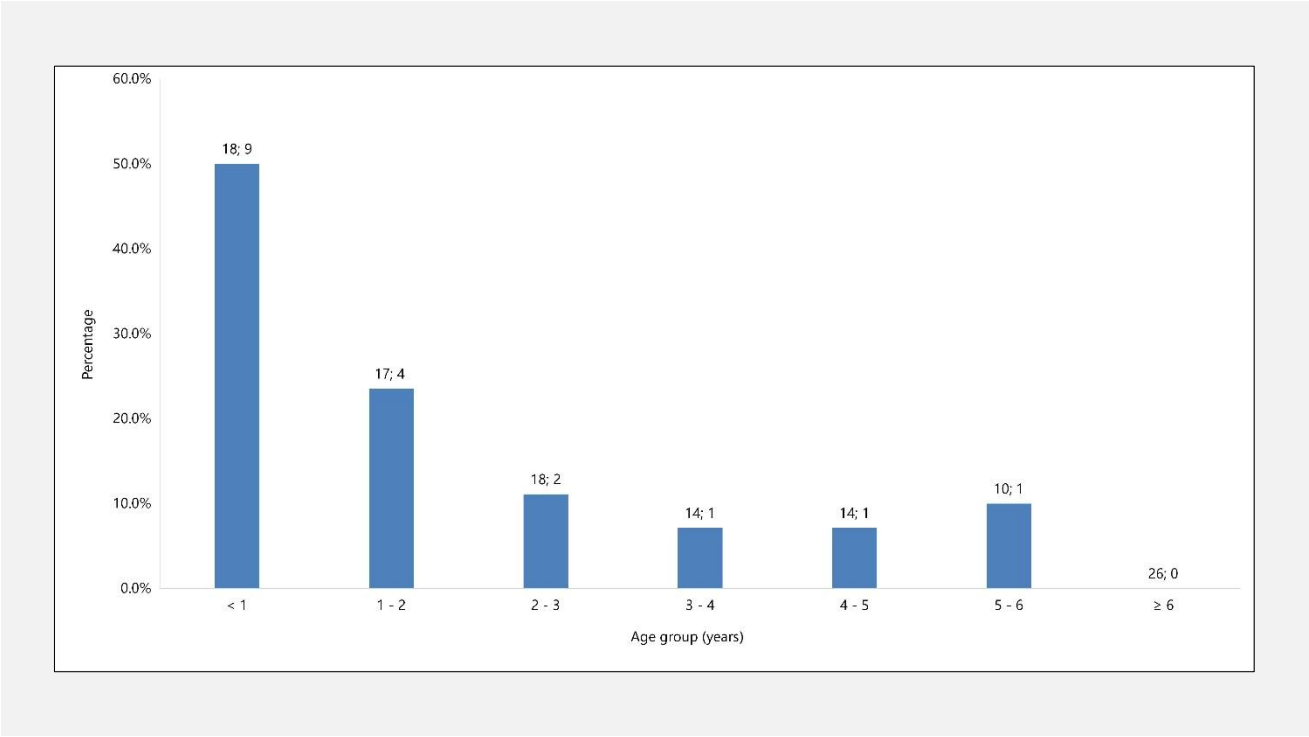

Figure 2. *Pneumocystis jirovecii* prevalence in different age groups in immunocompetent children with lower respiratory tract infection.

Numbers over the bars represent the number of children in each age group and the number of children with *Pneumocystis jirovecii.*

(1/14), 7.1% (1/14), and 10.0% (1/10) in patients 1 - 2, 2 - 3, 3 - 4, 4 - 5, 5 - 6 years of age, respectively. *Pneumocystis jirovecii* was not detected in 26 patients older than 6 years.

Comparison between the *Pneumocystis jirovecii*-positive and -negative groups

We compared the characteristics and pathogens detected between the *Pneumocystis jirovecii*-positive and -negative groups (Table 2). The median age of the *Pneumocystis jirovecii*-positive group was 0.8 years, which was significantly younger than that of the negative group (3.9 years) ($p = 0.008$).

Among the 18 *Pneumocystis jirovecii*-positive patients, 17 (94.4%) had respiratory co-infections. Viral co-infections occurred in all 17 patients, while bacterial and fungal co-infections were present in 8 (47.1%) and 1 (5.9%) of these patients, respectively. A significantly higher prevalence of CMV and RSV was observed in the *Pneumocystis jirovecii*-positive group than in the negative group (55.5% vs. 20.2% and 55.5% vs. 24.2%, respectively). Out of the 23 *Mycoplasmoides pneumoniae*-positive patients, none were positive for *Pneumocystis jirovecii* ($p = 0.008$). No significant differences in the prevalence of other pathogens were observed between the two groups.

Prevalence of *Pneumocystis jirovecii* in immunocompetent children with respiratory symptoms

We reviewed the literature on molecular detection of *Pneumocystis jirovecii* in immunocompetent children with respiratory symptoms (Table 3). Nine articles published between 2001 and 2023 were included. A total of 1,495 children with upper respiratory tract infection or lower respiratory tract infection were included. Sample types included mainly nasopharyngeal aspirates and BALF, and the molecular assays included PCR (nested PCR and qPCR) and NGS (mNGS and tNGS). The median age ranged from 1 month to 5 years, and the prevalence of *Pneumocystis jirovecii* ranged from 0.8% to 32.4%.

DISCUSSION

Primary infection with *Pneumocystis jirovecii* is a worldwide phenomenon occurring in children around the world. In the present study, *Pneumocystis jirovecii* was detected in the BALF of 15.4% of immunocompetent children with LRTI. Previously reported prevalence in immunocompetent children with respiratory symptoms ranged from 0.8% to 32.4% [5-7,19-24]. This discrepancy was difficult to evaluate because of differences in detection methods, sample types, populations,

and especially age distribution. As in our study, there was a significant difference in age distribution between the *Pneumocystis jirovecii*-positive and -negative groups (median age: 0.8 years vs. 3.9 years, p = 0.008). The prevalence was highest (50%) in patients aged < 1 year and lowest (0%) in patients aged ≥ 6 years. This might indicate that *Pneumocystis jirovecii* infection is an early event in life and clearance occurs with age.

While *Pneumocystis jirovecii* can cause life-threatening pneumonia in immunocompromised individuals, the clinical significance of detecting *Pneumocystis jirovecii* DNA in immunocompetent individuals remains controversial. It may represent colonization without causing any respiratory symptoms or be associated with bronchiolitis, self-limiting upper respiratory tract infection, respiratory distress syndrome, and abnormal lung development in children [6-9]. Immunocompetent adult patients with *Pneumocystis jirovecii* colonization might also present with milder symptoms than pneumonia, and treatment with trimethoprim-sulfamethoxazole resulted in symptom relief [25]. Out of the 18 children with *Pneumocystis jirovecii* infection in the present study, 3 were classified as putative PJP based on a composite clinical standard. Patient 1 had only *Pneumocystis jirovecii* infection, Patient 2 had a viral co-infection (RSV), and Patient 3 had viral (CMV and rhinovirus) and bacterial (*Streptococcus pneumoniae* and *Moraxella catarrhalis*) co-infections. All patients had persistent cough and wheezing for 2 - 7 months and were treated with trimethoprim-sulfamethoxazole for three weeks. Patients 1 and 2 recovered clinically and radiologically, but Patient 3 still had radiological abnormality 1 month after treatment. This might be due to the complex co-infection pattern, which deserves further investigation. All of the other 15 children diagnosed with *Pneumocystis jirovecii* colonization recovered well, despite the lack of specific therapy.

Pulmonary co-infections with *Pneumocystis jirovecii* and other pathogens have been widely described. Previous studies have reported that viral co-infections in PJP patients are associated with poor outcomes, especially with CMV and SARS-CoV-2 [17,26]. The present study found that co-infections were also very common in immunocompetent children with LRTI, including viruses and bacteria. However, only CMV and RSV were significantly associated with *Pneumocystis jirovecii*. It was not possible to determine whether there was a synergistic effect between these pathogens or whether the association was due to a similar age distribution. A systematic review found that CMV infection was associated with the development of PJP [27], but this might not explain the situation in immunocompetent children. In addition, as *Mycoplasmoides pneumoniae* infection occurs mostly in older children, a negative correlation with *Pneumocystis jirovecii* was expected in the present study. This indicated that *Mycoplasmoides pneumoniae* might not increase the risk of *Pneumocystis jirovecii* re-infection in older children with normal immunity.

tNGS technology combines multiplex PCR with next-generation sequencing to detect tens to hundreds of pathogens in a single test. Although it has a lower coverage than mNGS, it is cost-effective and more sensitive for targeted pathogens. It is more powerful than PCR for detecting co-infections. The present study provided more comprehensive co-infection patterns with *Pneumocystis jirovecii* by tNGS, and the prevalence revealed by tNGS was higher than that by mNGS in immunocompetent children with LRTI, mainly due to the lower copy numbers of *Pneumocystis jirovecii* at colonization.

The present study has several limitations. Due to the lack of standard of diagnosis and treatment of *Pneumocystis jirovecii* infection in immunocompetent children, we could not exclude the possibility that the 3 children diagnosed with putative PJP did not actually require treatment with trimethoprim-sulfamethoxazole. In addition, a cutoff value to help distinguish colonization from PJP based on tNGS results was not available due to small sample size. A healthy control group is also needed to provide baseline information on the prevalence of *Pneumocystis jirovecii* in Chinese children.

In conclusion, the present study demonstrated that *Pneumocystis jirovecii* infection was common in immunocompetent children with LRTI; it presented mainly as colonization and resolved with age. Infection might occur alone or be significantly associated with CMV and RSV. Further scientific evidence and guidelines are needed to inform on the management of this specific population, particularly for those who might benefit from specific therapy.

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Ethical Approval and Consent to Participate:
The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of Maternal and Child Health Hospital in Meizhou. Informed consent was waived by the Ethics Committee of Maternal and Child Health Hospital in Meizhou due to the retrospective nature of this study.

Availability of Data and Materials:
The main data generated or analyzed during this study are included in the manuscript. The raw data are not publicly available due to privacy or ethical restrictions but are available from the corresponding author upon reasonable request.

Declaration of Interest:

The authors declare no competing interests.

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