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Clinical Value of Metagenomic Next-Generation Sequencing From Blood Samples to Identify *Pneumocystis jirovecii* Pneumonia in Patients With Human Immunodeficiency Virus

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Background. The aim of this study was to evaluate the clinical value of metagenomic next-generation sequencing (mNGS) of blood samples for identifying *Pneumocystis jirovecii* pneumonia (PJP) in patients with human immunodeficiency virus (HIV).

Methods. A total of 76 people with HIV (PWH) with suspected lung infections were enrolled in the study. The patients were divided into two groups: the PJP group and the non-PJP group.All patients underwent pulmonary computed tomography scans, and blood or respiratory tract specimens were subjected to mNGS and conventional microbiological tests. Patient characteristics were collected from their medical records.

Results. Thirty patients were diagnosed with PJP and 46 were confirmed to have non-*P jirovecii* (*Pj*) infectious pneumonia. mNGS was conducted on bronchoalveolar lavage fluid samples from 25 patients and on blood samples from 59 patients. Twenty-one of 22 (95.5%) blood samples from the PIP group contained sequences of Pi, with the number of specific reads for circulating *Pj* sequences ranging from 2 to 2035. In the non-PJP group, 4 blood samples exhibited low *Pj* sequences, ranging from 1 to 2 reads. The sensitivity and specificity for blood samples were 95.5% (95% confidence interval [CI], 91.2%-98.4%) and 90.0% (95% Cl, 89.5%-100%), respectively.

Conclusions. Our study indicates that mNGS of blood samples exhibits high sensitivity and specificity for diagnosing PJP in PWH. Caution should be exercised when interpreting low *Pj* mNGS read counts in blood samples; the definitive diagnosis of PJP relies on the synthesis of clinical data with *Pj* mNGS results. Further studies are necessary to validate this finding.

Keywords. HIV infection; metagenomic next-generation sequencing; peripheral blood; *Pneumocystis jirovecii* pneumonia.

Pneumocystis jirovecii pneumonia (PJP) is a common opportunistic infection in people with human immunodeficiency virus (HIV). It is caused by the fungus *Pneumocystis jirovecii* (*Pj*). Due to the widespread use of antiretroviral therapy and preventive measures against PJP, most cases now occur in patients with advanced immunosuppression (ie, CD4 counts <100 cells/μL) and in those who are unaware of their HIV status or are not receiving ongoing care for the virus [1]. However, the treatment regimen for PJP has been associated with a 20%–40% mortality rate among patients with severe immunosuppression. Therefore, timely diagnosis and the immediate commencement of anti-*Pj* treatment are crucial in reducing mortality rates.

The definitive diagnosis of PJP relies on the microscopic visualization of fungal structures within respiratory samples. However, this method is limited by its sensitivity and specificity and is challenging to implement in critical clinical settings. (1,3)-β-D-glucan (BDG) is a sensitive (0.39–0.85) but unspecific serologic biomarker for PJP [2, 3], as it can also yield positive results for many other fungal diseases, especially in people with HIV (PWH). Presently, polymerase chain reaction (PCR) serves as an alternative diagnostic method for PJP. PCR demonstrates high sensitivity (ranging from 0.78 to 0.99) and specificity (ranging from 0.83 to 0.93) in detecting *Pj*; however, it cannot reliably differentiate between colonization and active disease. Additionally, mixed infections are prevalent among PWH, which hampers the diagnostic utility of *Pj* PCR in these complex cases. Therefore, there is an urgent need to explore

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more accurate, efficient, and comprehensive microbiological diagnostic tools for PJP in PWH.

Metagenomic next-generation sequencing (mNGS) has been utilized to identify specific infectious pathogens in a timely, unbiased, and hypothesis-free manner [4–10]. It also has the capability to recognize coinfecting microbes [5, 7]. Furthermore, studies have indicated that mNGS can identify hematologic dissemination through blood tests, which may obviate the need for invasive procedures, contain fewer colonization, and offer greater cost-effectiveness [4, 5, 9]. Recent data from people without HIV have shown that mNGS is highly effective in diagnosing PJP, exhibiting excellent accuracy, sensitivity, and specificity [4–11]. This also applies to recipients of biologic immunosuppression and tests performed exclusively on blood samples, thereby eliminating the need for bronchoalveolar lavage fluid (BALF) samples [9, 12, 13]. In individuals with HIV-associated immunosuppression, Chen et al [14] also demonstrated that BALF mNGS is a powerful technique for rapid diagnosis of PJP, exhibiting high specificity (100%) and sensitivity (85.86%). The sensitivity of BALF PCR (84.85%) was comparable to that of mNGS. However, disseminated infection is the most common manifestation in individuals with advanced HIV disease. It is worth noting that the diagnostic value of blood mNGS for PJP in PWH still requires further exploration. There are few studies in this area.

In this study, we hypothesized that blood mNGS could serve as a complementary diagnostic tool for PJP in PWH, especially when BALF samples are not easily obtainable or when disseminated infection is suspected. Our aim was to describe the clinical features of PJP and to explore the application of blood mNGS in identifying PJP among PWH.

MATERIALS AND METHODS

Study Design and Subjects

In this retrospective study, we consecutively enrolled 76 PWH with suspected lung infections at the Department of Infectious Diseases of the Second Xiangya Hospital, Central South University (Changsha, China) from April 2021 to August 2024. Patients were eligible for enrollment provided they met all the following criteria: (1) consent to undergo the plasma mNGS examination (due to the high cost) and (2) suspected lung infections. Patients were excluded based on the following criteria: (1) incomplete medical records, (2) mNGS not performed, and (3) patients already receiving anti-PJP therapy (Figure 1). All patients underwent pulmonary computed tomography (CT) scans and mNGS for pathogen identification upon admission. Infections were suspected in patients exhibiting fever, respiratory symptoms, or new lung lesions. Pathogens were identified using a panel of conventional microbiological tests and mNGS. *Pneumocystis jirovecii* sequences were detected in plasma cell-free DNA (cfDNA) or respiratory samples through mNGS in patients.

Patients meeting the following criteria were classified into the PJP group: (1) positive microscopy/positive mNGS in the BALF or blood sample; (2) clinical manifestations, including fever or dry cough, or shortness of breath; and (3) lung radiological signs of diffuse ground glass opacities or diffuse interstitial infiltration. In the absence of microbiological evidence, the attending physician may diagnose PJP clinically by correlating the patient's clinical manifestations and imaging results to exclude other diseases, along with the patient's confirmed responsiveness to anti-PJP treatment after 1 month of follow-up. Patients with a positive mNGS result in the BALF or blood sample, but no symptoms or radiological signs of PJP, were classified into the non-PJP group. The clinical diagnosis was conducted by 2 experienced clinicians who evaluated the patients' immunity, clinical manifestations, laboratory results, radiographic imaging, mNGS report, and response to anti-*Pj* treatment. This study was approved by the Research Ethics Committee of the Second Xiangya Hospital, and the need for written informed consent was waived due to its retrospective design.

Clinical Data Collection

Data were collected from the patients' medical records. The following details at diagnosis were gathered: sex, age, primary disease and treatment, epidemic history, and anti-infection therapeutic regimen; clinical manifestations, symptoms, and signs, including cough, fever, shortness of breath, dyspnea, and results from general physical examinations; laboratory test results (performed within 24 hours of the onset of symptoms), including those obtained from routine blood tests, C-reactive protein (CRP) levels, procalcitonin (PCT) levels, lactate dehydrogenase (LDH), BDG, galactomannan test, $CD4^+$ T cells, $CD8^+$ T cells, HIV RNA, and chest CT; and outcome data. BALF *Pj* PCR for identifying PJP cannot be collected because it is not commonly used in our hospital.

Sample Processing and DNA Extraction for mNGS

Specimens were collected from patients using standard procedures. In brief, 3 mL of blood or 0.6 mL of BALF was collected in sterile, DNase-free tubes. Plasma was separated by centrifuging the blood at 1600*g* at 4°C for 10 minutes within 8 hours of collection. The BALF was mixed with lysozyme and 1 g of 0.5-mm glass beads, and then the mixture was attached to a horizontal platform on a vortex mixer and agitated vigorously at 2800–3200 rpm for 30 minutes. For nucleic acid extraction, 300 μL of supernatant or plasma was transferred to a 2-mL centrifuge tube. Nucleic acid was extracted using a magnetic bead–based DNA/RNA extraction kit (Genskey Medical Technology Co, Ltd, Beijing, China) following standard procedures.

Library Preparation and Sequencing Construction

The DNA library was constructed using DNA fragmentation, end repair, adapter ligation, and PCR amplification through the MGIEasy Cell-free DNA Library Prep Set (MGI Tech,

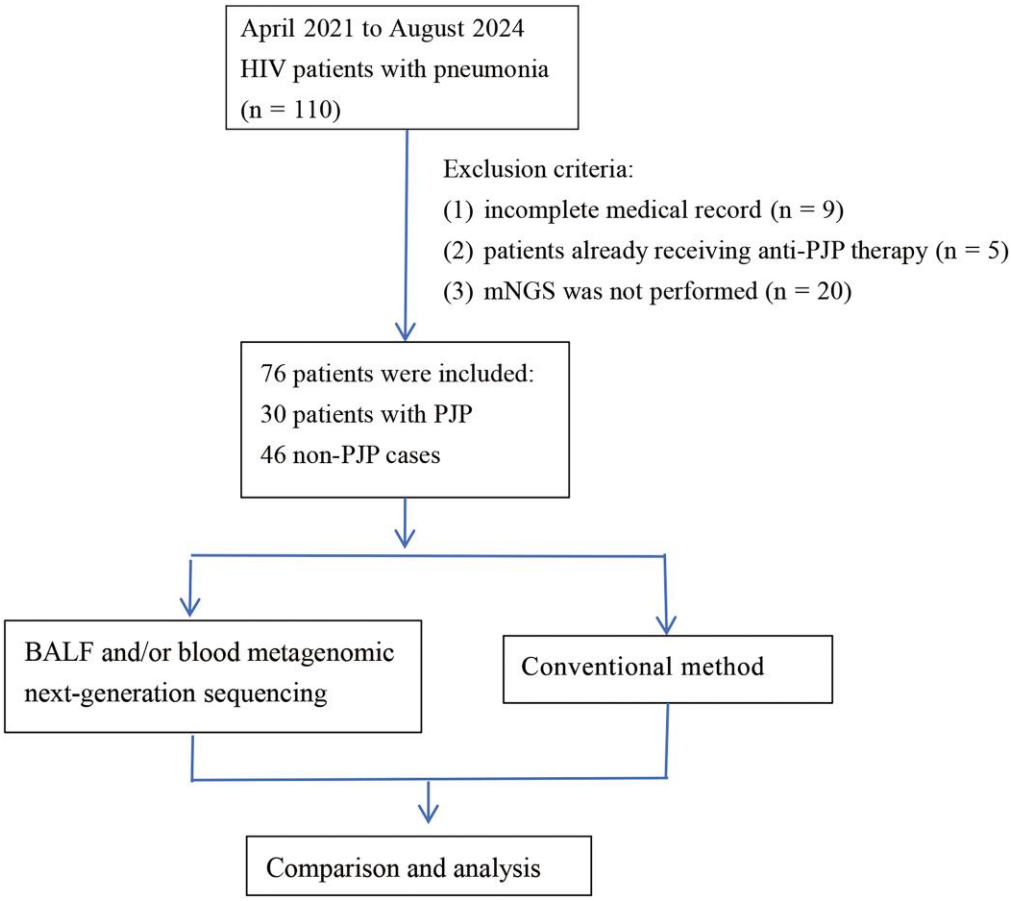

Figure 1. Flowchart of case selection. A total of 76 patients with HIV and suspected lung infections were selected for further analysis. Abbreviations: BALF, bronchoalveolar lavage fluid; HIV, human immunodeficiency virus; mNGS, metagenomic next-generation sequencing; PJP, *Pneumocystis jirovecii* pneumonia.

Shenzhen, China). Quality control of the library was conducted using an Agilent 2100. The double-stranded DNA library was converted into single-stranded circular DNA through DNA degradation and circularization. The DNA Nanoballs were produced using rolling circle amplification technology. Qualified DNA Nanoballs were then sequenced by the MGISEQ-200 sequencing platform (MGI Tech).

Bioinformatic Analysis

High-quality sequencing data (>10 million) were generated by removing low-quality and short (length <35 bp) reads using fastp software [15]. Human host sequences were subtracted by mapping to the human reference genome sequences (hg19) using the Burrows-Wheeler Aligner [16]. The remaining data, after the removal of low-complexity reads, were classified by simultaneously aligning with the Pathogen Metagenomics Database, which includes bacteria, fungi, viruses, and parasites. The classification reference databases were downloaded from the National Center for Biotechnology Information. RefSeq contains 11 958 bacterial genomes or scaffolds, 1714 fungi related to human infection, 7373 whole-genome sequences of viral taxa, and 343 parasites associated with human diseases. The coverage ratio and the depth of each microorganism were calculated using BEDTools [17]. The criteria for an infectious pathogen by mNGS were defined as follows: for bacteria, virus, and parasites, if a microbe (at the species level) had a coverage rate 10-fold greater than other microbes of the same type [18]; for fungi, a microbe (at the species level) had a coverage rate 5-fold higher than that of any other fungus, due to its low biomass in DNA extraction [19]; and for *Mycobacterium tuberculosis*, when at least 1 read was mapped to either the species or genus level, considering the difficulty of DNA extraction and the low possibility for contamination [2].

Statistical Analysis

Continuous data are presented as mean ± standard deviation, and nonnormally distributed measures are expressed as median (interquartile range). Categorical variables are presented as frequencies and percentages. Differences between groups were assessed using 1-way analysis of variance for continuous data and the χ^2 test or Fisher exact test for categorical data, depending on the type of data and distribution. Statistical significance was set

Table 1. Clinical Characteristics and Laboratory Examination Results of the Patients on Admission

Characteristics	PJP (n = 30)	Non-PJP (n = 46)	*P* Value
Age, y	49.00 (34.00–58.75)	48.05 (27.75–56.0)	.29
Male sex	22 (73.3)	38 (82.6)	.613
Clinical symptoms			
Dyspnea	28 (93.3)	9 (19.6)	**.001**
Fever	23 (76.6)	32 (69.6)	.76
Cough	26 (86.7)	23 (50)	.025
Serum BDG, pg/L	133.85 (83.12–330.75)	54.20 (37.50–132.70)	.043
Serum BDG >70 pg/L	25 (83.3)	19 (41.3)	**.001**
Galactomannan	0.07 (0.06–0.11)	0.14 (0.06–2.05)	.177
LDH, U/L	367.25 (277.33–541.80)	293.50 (237.50–491.20)	**.009**
CRP, mg/L	33.00 (19.71–49.50)	25.40 (0.60–49.53)	.601
PCT, ng/mL	0.14 (0.06–0.24)	0.15 (0.07–0.19)	.319
CD4 count, cells/μL	29.5 (13.8–55.25)	22 (11.00–56.00)	.512
CD8 count, cells/μL	418.5 (154.50–612.85)	372 (175.00–833.00)	.998
Viral load, × 10^5, IU/mL	6.95 (1.77–22.50)	6.85 (0.23–32.35)	.939
Pj reads by mNGS of blood samples	80 (6–385.00)	0 (0–2)	**.001**
Pj reads by mNGS of BALF samples	13 153.50 (95.00–45 450.25)	4 (0–108)	.005

Data are presented as No. (%) or median (interquartile range) unless otherwise indicated. Values in bold indicate statistical significance.

Abbreviations: BALF, bronchoalveolar lavage fluid; BDG, (1,3)-β-D-glucan; CRP, C-reactive protein; LDH, lactate dehydrogenase; mNGS, metagenomic next-generation sequencing; PCT, procalcitonin; *Pj*, *Pneumocystis jirovecii*; PJP, *Pneumocystis jirovecii* pneumonia.

at $P < .05$. A method of listwise deletion techniques is used to remove missing data. All analyses were performed using IBM SPSS statistical software version 26.0.

RESULTS

Patient Profiles

The patients were categorized into 2 groups: PJP and non-PJP. Thirty patients were diagnosed with confirmed PJP. The non-PJP group comprised 46 patients with non-*Pj* infectious pneumonia. The demographic and clinical characteristics of the patients are summarized in Table 1. The median age (49 vs 48 years) and sex composition of the 2 cohorts were similar. The most common symptoms of PJP included dyspnea (93.3%), fever (76.6%), and cough (86.7%). Compared to those with non-PJP pneumonia, patients with PJP more frequently exhibited cough and dyspnea. The median serum levels of LDH (367.25 vs 293.50 U/L, $P = .009$) and BDG (133.85 vs 54.20 pg/mL, $P = .043$) were notably higher in the PJP group compared to the non-PJP group. There were no significant differences between the 2 groups in terms of age, sex, glucose metabolism, CRP, PCT, CD4 count, CD8 count, or HIV RNA.

mNGS for Detection of *P jirovecii* in Blood and/or BALF Samples

MNGS was conducted on BALF samples from 25 patients and on blood samples from 59 patients. Within the PJP group, 9 individuals had only mNGS of the BALF sample, 17 had only mNGS of the blood sample, and 4 had both (Table 2). One patient had negative blood and positive BALF results. In the non-PJP group, 8 had only mNGS of the BALF sample, 35 had only mNGS of the blood sample, and 5 had both (Table 2). No patients had negative blood and positive BALF results. *Pneumocystis jirovecii* was detected by mNGS in both BALF and blood samples, achieving a concordance rate of 88.9%.

Twenty-one of 22 (95.5%) blood samples contained *Pj* sequences in the PJP group (Table 1 and Supplementary Table 1). The specific reads of *Pj* ranged from 7 to 2035 in the PJP group, as determined by circulating cfDNA sequencing. Conversely, only a few *Pj* sequences (ranging from 1 to 2 reads) were detected in 4 blood samples from the non-PJP group. The 4 patients with positive mNGS results, normal BDG test levels, and no PJP radiological signs, who only received prophylactic therapy and recovered without anti-PJP therapy, were categorized into the non-PJP group. This indicates that circulating *Pj* sequencing contributed to an improvement in the diagnosis of PJP in PWH, with a sensitivity of 95.5% and specificity of 90.0% (Table 3). In a patient where plasma cfDNA sequencing did not detect *Pj*, respiratory tract samples were used to identify the PJP infection. The proportion of *Pj*-specific reads in BALF samples was 100% in 13 patients in the PJP group. Additionally, the *Pj* load in BALF samples was higher in the PJP group, with a median mNGS read number of 13 153 compared to 4 (range, 0–612) in the non-PJP group ($P = .005$), resulting in a sensitivity of 100% and specificity of 61.5% (Table 3).

Diagnostic Performance of mNGS and BDG Assay in Confirmed PJP

We assessed the diagnostic performance of mNGS and BDG methods in comparison with the clinical final diagnosis of PJP (Table 3, Figure 2). Among the 30 patients with confirmed

Table 2. Metagenomic Next-Generation Sequencing for Detection of *Pneumocystis jirovecii* in Blood and/or Bronchoalveolar Lavage Fluid Samples

PJP				Non-PJP			
Specimen	No. of Patients	Positive	Negative	Specimen	No. of Patients	Positive	Negative
BALF only	9	9	0	BALF only	8	4	0
Blood only	17	17	0	Blood only	35	3	0
Both	4	3 (double +)	1[a]	Both	5	1 (double +)	4 (double –)

Abbreviations: BALF, bronchoalveolar lavage fluid; PJP, *Pneumocystis jirovecii* pneumonia.

[a]One patient had negative blood and positive BALF results.

Table 3. Diagnostic Performance of Plasma Metagenomic Next-Generation Sequencing (mNGS), Bronchoalveolar Lavage Fluid mNGS, and (1,3)-β-D-Glucan in Confirmed *Pneumocystis jirovecii* Pneumonia

Diagnostic Test	Result	PJP Patients, No.	Non-PJP Patients, No.	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
Plasma mNGS	+	21	4	95.5 (91.2–98.4)	90.0 (89.5–100)	84.0 (71.2–91.3)	97.3 (90.5–100.0)
	–	1	36				
BALF mNGS	+	13	5	100.0 (92.0–95.0)	61.5 (56.5–76.6)	72.2 (58.2–85.5)	100.0 (95.4–99.8)
	–	0	8				
BDG	+	24	19	80.0 (70.2–85.5)	56.8 (45.6–67.2)	55.8 (45.2–60.5)	80.6 (75.4–86.2)
	–	6	25				

Abbreviations: BALF, bronchoalveolar lavage fluid; BDG, (1,3)-β-D-glucan; CI, confidence interval; mNGS, metagenomic next-generation sequencing; NPV, negative predictive value; PJP, *Pneumocystis jirovecii* pneumonia; PPV, positive predictive value.

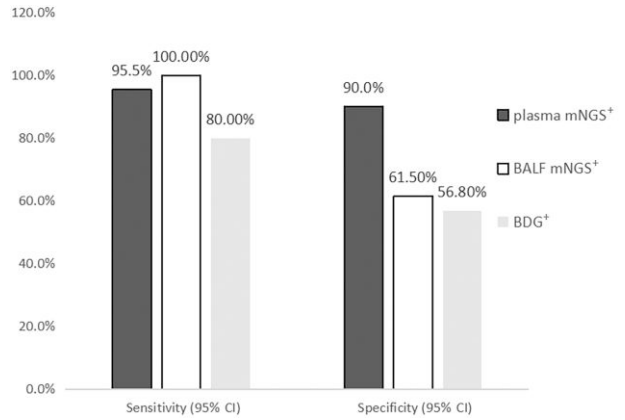

Figure 2. Diagnostic performance of plasma metagenomic next-generation sequencing (mNGS), bronchoalveolar lavage fluid mNGS, and (1,3)-β-D-glucan in patients with confirmed *Pneumocystis jirovecii* pneumonia. Abbreviations: BALF, bronchoalveolar lavage fluid; BDG, (1,3)-β-D-glucan; CI, confidence interval; mNGS, metagenomic next-generation sequencing.

PJP, mNGS detected the *Pj* sequence in 21 of 22 blood samples, yielding a positive rate of 95.5%. In BALF samples, mNGS identified all 13 cases of PJP, resulting in a positive rate of 100%. Conversely, elevated BDG levels were detected in 20 of 25 cases, yielding a positive rate of 80%. Among these 25 patients, the positive rates of BDG and mNGS were ($P < .05$) statistically different. Using clinically confirmed PJP as the reference standard, blood mNGS exhibited a clinical sensitivity of 95.5%, clinical specificity of 90.0%, positive predictive value (PPV) of 84.0%, and negative predictive value (NPV) of 97.3%. BALF mNGS demonstrated a clinical sensitivity of 100%, clinical specificity of 61.5%, PPV of 72.2%, and NPV of 100%. In comparison, the BDG assay had a clinical sensitivity of 80%, clinical specificity of 56.8%, PPV of 55.8%, and NPV of 80.60%.

Pathogen Characteristics and Treatment Impact

All patients had BALF or blood samples collected and underwent mNGS. Data indicated that mNGS detected >1 pathogen in 86.7% (26/30) of patients with PJP, including *Talaromyces marneffei*, tuberculosis, *Cryptococcus*, *Toxoplasma gondii*, *Klebsiella pneumoniae*, parvovirus B19, Epstein-Barr virus, herpes simplex virus, cytomegalovirus (CMV), and *Candida albicans* (Figure 3*A*). Additionally, in patients without PJP, the most frequently identified pathogens were *T marneffei*, CMV, and tuberculosis (Figure 3*B*). Oral trimethoprim-sulfamethoxazole therapy was administered to all patients, with 36.8% receiving it in combination with intravenous caspofungin, which is currently used to treat PJP. The all-cause mortality rate among patients with PJP was 13.3% (Supplementary Table 1).

DISCUSSION

In this study, we investigated the diagnostic value of mNGS for identifying PJP in PWH. Our findings indicated that mNGS exhibited a sensitivity ranging from 95.5% to 100% in PWH with

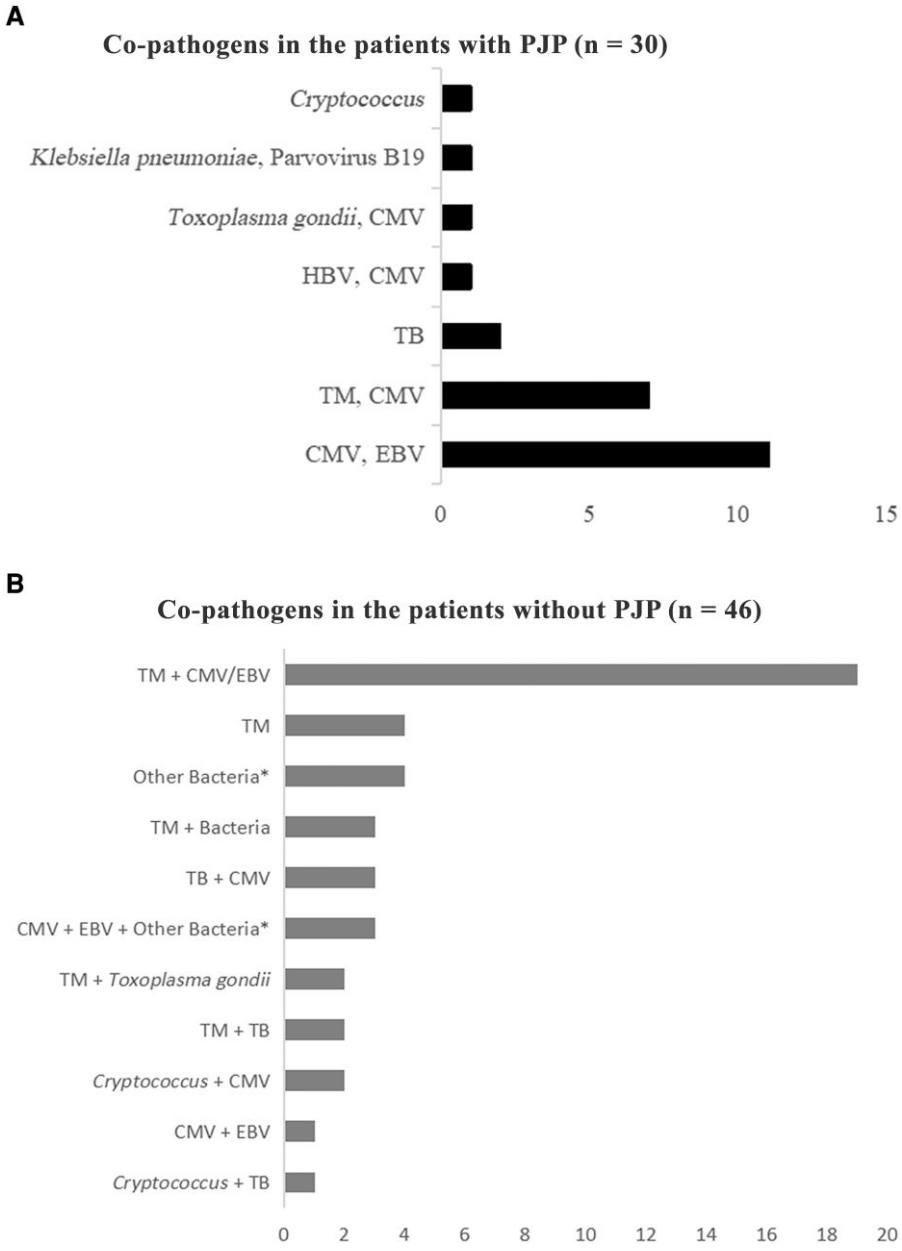

Figure 3. A pathogen spectrum of patients with *Pneumocystis jirovecii* pneumonia (PJP) and those without PJP. *A,* Co-pathogens in the patients with PJP. *B,* Co-pathogens in patients without PJP. *Including *Salmonella, Klebsiella pneumoniae, Escherichia coli,* and *Legionella.* Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; PJP, *Pneumocystis jirovecii* pneumonia; TB, tuberculosis; TM, *Talaromyces marneffei.*

PJP, which was significantly higher than that of serum BDG. Moreover, blood mNGS demonstrated a specificity of 90.0%, which was considerably better than BALF mNGS and serum BDG. mNGS also proved advantageous for identifying co-pathogens in mixed infections. Additionally, there was a good concordance between mNGS results from blood and BALF samples for the detection of *Pj*.

Traditionally, the diagnosis of PJP relied on positive Grocott methenamine silver (GMS) staining of induced sputum or BALF, and/or a positive PCR test on a BALF specimen. However, the sensitivity of GMS staining is relatively low. Quantitative PCR on BALF has shown higher sensitivity, ranging from 0.78 to 0.98, and adequate specificity, between 0.83 and 0.89 [2, 3]. Nevertheless, it requires the collection of adequate respiratory specimens and offers little value in diagnosing mixed infections. Serum BDG is also a commonly reported diagnostic marker for PJP. BDG is 1 of the major components of the fungal cell wall and has been widely used for the diagnosis of PJP. In our study, we observed significantly high serum levels of BDG in patients with PJP. BDG demonstrated a relatively

good sensitivity in diagnosing PJP (80.0%). However, when diagnosing PJP, the BDG detection results should be interpreted with caution, as other fungal infections can also yield positive results, particularly in PWH.

Compared to traditional diagnostic methods, mNGS enables sequence-based identification of all potential pathogenic microorganisms in clinical samples within a relatively short time. Our findings indicated that 21 blood samples contained *Pj* sequences from 22 individuals diagnosed with PJP, demonstrating that mNGS of blood had a high sensitivity rate of 95.5%. Additionally, circulating cfDNA sequencing enhanced the diagnosis of PJP in PWH. Consistent with our findings, several studies [4, 12, 20, 21]. have also revealed that the potential advantage of mNGS is its ability to detect *Pj* in blood samples from immunocompromised patients without HIV, thereby avoiding the need for invasive procedures. This is especially important for PWH when respiratory samples are unavailable. Interestingly, in our study, a small number of plasma *Pj* sequences were detected in 4 non-PJP patients, with a specificity of 90.0%. This could affect the specificity of plasma cfDNA. These results were inconsistent with those reported for immunocompromised patients without HIV [5, 20]. In a study by Li et al [4], serum cfDNA sequencing achieved a detection specificity of 100% for the diagnosis of PJP in immunosuppressed individuals. It is possible that *Pj* in human lungs could release a small amount into the bloodstream under conditions of pulmonary *Pj* colonization, particularly in the event of HIV infection. Further studies are required due to the limited sample size. The false positives of blood mNGS should be carefully monitored and evaluated. *Pneumocystis jirovecii* is not a common background bacterium in laboratory settings; the likelihood of contamination with *Pj* in blood samples is lower than in other types of samples. Therefore, even when a small specific read of a taxon is mapped to either the species or genus level, positive results should be considered. To minimize the risk of contamination prior to mNGS procedures, we implemented a stringent sterile protocol during sample collection and established guidelines for nucleic acid–free specimen preparation. Additionally, 2 independent, experienced clinicians analyzed the mNGS results to ensure they were consistent with the patient's clinical manifestations and diagnosis. In our study, patients with positive blood mNGS results who did not exhibit radiological signs received only prophylactic therapy and recovered without anti-PJP treatment, which suggested that caution should be exercised when interpreting low-specificity findings in blood samples.

We also investigated the diagnostic value of mNGS in BALF samples for identifying patients with PJP. Our findings indicated that the percentage of *Pj*-specific reads in BALF samples was 100% in 13 patients. The *Pj* load in BALF samples was higher in the PJP group, with a sensitivity of 100%. These results align with those reported for immunocompromised patients without HIV [4, 5], which indicated that mNGS of BALF exhibited a high sensitivity for detecting *Pneumocystis*. However, distinguishing between colonization and true infections remains challenging. In this study, we found that some *Pj* sequences were detected in 5 of 13 BALF samples from non-PJP groups, with a specificity of 61.5%. A study revealed the excellent ability of BALF mNGS to discriminate PJP from colonization with a suggested cutoff of 14 reads in patients without HIV [22]. Our findings, which diverge from previous studies, indicate that 4 patients with non-PJP pneumonia exhibited higher *Pj* read counts (105, 111, 320, and 612 reads, respectively). This suggests that the burden of *Pj* colonization in the lungs may be greater in PWH compared to those without HIV infection.

When evaluating the diagnostic value of PJP in PWH, mNGS has been shown to be superior to BDG testing, and it may also provide cost-effectiveness benefits. In China, the cost of an mNGS test ranges from $300 to $450 per sample, whereas BDG testing costs approximately $22 to $27. However, BDG testing lacks specificity, as elevated serum BDG levels can also be observed in PWH with other fungal infections, such as those caused by *T marneffei*. mNGS offers a more precise diagnosis by directly detecting the *Pj* genome, thereby reducing the likelihood of misdiagnosis. Furthermore, mNGS has the advantage of being able to identify multiple pathogens simultaneously, which is particularly beneficial in PWH, in whom coinfections are common. Despite the higher initial cost per test, the overall cost-effectiveness of mNGS may be superior due to the reduced need for additional diagnostic tests and shorter hospital stays associated with more accurate and timely diagnoses. Blood mNGS offers a wide diagnostic scope, especially for immunocompromised patients, such as those with HIV, and is cost-effective for initial testing. BALF mNGS has higher sensitivity for PJP detection in PWH, but its invasiveness and cost ($600–$750 per sample) may limit its use as a first-line test, particularly in resource-limited settings. A balanced diagnostic strategy considering clinical presentation and test cost-effectiveness is essential.

This study has some limitations. First, the sample size was limited, which may affect the accuracy of mNGS and be underpowered in diagnosing PJP. Second, there was an unavoidable intrinsic bias due to the selection and referral processes, as these investigations were of a retrospective design and conducted in single centers. Third, the patients involved in the study did not undergo PCR for *Pj*, as it is not commonly utilized in our hospital; therefore, comparative analysis with PCR was limited due to insufficient data. Fourth, stratified analyses based on CD4 count or coinfections cannot be performed, as 100% of the study group and 91% of the control group have lower CD4 count levels (<200 cells/μL), and 89.4% of patients have coinfections. This may affect statistical power and the ability to detect significant differences between groups. Despite some limitations, this study provides useful insights into the clinical

value of mNGS for identifying PJP in PWH. A large-scale, multicenter investigation is needed to verify our findings.

In conclusion, our study found that mNGS of blood samples exhibits excellent sensitivity and specificity for diagnosing PJP in PWH patients, particularly when respiratory samples are unavailable. Caution should be exercised when interpreting low numbers of *Pj*-specific mNGS reads in blood samples, as the definitive diagnosis of PJP relies on the integration of clinical data with mNGS results. Further research is needed to confirm these findings.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. H. L. collected the data and drafted the manuscript. Y. H. and Y. J. contributed to the analysis and interpretation of the data, provided critical revision of the manuscript, and supervised the study. H. Z. designed the study and revised the manuscript. All authors gave final approval for the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Data availability. The data presented in the study are included in the article and the Supplementary Material and are available upon written request to the corresponding author.

Ethics approval. This study was approved by the Ethics Committee of the Second Xiangya Hospital of the Central South University (LYF20240235). All procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The requirement of informed consent was waived due to the retrospective nature of the study, the anonymization of data, and the absence of any risk.

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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