

Study on mNGS Technique in Diagnosing *Pneumocystis jirovecii* Pneumonia in Non-HIV-Infected Patients

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Objective: To investigate the value of metagenomic Next-Generation Sequencing (mNGS) in diagnosing *Pneumocystis jirovecii* pneumonia (PJP) in non-human immunodeficiency virus (HIV)-infected patients.

Methods: In this retrospective study, non-HIV-infected patients with PJP and those diagnosed with non-PJP from August 2022 to December 2024 were selected as subjects. The presence of *Pneumocystis jirovecii* (PJ) and other co-pathogens in bronchoalveolar lavage fluid (BALF) was analyzed, and the diagnostic efficacy of NGS, polymerase chain reaction (PCR) and serum 1,3- β -D-glucan (BDG) in PJP was compared with the reference standard of clinical compound diagnosis.

Results: Eighty-nine non-HIV-infected patients were recruited, with dyspnea as the primary symptom (69.66%) and solid malignant tumor as the most common underlying disease (20.22%). Taking clinical compound diagnosis as the reference standard, the sensitivity, specificity, negative predictive value and positive predictive value of mNGS were higher than those detected by PCR and serum BDG. Among 42 non-HIV-infected patients with PJP who underwent mNGS and conventional pathogen detection of BALF, 6 had simple PJ infection and 36 had combined PJ infection. The detection rate of mNGS in mixed infections was significantly higher than that of conventional pathogen detection (85.71 vs 61.70%, $P = 0.012$). A total of 127 pathogens were detected in BALF using mNGS, among which fungi had the highest detection rate (46.46%). The fungi, viruses and bacteria detected were mainly *Pneumocystis jirovecii*, human gammaherpesvirus 4 and *Acinetobacter baumannii*.

Conclusion: mNGS is highly effective in diagnosing non-HIV-infected patients with PJP and exhibits ideal performance in the detection of co-pathogens. In addition, it has certain value for clinical diagnosis and guidance of targeted anti-infective drug treatment.

Keywords: metagenomic next-generation sequencing, *Pneumocystis jirovecii* pneumonia, polymerase chain reaction, non-HIV infection

Introduction

Pneumocystis jirovecii pneumonia (PJP) is an acute or subacute pneumonia resulting from infection with *Pneumocystis jirovecii* (PJ). It may initially cause dyspnea, fever, and dry cough, and in severe cases, death.¹ As an opportunistic infection, it occurs in immunocompromised or compromised hosts and is one of the most common complications of human immunodeficiency virus (HIV). It also frequently makes inroads on non-HIV immunocompromised people with hematological malignancies, hematopoietic stem cell transplantation, autoimmune diseases, inflammatory diseases, and immunosuppressive therapy.² Recent decades have witnessed a decrease in the incidence and mortality of PJP in HIV-infected patients due to routine use of chemoprophylaxis and antiretroviral therapy.³ In contrast, the incidence of PJP in non-HIV-infected patients increased significantly.⁴ Studies have shown that the mortality rate of PJP in HIV-infected patients is less than 10%, while it is as high as 25% to 55% in non-HIV-infected patients.⁵ Delayed diagnosis and treatment of PJP will affect the prognosis of patients and bring a huge economic burden to families and society.

Therefore, pathogen identification and clinical intervention of PJP should be carried out at an early stage, which is crucial to reduce mortality and improve the prognosis of patients.

In the previous practice, the diagnosis of PJP depended on observing respiratory specimens under a microscope after routine or immunofluorescence staining. However, the low fungal load and low sensitivity of routine staining resulted in negative reports that were far from sufficient to rule out a diagnosis of PJP. In addition, immunofluorescence staining is not routinely performed in many hospitals.⁶ 1,3- β -D-glucan (BDG) is currently a common serological biomarker for auxiliary diagnosis of PJP, but it lacks specificity.⁷ Polymerase chain reaction (PCR) is widely used to detect PJ because of its good sensitivity and specificity, but it has limited value in single detection of mixed infection.⁸ Therefore, there is an urgent need to explore a more accurate and efficient microbial diagnostic tool for detecting PJP in non-HIV populations.

With the advent of the metagenomic Next-Generation Sequencing (mNGS) technique, pathogen detection approaches are increasingly diverse. mNGS refers to the use of high-throughput sequencing and bioinformatics analysis technology for gene sequencing of all microbial nucleic acids extracted from different types of clinical samples. It can be used to obtain all microbial information in samples in an efficient, objective and accurate way and provide a new means for identifying pathogenic infection.⁹ At present, the vast majority of studies on mNGS focus on the diagnosis and treatment of bacterial infections, but rarely on the diagnosis and treatment of fungal infections, especially non-HIV-infected PJP. To this end, we specifically designed this study to investigate the value of mNGS in the diagnosis and treatment of non-HIV-infected patients with PJP.

Materials and Methods

Subjects

This retrospective study selected non-HIV-infected PJP patients and non-PJP patients diagnosed in the People's Hospital of Xinjiang Uygur Autonomous Region from August 2022 to December 2024 as the research objects. Forty-two PJP patients without HIV infection were selected as the case group, and 46 patients with pulmonary infection who were admitted to the hospital during the same period and subsequently diagnosed as non-PJP were selected as the control group. Inclusion criteria for non-HIV-infected PJP patients: (1) *Pneumocystis jirovecii* was detected in lower respiratory tract specimens;¹⁰ (2) Immunocompromised patients, including but not limited to hematological diseases, rheumatic connective tissue diseases, long-term use of corticosteroids or immunosuppressants, solid organ transplantation, solid tumors, etc.; (3) The patient was definitely diagnosed as non-HIV-infected person; (4) Those with PJ nucleic acid positively detected by PCR or PJ gene sequence detected by mNGS. A clinical compound diagnosis of PJP or non-PJP was made jointly by two experienced clinicians. Inclusion criteria for non-PJP patients: (1) Patients with fever, dyspnea, cough, expectoration and other respiratory symptoms; (2) The patient's imaging findings were pale patchy shadows with increased density with blurred edges, which could also be manifested as patchy consolidation shadows or ground glass shadows; (3) PCR detection of PJ nucleic acid was negative, and mNGS detection of PJ gene sequence was negative; (4) Sputum culture or bronchoalveolar lavage fluid specimens produced a clear pathogen. The exclusion criteria were as follows: (1) Patients with missing important case data; (2) Those with infection in other sites; (3) Those aged <18 years old; (4) Those co-infected with HIV. This study was approved by the Ethics Committee of our hospital. All patients or family members have signed an informed consent form, this was conducted in accordance with the Declaration of Istanbul, and all organs were donated voluntarily with written informed consent.

Study Methods

All patients underwent fiberoptic bronchoscopy, and their bronchoalveolar lavage fluid (BALF) samples were collected. Subsequently, each BALF sample was divided into aliquots for routine microbial tests (including PCR detection and traditional culture) and mNGS, and blood samples were sent for related tests.

Sample Processing and DNA Extraction for mNGS Detection

Enzyme and 100 g of 0.5 mm glass beads were added to the 600 μ L sample taken and vortexed for 30 min [(1200–1500) \times g]; Then, 300 μ L of the shaken supernatant was transferred to a 1.5 mL centrifuge tube for nucleic acid extraction. Three to five milliliters of anticoagulated venous blood samples were centrifuged for 10 min at 4°C and 1600 g, and the

plasma was transferred to a 2 mL centrifuge tube for DNA extraction. DNA was extracted using the Huada Nucleic Acid Extraction Kit.

DNA Library Construction and Sequencing

MGIEasy Cell-free DNA Library Prep Set (batch No.: 108420230) was utilized to construct the DNA library by DNA fragmentation, end repair and PCR amplification. This technique transforms double-stranded DNA into single-stranded circular DNA via DNA degradation and cyclization and then loads the qualified DNA onto the chip. DNA library nanospheres were prepared by using the MGISP-100 automated sample preparation system. After preparation, the concentration was determined for quality control, which was required to be ≥ 8 ng/ul. The nanospheres with qualified concentration, nanospheres loading buffer and nanospheres polymerase mixed solution were configured into a loading system and were put into the MGISEQ-2000 platform for gene sequencing together with sequencing slides.

Bioinformatics Analysis

The results of gene sequencing were preliminary data, which need to be further processed. First, high-quality data were obtained by removing low-quality data and data with reads of length less than 35bp.⁹ The high-quality data were aligned to the human genome (hg19) by using the Burrows-Wheeler Aligner software (BWA: <http://bio-bwa.sourceforge.net/>), and then the data containing the host human nucleic acid sequence were removed. The remaining data were compared with a dedicated microbial database after removing low-complexity sequences, and the sequenced data were classified and arranged according to viruses, bacteria, fungi and parasites. The classification reference database can be downloaded from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>). As to the threshold criteria for interpretation of mNG, the microbial species-specific read number (SSRN) method was used to interpret the sequencing results. For *Pneumocystis jirovecii*, SSRN, that is, Pj reads ≥ 1 , was used as the positive criterion in this study to ensure sensitivity.

Data Collection

The basic information (demographics, clinical symptoms, underlying diseases, etc.), laboratory indicators, BALFmNGS, PCR, serum BDG, etc. of the patients were collected.

Statistical Analysis

All data collected in this study were statistically analyzed using SPSS 26.0 software and plotted by GraphPad 8.0. Measurement data conforming to normal distribution were presented as mean \pm standard deviation ($\bar{x} \pm s$), and the independent sample *t*-test was used for comparison between the two groups. Non-normally distributed measurement data were expressed as median (interquartile interval) [M (P25, P75)], and the Mann-Whitney *U*-test was used for comparison between the two groups. Count data were expressed as frequency (%), and χ^2 test or Fisher's exact test was used for comparison between groups. Taking the clinical compound diagnosis (whether *Pneumocystis jirovecii* was detected in lower respiratory tract specimens) as the reference standard,¹⁰ the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated, and $P < 0.05$ was considered a statistically significant difference.

Results

Clinical Data

Eighty-nine non-HIV-infected patients were recruited in this study, including 57 males (64.04%) and 32 females (35.96%), aged from 22 to 83, with an average age of 58.34 ± 17.44 . Most patients had dyspnea as the primary symptom (69.66%) and solid malignant tumor (20.22%) as the most common underlying disease. Laboratory examination showed that the absolute value of the median lymphocyte count was $0.41 \times 10^9/L$, and the median CD4 + T lymphocyte count was 117.0/L, both of which were lower than the lower limit of the normal reference range. See Table 1 for details.

Table 1 Clinical Data of Non-HIV Patients

	Item	Data
Age [cases (%)]	< 60 years old	40(44.94)
	≥ 60 years old	49(55.06)
Gender [cases (%)]	Male	57(64.04)
	Female	32(35.96)
Symptoms of onset [cases (%)]	Dyspnea	62(69.66)
	Cough	54(60.67)
	Expectoration	37(41.57)
	Fever	41(46.07)
Accompanied disease [cases (%)]	Chest tightness	23(25.84)
	Diseases of the blood system	17(19.10)
	Rheumatoid connective tissue disease	11(12.36)
	Solid organ transplantation	15(16.85)
	Solid tumor receive radiotherapy and chemotherapy	18(20.22)
	Neurological disorders	7(7.87)
Laboratory examination	Immunosuppression of chronic kidney disease	9(10.11)
	Interstitial lung disease	13(14.61)
	Leukocyte ($\times 10^9/L$)	5.85(4.24,8.16)
	Absolute neutrophil count ($\times 10^9/L$)	5.45(3.42,5.65)
	Lymphocyte absolute value ($\times 10^9/L$)	0.41(0.26,0.60)
	Hemoglobin (g/L)	102.25(82.19,120.00)
	Platelets ($\times 10^9/L$)	161.35±69.83
	Procalcitonin (ng/mL)	0.19(0.06,0.59)
	C-reactive protein (mg/L)	63.39(16.78,137.40)
	CD4+T lymphocyte count (pcs/pL)	117.0(54.5,230.5)

Abbreviation: HIV, human immunodeficiency virus.

Diagnostic Efficacy of mNGS, PCR and Serum BDG Detection in PJP

Taking clinical compound diagnosis as the reference standard, the sensitivity, specificity, negative predictive value and positive predictive value of mNGS were all 100%, those of PCR detection were 90.48%, 91.49%, 90.48% and 91.49%, respectively, and those of BDG detection were 73.81%, 80.85%, 77.50% and 77.55%, respectively. It can be seen that the diagnostic efficiency of mNGS is significantly higher than that of PCR detection and serum BDG detection (Table 2).

Detection of Mixed Infections by mNGS and Conventional Pathogen Detection Technologies

Among 42 non-HIV-infected patients with PJP who underwent mNGS and conventional pathogen detection of BALF, 6 (14.29%) had simple PJ infection and 36 (85.71%) had combined PJ infection. The detection rate of mNGS in mixed

Table 2 Diagnostic Efficacy of mNGS, PCR and Serum BDG Detection in Non-HIV-Infected PJP

Item	PJP Group	Non-PJP Group	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
mNGS+	42	0	100.00	100.00	100.00	100.00
mNGS-	0	47				
PCR+	38	4	90.48	91.49	90.48	91.49
PCR-	4	43				
BDG+	31	9	73.81	80.85	77.50	77.55
BDG-	11	38				

Abbreviations: mNGS, metagenomic Next-Generation Sequencing; PCR, polymerase chain reaction; BDG, 1,3- β -D-glucan; HIV, human immunodeficiency virus; PJP, *Pneumocystis jirovecii* pneumonia.

Table 3 Comparison Between mNGS and Traditional Detection of Infection

Item	mNGS	Conventional Detection	χ^2	P
Single infection	6(14.29)	18(33.30)	6.017	0.012
Mixed infection	36(85.71)	29(61.70)		

Abbreviation: mNGS, metagenomic Next-Generation Sequencing.

infections was significantly higher than that of conventional pathogen detection (85.71 vs 61.70%, $P = 0.012$) (Table 3). Among mixed infections, fungi combined with bacteria and viruses were the most common (20 cases) (Figure 1).

Distribution of Pathogens of MNGS in Non-HIV-Infected Patients with PJP

In 42 non-HIV-infected patients with PJP, 127 pathogens were detected by mNGS in alveolar lavage fluid, among which the detection rate of fungi was the highest (46.46%). The fungi detected were mainly *Pneumocystis jirovecii* (42 cases) and *Streptococcus albicans* (8 cases) (Figure 2). The main bacteria detected were *Acinetobacter baumannii* (6 cases) and *Staphylococcus aureus* (4 cases) (Figure 3). The viruses detected are mainly human gammaherpesvirus 4 (12 cases) and human betaherpesvirus 5 (8 cases) (Figure 4).

Discussion

Pneumocystis jirovecii is an opportunistic pathogenic fungus that enters the lungs through the respiratory tract, mostly presenting as an asymptomatic infection. If left untreated on time, it will easily develop into acute respiratory distress syndrome, which is also one of the primary causes of death among AIDS patients.¹¹ Given the possible variation in the host immune response to PJ, PJP is a relatively inert process in HIV-infected patients, which usually develops into a subacute or chronic course. In contrast, PJP in non-HIV-infected patients is mostly acute, with rapid progress of lung lesions, high risk of respiratory failure and high mortality.¹² Ko et al¹³ found that delayed initiation of specific anti-

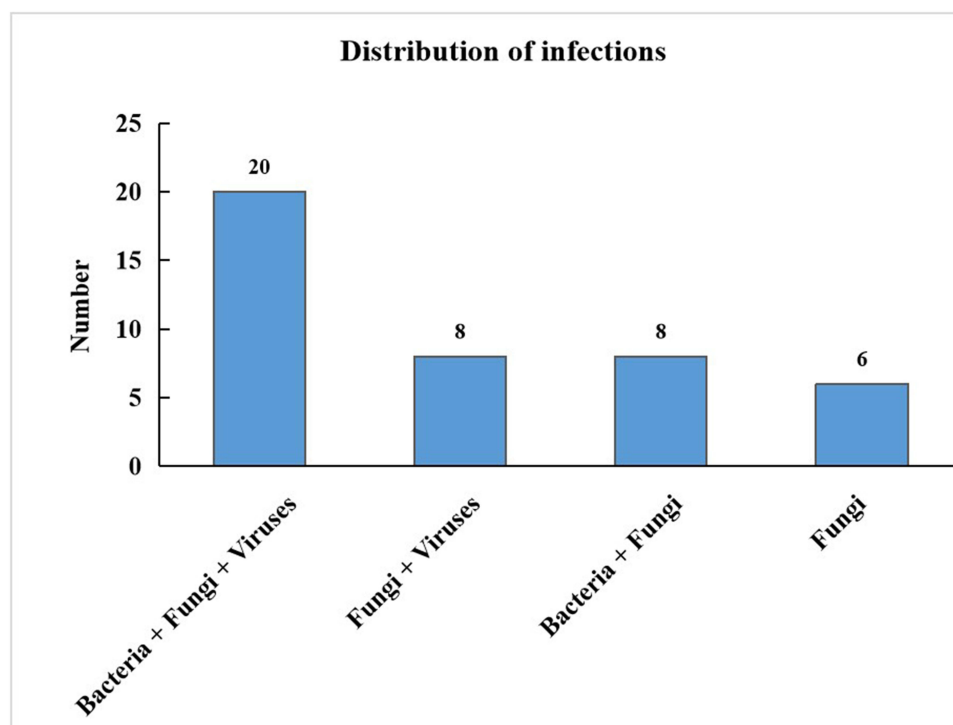


Figure 1 Distribution of mixed infections in patients with PJP.

Abbreviation: PJP, *Pneumocystis jirovecii* pneumonia.

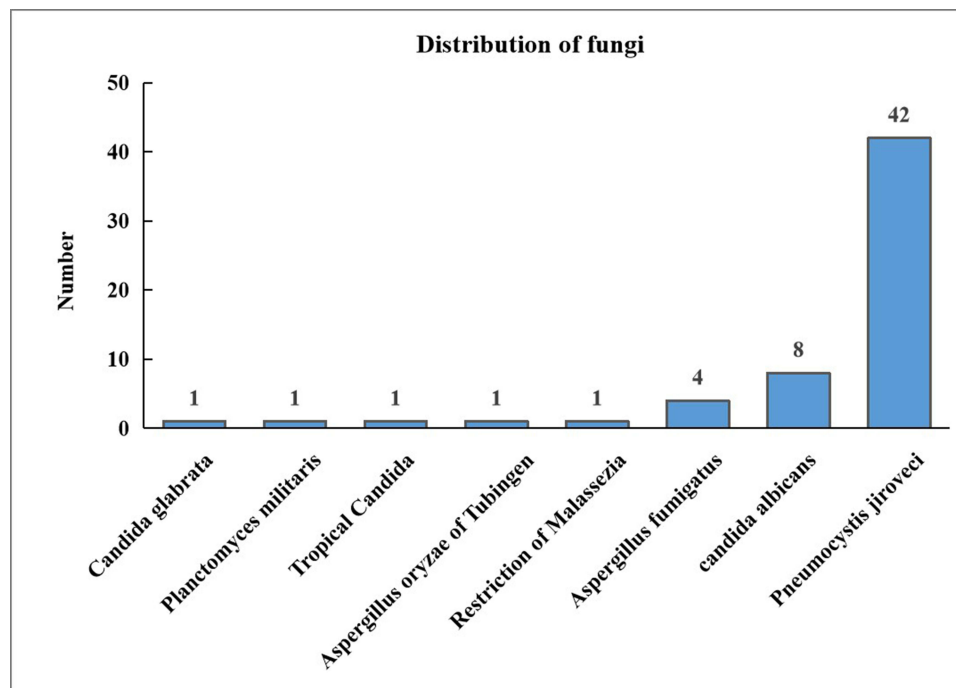


Figure 2 Distribution of fungi in non-HIV-infected patients with PJP.

Abbreviations: PJP, *Pneumocystis jirovecii* pneumonia; HIV, human immunodeficiency virus.

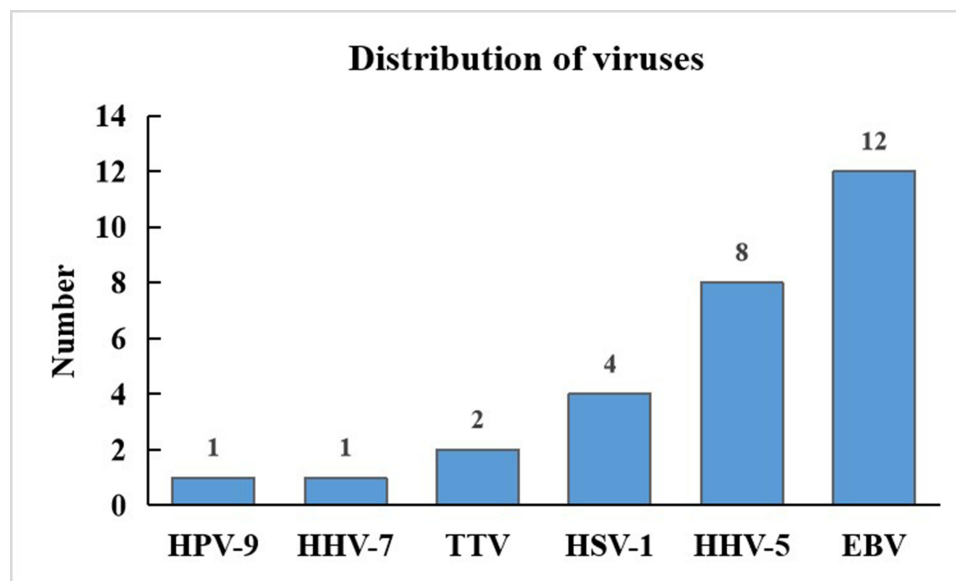


Figure 3 Distribution of virus in non-HIV-infected patients with PJP.

Abbreviations: PJP, *Pneumocystis jirovecii* pneumonia; HIV, human immunodeficiency virus; HPV, human papillomavirus; HHV, human herpesvirus; TTV, torque teno virus; HSV, herpes simplex virus; HHV, human herpesvirus; EBV, Epstein-Barr virus.

infective therapy or initially unsuccessful antimicrobial therapy resulted in acute respiratory failure with poor prognosis in non-HIV-infected patients with PJP. Rapid diagnosis and early initiation of treatment for PJP yields a better prognosis.

PJ has difficulty in being diagnosed due to a low inoculation amount in the respiratory tract of patients, which is usually negative by direct microscopic examination of smears.¹⁴ In the current microbiological diagnosis of PJP, two detection technologies, PCR and serum BDG, are preferred. In particular, PCR technology can quantitatively detect PJ in respiratory secretions. It was found in the present study that PCR has high sensitivity (90.48%) and specificity (91.49%).

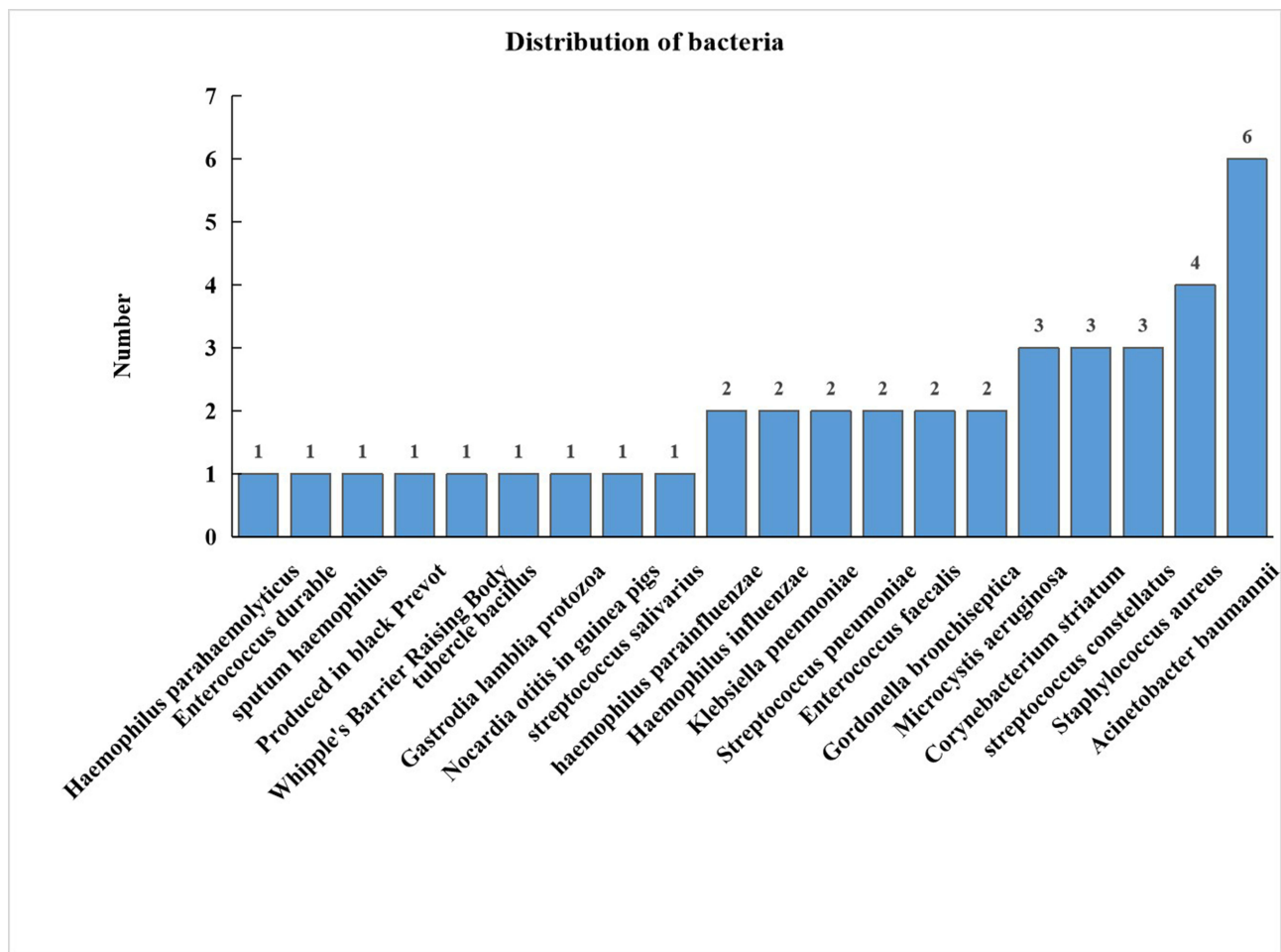


Figure 4 Distribution of bacteria in non-HIV-infected patients with PJP.
Abbreviations: PJP, *Pneumocystis jirovecii* pneumonia; HIV, human immunodeficiency virus.

Salzer et al¹⁵ concluded that the sensitivity of PJ in BALF detected by PCR was 94%–99%, and the specificity was 89%–91%, which was consistent with the results of this study. However, PCR has limitations in the diagnosis of PJP. Contamination, colonization, etc. may cause false-positive results. Studies have shown that quantitative PCR (qPCR) seems to improve diagnostic efficiency, but there is controversy regarding the threshold required to distinguish PJ colonization from infection.¹⁶ Furthermore, PCR has limited value in the single detection of mixed infections, despite its higher detection value in single infections. In contrast, mNGS has higher diagnostic efficiency in PJP and higher diagnostic value in mixed infections. mNGS is a good choice when the conventional anti-infection treatment is not effective and mixed infection is considered. BDG is a structural polysaccharide produced in the process of cell wall synthesis of many pathogenic fungi. At present, it is primarily used in the auxiliary diagnosis of pulmonary invasive aspergillosis and invasive candidiasis.¹⁷ Several observational studies in recent years have described the use of BDG detection for the presumptive diagnosis of PJP. Studies have shown that BDG detection can be used as a minimally invasive screening test for the presumed diagnosis of PJP without invasive fibrobronchi.¹⁸ However, BDG is prone to false-positive results in the diagnosis of PJP, resulting in the termination of the diagnosis (in the case of positive results, the diagnosis is generally not terminated), especially in critically ill patients. Therefore, further confirmatory testing is required when performing BDG detection.¹⁹ mNGS is superior to BDG in that it has better diagnostic performance and can identify pathogenic microorganisms quickly, objectively and without bias.

mNGS is a novel microbial detection and identification technique that can identify pathogenic microorganisms quickly, impartially and objectively compared with conventional detection techniques. It has obvious advantages in the early diagnosis of

unknown pathogens, pathogens that are difficult to culture, conditional pathogens, mixed pathogen infections and difficult infections, and provides a new means for accurate anti-infection treatment.²⁰ However, there is currently no complete knowledge of the diagnostic performance of mNGS in PJP. In this study, the sensitivity and specificity of mNGS in non-HIV-infected patients with PJP were both 100%, which was significantly better than that of serum BDG and PCR detection. These results show that early application of mNGS is beneficial to promote the rapid and accurate diagnosis of PJP. Besides, the detection rate of mixed infection in BALF using mNGS was significantly higher than that of conventional pathogen detection, suggesting that mNGS has advantages in identifying co-pathogens in patients with PJP mixed infection. This is consistent with the results by Duan et al.²¹ More studies by Li et al¹⁰ explored the diagnostic value of mNGS in AIDS patients complicated with PJ infection, and the results showed that the coincidence rate of mNGS detection was 100%. This is consistent with the results of this study. In the present study, the mNGS technique was used to detect not only the target pathogen but also the mixed infection of other pathogens, among which mixed infections with herpes viruses were the most common. Meanwhile, the infection of bacteria, viruses and fungi is also common, which can provide guidance for effective anti-infection treatment.

There are still certain limitations in this study: this is a single-center study with bias in patient group selection; Besides, this is also a retrospective study with limited sample size. In response to this, further prospective study is needed to confirm the application value of mNGS in the diagnosis and treatment of PJP infection in non-HIV patients.

Conclusion

To sum up, compared with serum BDG and PCR detection, mNGS has obvious advantages in the diagnosis of non-HIV-infected PJP, with its rapid detection speed, short reporting time, high sensitivity and specificity, and high detection rate of mixed infection. It has a certain value for early clinical diagnosis and guiding targeted treatment.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region. Due to the nature of retrospective study and anonymized patient's information, informed consent is waived with the approval of Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region. All methods were carried out in accordance with relevant guidelines and regulations.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of 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.

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Disclosure

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