Infection and Drug Resistance

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ORIGINAL RESEARCH

The Clinical Value of Metagenomic Next-Generation Sequencing in *Pneumocystis jirovecii* Pneumonia

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Background: The incidence of *Pneumocystis jirovecii* pneumonia (PJP) is increasing.

Methods: 108 patients were analysed retrospectively at the Wuhan Union Hospital. The patients were classified into the PJP group or the *P. jirovecii* colonisation (PJC) group based on clinical diagnosis. Clinical data included demographics, laboratory examinations, treatment, and outcomes.

Results: A notable difference in the fungal load was seen between two groups, with median reads of 3215.79 vs. 5.61 in two groups, respectively ($P<0.001$). The optimal threshold value for discriminating *P. jirovecii* infection between colonisation for mNGS was six, and serum (1,3)-β-D-glucan (BDG) was 47.6 pg/mL. Besides, the positive detection rate of mNGS for co-pathogens in PJP patients was significantly higher than that of culture (88.16% vs. 22.37%, $P<0.0001$). *Epstein-Barr virus* and *cytomegalovirus* were the most common pathogens of co-infection in PJP patients. The antibiotic therapy in PJP patients was adjusted according to the mNGS results, of which seventeen (22.37%) were downgraded, 38 (50.0%) patients were upgraded, and 21 (27.63%) were unchanged. And almost all patients showed significant improvement in C-reactive protein.

Conclusion: mNGS is a promising and valuable technique with good performance for differentiating *P. jirovecii* infection and colonisation, the detection of pathogens, and antibiotic treatment.

Keywords: *Pneumocystis jirovecii* pneumonia, *Pneumocystis jirovecii*, clinical value, metagenomic next-generation sequencing, non-HIV

Introduction

P. jirovecii is a common fungal pathogen that commonly causes opportunistic lung infections in individuals with impaired immune systems.[1] PJP was initially considered a human immunodeficiency virus (HIV)-related disease associated with the organism's T-cell immunity. The incidence of PJP among HIV patients has gradually declined due to the implementation of highly effective antiretroviral therapy and chemoprophylaxis for HIV, especially in countries and regions where routine HIV testing and timely treatment are available.[2,3] Meanwhile, the prevalence of PJP has increased in non-acquired immunodeficiency syndrome (AIDS) groups with a combination of immunocompromise, owing to the growing proportion of immunosuppressive drugs and corticosteroids applied in clinical practice and the parallel increase in transplant patients with advances in medical technology.[4] Concerningly, patients with non-AIDS combined with immunocompromised PJP usually have a poor prognosis as a result of their severe symptoms, acute disease course, and rapid progression.[5] Furthermore, the mortality rate increases as well. Hematologic tumours, autoimmune disorders, and taking corticosteroids or immunosuppressants for a long time are risk factors for PJP. Fever, a dry cough, hypoxemia, and dyspnea are the typical clinical symptoms of PJP. Diffuse ground glass shadows are considered the characteristic images of the lungs in PJP patients, with changes such as cysts, solids, nodules, and spontaneous

Received: 31 October 2023
Accepted: 22 December 2023
Published: 9 January 2024

pneumothorax followed by others.[7] Trimethoprim-sulfamethoxazole (TMP-SMZ) is currently the preferred first-line prophylactic and therapeutic regimen.[8]

Detection of *P. jirovecii* mostly relies on the staining of clinical specimens with Gomori methenamine silver (GMS) stains and finding characteristic cysts or their trophic forms under the microscope. Thus, the microbiological diagnosis was limited. Meanwhile, delayed diagnosis means a lag in treatment and worsening of symptoms. The insufficient detection performance of traditional methods has promoted the speedy development of modern diagnostic methods. The serum BDG assay and the serum level of S-adenosylmethionine (SAM) are regarded as supplements for comprehensive diagnosis.[9] Real-time quantitative fluorescent PCR assays can detect extremely low fungal loads,[10] but they have not been widely validated and used. Recent studies have also been conducted to distinguish between colonisation and infection by thresholds determined by quantitative PCR.[11] mNGS is an emerging method for discovering rare pathogens through high-throughput sequencing platforms. *P. jirovecii* cannot be cultured outside the lung, so mNGS has been proven to have excellent performance compared to conventional approaches.[12,13] mNGS enables the discovery of *Pneumocystis* nucleic acid sequences. It can also detect fungi, viruses, parasites, and some rare bacteria that cannot be detected by regular culture, which is beneficial for diagnosing mixed infections.[14,15]

In our study, we evaluated the diagnostic performance of mNGS in non-HIV-infected patients, differentiating between colonisation and infection with *P. jirovecii*. Besides, we also analysed the clinical value of mNGS for pathogenic detection and antibiotic adjustment.

Methods

Study Participants and Study Design

The 132 patients were enrolled at Wuhan Union Hospital from October 2018 to October 2022. Patients were eligible for enrollment if they met all the following criteria: (1) consented to undergo the mNGS examination; (2) had a positive mNGS result for *P. jirovecii*; (3) clinical manifestations (fever, cough, dyspnea, and progressive hypoxemia) or radiologic findings (diffuse ground glass shadows or diffuse infiltration in the interstitium). The patients were excluded if they met any of the following criteria: (1) HIV infection; (2) age < 18 years old; (3) medical record was incomplete.

The patients who met the following criteria were classified into PJP: (1) had a positive mNGS result for *P. jirovecii*; (2) clinical manifestations—fever, cough, dyspnea, and progressive hypoxemia; (3) lung radiological signs—diffuse ground glass shadows or diffuse infiltration in the interstitium.[16] The patients with a positive mNGS result but with no symptoms or radiological signs of PJP were clinically diagnosed with PJC. The clinical diagnosis was conducted by two experienced clinicians who evaluated the patients' immunity, clinical manifestations, laboratory results, radiographic imaging, the mNGS report, and the response to anti-*P. jirovecii* treatment. A total of 108 individuals were eventually included, which were divided into the PJP group (n = 76) and the PJC group (n = 32) based on clinical diagnosis. This study was approved by the research ethics committee of Wuhan Union Hospital (2023-0177), and the need for obtaining written informed consent was waived.

Clinical Data Collection

A standardised data collection form was utilised to gather data elements, including demographics, laboratory results, radiographic imaging, treatment, and clinical outcomes, from the electronic medical records of Wuhan Union Hospital. Data included pre-admission treatment, initial antibiotic administration upon admission, and subsequent adjustments based on mNGS results. Except for adding TMP/SMZ, most escalation events were related to increasing the number of antimicrobial agents against specific pathogens, such as antiviral and antifungal drugs, and other escalation events were related to expanding the spectrum of agents. Similarily, escalation events included reducing the number of agents and the spectrum of agents.

Electronic Bronchoscopy and Bronchoalveolar Lavage Fluid Samples Collection

All patients in the cohort underwent electronic bronchoscopy after being fully evaluated. Everyone signed an informed consent form. The operation is performed by an experienced bronchoscopist following a standardized procedure. To enhance the diagnostic accuracy, the sample was collected based on the precise location of the lesion according to chest

radiography. Patients received local anaesthesia with 2% lidocaine before the examination. All bronchial tubes were examined in detail using electronic bronchoscopy, especially at the lesion site. After that, the distal portion of the bronchoscope was introduced into the designated bronchi, followed by the instillation of 20 mL of sterile saline solution (0.9% concentration) at a temperature of 37°C for lavage purposes. Bronchoalveolar lavage fluid (BALF) was obtained through suction and then subjected to mNGS or other diagnostic evaluations for the purpose of assessment.

mNGS and Analysis

A total of 108 BALF samples were obtained and sent for sequencing, either using DNA alone or a combination of DNA and RNA. The samples for mNGS were sent to testing companies for nucleic acid extraction, library construction, high-throughput sequencing, bioinformatics analysis, result presentation, and pathogen data interpretation. The detection of pathogenic microbial metagenomics relied on the use of high-throughput sequencing technology. This approach enables the identification of microbial species by comparing their nucleic acid sequences with those of known bacteria stored in the database. The detection scope covered the entire genome sequences of 9218 bacteria, which includes 102 species of mycoplasma/chlamydia. Additionally, it included 9194 viruses, 721 fungi, 96 rickettsia, and 180 parasites. Various parameters of the sequencing platform included mapping read number (species and genus level), abundance (species and genus level), depth, and coverage rate.[17–19] These factors were applied to eliminate any potential interference from other microorganisms. The mNGS detection was conducted according to the manufacturer's protocol, which is reported in detail in Supplementary File 1.

Pathogens detected by mNGS other than PJP were considered co-infectious pathogens if they met one of the following criteria: (1) both culture and mNGS identified the same microbe, and mNGS reads exceeded 50 for a single species;[20] (2) A positive identification of Mycobacterium tuberculosis was established if at least one read was successfully observed;[21] (3) >30% relative abundance at the genus level in bacteria, viruses, or fungi.[20]

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 26.0 software package (IBM, Armonk, NY, USA). The continuous variables that corresponded to a normal distribution were expressed as the mean ± standard deviation (SD), and non-normally distributed measures were expressed as the median (25th–75th percentiles). Continuous measures were statistically analysed using a *t*-test or Mann-Whitney *U*-test to compare the differences between the two groups. Categorical variables are expressed as n (%). Categorical variables were compared using the χ^2 test or Fisher's test. To evaluate the sensitivity and specificity of serum BDG and mNGS for the diagnosis of PJP, a ROC was constructed. Statistical significance was set at a two-tailed *P*-value of < 0.05.

Results

Population Characteristics

All patients were divided into the PJP group and the PJC group based on the final clinical diagnosis. The median age of patients with PJP was 58 years, of which 64.47% (49/76) were male. The median age of patients in the PJC group was 62 years, and 53.13% (17/32) were male. Except for hematologic tumours, complications were more common in the PJP group than in the PJC group. However, only in chronic kidney disease was there a statistically significant difference between the two groups. Thirty-five PJP patients and only one PJC patient were receiving glucocorticoids before admission, and there was a significant difference between the two groups ($P<0.001$). Besides, immunosuppressive therapy was more common in the PJP group than in the PJC group (39.47% vs. 6.25%, $P=0.001$). The common symptoms of PJP patients included dyspnea (75.00%), cough (65.79%), and fever (63.16%). The median hospital day of PJP group was 16d, but PJC was 10d. There was a statistical difference between them. As expected, PJP patients developed more severe pneumonia than PJC patients. In terms of mechanical ventilation, the number of PJP patients receiving non-invasive mechanical ventilation was higher than that of PJC patients, and there was a statistical difference, while there was no statistical difference in endotracheal intubation.

The white blood cell counts and neutrophil counts exhibited lower values in PJP patients compared to PJC patients, although no statistically significant difference was detected. All PJP patients showed lymphopenia in peripheral blood and significantly lower hemoglobin levels. Median serum levels of lactate dehydrogenase (415 U/l vs. 261.5 U/l) and C-reactive protein (56.4 mg/l vs. 29.3 mg/l) were remarkably increased in both groups, and there were significant differences between the two groups (P=0.01 and P=0.033, respectively) (Table 1). The median PaO2/FiO2 was 228 mmHg in PJP patients. The median read count of mNGS and the median titer of serum BDG were found to be considerably higher in the PJP group. The

Table 1 Clinical Characteristics of 108 Patients

Characteristic	PJP Group (n=76)	PJC Group (n=32)	*P*-value
Age, years	58 (50–65)	62 (53.5–67.75)	0.181
Gender, male/female, n	49/27	17/15	0.269
Smoking history, n (%)	29 (38.16%)	10 (31.25%)	0.71
Comorbidities, n (%)			
Hypertension	27 (35.53%)	11 (34.38%)	0.909
Diabetes	16 (19.75%)	2 (6.25%)	0.059
Chronic kidney diseases	24 (31.58%)	3 (9.38%)	0.015
Connective-tissue disease	19 (25.00%)	6 (18.75%)	0.482
Solid organ tumor	22 (28.95%)	8 (25%)	0.676
Hematologic tumor	2 (2.63%)	2 (6.25%)	0.838
Chemotherapy, n (%)	17 (22.37%)	4 (12.5%)	0.237
Immunosuppressive therapy, n (%)	30 (39.47%)	2 (6.25%)	<0.001
Corticosteroid therapy, n (%)	35 (46.05%)	1 (3.13%)	<0.001
Clinical presentation			
Fever, n (%)	48 (63.16%)	10 (31.25%)	0.002
Cough, n (%)	50 (65.79%)	26 (81.25%)	0.108
Dyspnea, n (%)	57 (75.00%)	21 (65.63%)	0.321
Hospital LOS, days	16 (11.25–22)	10 (8–14.75)	<0.001
The time from onset to admission, days	10 (7–30)	15 (7–30)	0.197
Mechanical ventilation			
Noninvasive ventilation, n (%)	36 (47.37%)	6(18.75%)	0.005
Endotracheal intubation, n (%)	13(17.11%)	2 (6.25%)	0.236
Outcomes			
Severe pneumonia, n (%)	39 (48.15%)	3 (10.34%)	<0.001
30-day mortality, n (%)	15 (18.52%)	3 (9.38%)	0.231
Laboratory findings			
WBC count (10^9/L)	6.05 (4.41–8.99)	7.2 (5.46–9.82)	0.155
HB (g/l)	103.78 ± 22.43	121.31 ± 20.49	<0.001
PLT (10^9/L)	162.5 (110–240.5)	266 (186.5–349.5)	<0.001
Neutrophil count (10^9/L)	4.73 (3.41–7.73)	5.29 (3.25–8.08)	0.803
Lymphocyte count (10^9/L)	0.59 (0.40–0.94)	1.18 (0.75–1.58)	<0.001
NLR	8.12 (3.88–15.31)	4.69 (2.94–8.70)	0.007
[a]CRP (mg/l)	56.4 (20.70–129.03)	29.3 (6.10–60.05)	0.033
[b]PCT (ng/ml)	0.22 (0.13–0.95)	0.13 (0.13–0.133)	0.004
Albumin (g/L)	29.58 ± 4.85	35.84 ± 6.67	<0.001
LDH (U/L)	415 (308.5–579)	261.5 (194.75–458.75)	0.001
[c]PaO2/FiO2 (mmHg)	228.00 ± 98.86	302.25 ± 231.22	0.034
mNGS	796 (23.75–3339.91)	2 (1–3.5)	<0.001
[d]BDG (pg/ml)	346.7 (55.23–970.03)	9.6 (8–37.38)	<0.001

Notes: [a]75 PJP patients completed CRP examination and 28 PJC patients completed CRP examination. [b]69 PJP patients completed PCT examination and 26 PJC patients completed PCT examination. [c]50 PJP patients completed PaO2/FiO2 examination and 13 PJC patients completed PaO2/FiO2 examination. [d]32 PJP patients completed serum BDG examination and 10 PJC patients completed serum BDG examination.

Abbreviations: LOS, length of stay; WBC, White blood cell; HB, Hemoglobin; PLT, Platelet; NLR, Neutrophil count/Lymphocyte count; CRP, C-reactive protein; PCT, Procalcitonin; LDH, Lactic dehydrogenase; BDG, β-D-glucan assay; mNGS, metagenomic next-generation sequencing; PaO2, Arterial partial pressure of oxygen; FiO2, Fraction of inspired oxygen.

median mNGS read was 796, while the median titer of serum BDG was 346.7 pg/mL. The median number of mNGS was 2 in PJC patients, and the median titer of serum BDG was 9.6 pg/mL.

Diagnostic Efficacy of mNGS and BDG for PJP

The area under the curve (AUC) for mNGS in BAL was 0.9641 (Figure 1A), with a 95% confidence interval (CI) ranging from 0.868 to 1.007. Similarly, the AUC for serum BDG was 0.85 (Figure 1B), with a 95% CI ranging from 0.769 to 0.989. The study observed that the optimal cut-off values for distinguishing between *P. jirovecii* infection and colonization was six reads (sensitivity, 90.63%; specificity, 90%; positive likelihood ratio, 9.063). According to the manufacturer's instructions, the result was positive when the BDG value exceeded 100.5 pg/mL. When serum BDG > 100.5 pg/mL, the sensitivity was only 47.5%, and the specificity was 90%. The optimal threshold for distinguishing *P. jirovecii* infection from colonization was 47.6 pg/mL (sensitivity, 68.75%; specificity, 90%; positive likelihood ratio, 6.875) for serum BDG.

Diagnostic Performance of mNGS in Co-Pathogens

PJP patients often experience mixed infections as a result of their weak immunity. In our study, it was observed that out of the total number of PJP patients, only nine individuals did not exhibit coinfection with any other pathogens. The number of cases with mixed infections detected by mNGS are summarized. The most common pathogens coinfection was bacteria (43/119), followed by viruses (42/119) and fungi (34/119). *P. jirovecii*-virus coinfection, *P. jirovecii*-bacteria coinfection, and *P. jirovecii*-fungi coinfection were identified by mNGS in 19 (25%), 8 (30.77%), and 5 (6.58%) of 76 patients. The top two bacteria identified were *Acinetobacter baumannii* and *Enterococcus faecium*. *Cytomegalovirus* and *Candida albicans* were the most common viruses (29/119, 24.37%) and fungi (11/119, 9.24%), respectively. Among the PJP patients, six cases were identified by mNGS as having coinfections involving *P. jirovecii*, viruses, bacteria, and fungi. *Cytomegalovirus, Epstein-Barr virus, Candida albicans, Aspergillus fumigatus*, and *Human herpesvirus 7* were the top five co-pathogens identified by mNGS (Figure 2A–C).

Comparison of mNGS with Culture in Co-Pathogens

We compared the positive rate of mNGS and culture methods. The positive rate of pathogens of mNGS in PJP patients was much greater than culture (88.16% vs 21.05%, P <0.001). We analyzed the consistency of pathogens between mNGS

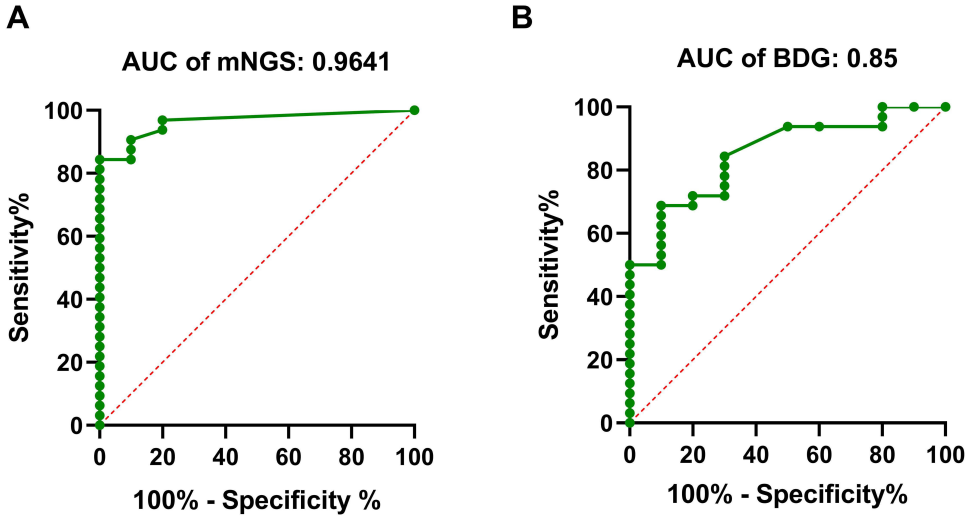

Figure 1 ROC curves for mNGS and serum BDG for discrimination between PJP and PJC. (**A**) ROC curves for mNGS. The area under the curve value of mNGS was 0.9641; (**B**) ROC curves for serum BDG, The area under the curve value of serum BDG was 0.85.
Abbreviations: ROC, Receiver operating characteristic; mNGS, metagenomic next-generation sequencing; BDG, β-D-glucan assay.

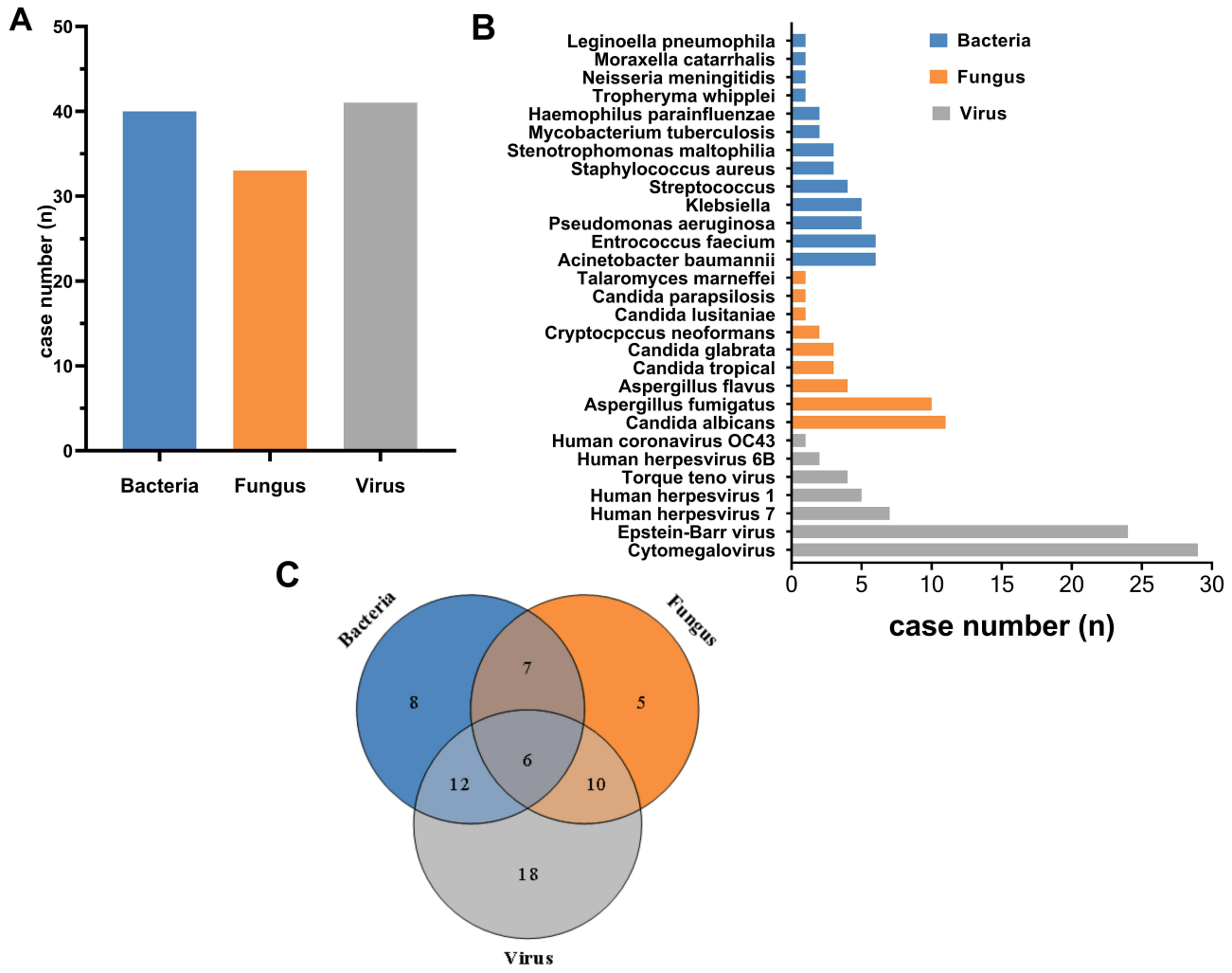

Figure 2 Diagnostic performance of mNGS in co-pathogens in PJP patients. (**A**)Composition of co-pathogens in PJP patients with positive mNGS result; (**B**) Distribution of co-pathogens in PJP patients identified by mNGS; (**C**) The number of PJP patients for co-infection identified by mNGS.
Abbreviations: mNGS, metagenomic next-generation sequencing; PJP, Pneumocystis jirovecii pneumonia.

and culture for PJP patients. The results of mNGS and culture methods were both positive in 15 of 76 cases (18.74%) and both negative in 7 cases (9.21%). The 52 cases (68.4%) were only positive in mNGS, and two patients' result was only positive by culture. To examine the results' consistency of mNGS and culture, we further compared the detected pathogens between mNGS and culture for fifteen patients whose mNGS result and culture result were both positive. The detected pathogens of mNGS were identical to the results of the culture method in two patients and partly matched with culture results in eight patients. For five patients, the pathogens were mismatched between mNGS and culture methods (Figure 3A–D).

Impact on Antibiotic Treatment

Seventy-four PJP patients received antibiotic therapy before the mNGS results were available, of which 42 patients were treated with TMP/SMZ. Besides, twenty-three individuals received a combination of antibacterial, antiviral, and anti-fungal drugs (Table 2). According to the mNGS results, a total of 31 out of 76 PJP patients were added to TMP/SMZ. The antibiotic regimen remained unchanged after combining the mNGS results in 21 (27.63%) cases. Based on the mNGS results, the initial empiric antimicrobial therapy were de-escalated in seventeen (22.37%) patients. Conversely, the antimicrobial agents were escalated in 38 (50.00%) individuals, including 26 cases in which the type of antibacterial drug was increased and 12 cases in which the antibacterial spectrum was expanded (Table 3).

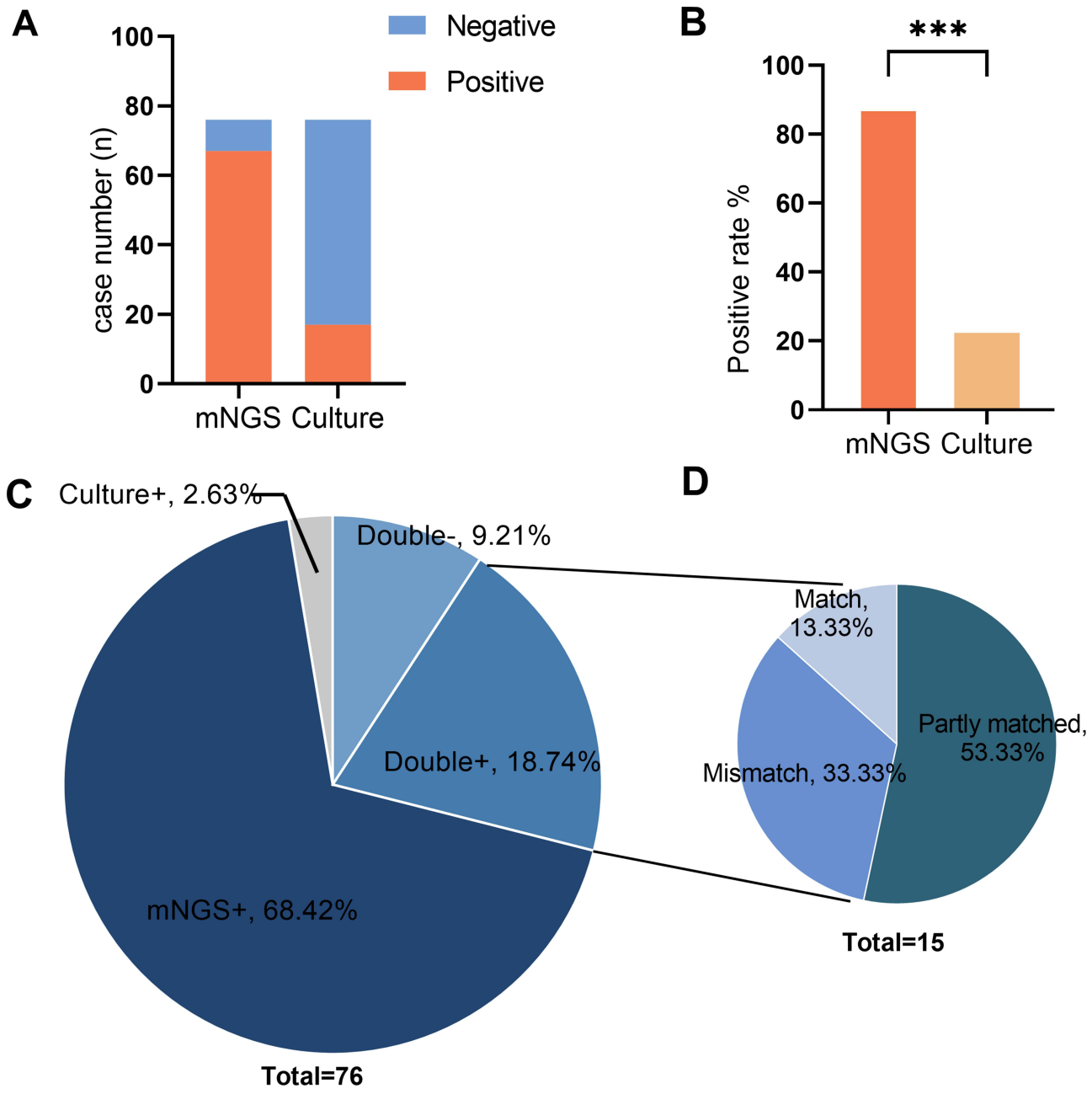

Figure 3 Comparison of mNGS with culture. (**A**)The detection performance of mNGS and culture in PJP patients; (**B**) Comparison of positive detection rate of co-infection for mNGS and culture in PJP patients; (**C**) Consistency analysis between mNGS and culture; (**D**) Consistency of detection in PJP patients who were positive for both mNGS and culture. ***$P<0.001$.

Abbreviations: mNGS, metagenomic next-generation sequencing; PJP, Pneumocystis jirovecii pneumonia.

After adjusting treatment based on the mNGS results, nearly all PJP patients revealed a significant improvement in some laboratory parameters (Table 4). The analysis results showed that the relevant inflammatory indicators such as ESR, CRP and PCT got improved in the PJP patients, among which CRP was statistically significant (Table 4, Figure 4).

Discussion

P. jirovecii is a fungal pathogen causing opportunistic lung infections, especially in individuals with weak immunity. The diagnosis of PJP remains challenging due to its clinical presentations lacking specificity, as well as the limited sensitivity and specificity of existing conventional diagnostic techniques.[22] Therefore, there is a need for the development of novel

Table 2 Application of Antibiotics Before mNGS

Application of Antibiotics Before mNGS	Case Number
TMP/SMZ	42(55.26%)
Antibacterial therapy	20(26.32%)
Antifungal therapy	2(2.63%)
Antiviral therapy	1(1.32%)
Antibacterial-antifungal therapy	11(14.47%)
Antibacterial-antiviral therapy	15(19.74%)
Antifungal-antiviral therapy	2(2.63%)
Antibacterial-antifungal-antiviral therapy	23(30.26%)

Table 3 Impact of mNGS on Antibiotic Therapy

Modifications	Case Number
No change	21(27.63%)
Add TMP/SMZ	31(40.79%)
Escalation	38(50.00%)
Increase spectrum of agents	12(15.79%)
Increase number of agents	26(34.21%)
De-escalation	17(22.37%)
Reduce number of agents	9(11.84%)
Reduce spectrum of agents	8(10.53%)

Abbreviation: TMP/SMZ, Trimethoprim-sulfamethoxazole.

Table 4 Comparison of Indicators Before and After Adjusting Treatment

Laboratory Findings	Before	After	*P*-value
WBC count[a]	5.71(4.24–8.14)	6.33(3.85–8.59)	0.42
PLT[a]	173±75.14	188.46±101.86	0.518
Neutrophil count [a]	4.61(3.41–7.11)	4.15(2.67–6.95)	0.733
Lymphocyte count [a]	0.62(0.36–1.00)	0.80(0.45–1.18)	0.046
ESR (mm/h)[b]	46.76±37.07	39.43±26.19	0.331
CRP (mg/l)[c]	55.4(19.6−126.75)	9.03(3.3–26.65)	<0.001
PCT (ng/mL)[d]	0.23(0.13–0.82)	0.13(0.13–0.34)	0.09

Notes: [a]52 PJP patients completed blood routine examination. [b]21 PJP patients completed ESR examination. [c]49 PJP patients completed CRP examination. [d]36 PJP patients completed PCT examination.

Abbreviations: WBC, White blood cell; PLT, Platelet; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein; PCT, Procalcitonin.

detection methods to assist in differentiating between *P. jirovecii* colonization and infection. mNGS has been used for detecting infectious agents, particularly in cases involving multiple pathogens and the detection of microorganisms that cannot be identified using conventional testing. Advanced mNGS technology for pathogen detection by detecting and characterizing microorganism DNA and RNA from specimens has become increasingly available as a method to identify pathogens, having a revolutionary impact on microbiological diagnosis.[14] We conducted a retrospective study to assess the efficacy of mNGS in differentiating *P. jirovecii* infection from colonization in a cohort of 108 patients who tested positive for *P. jirovecii*. Our study indicated that mNGS demonstrated a high level of effectiveness in differentiating between *P. jirovecii* infection and colonization. Specifically, the AUC for mNGS was found to be 0.9641, whereas the

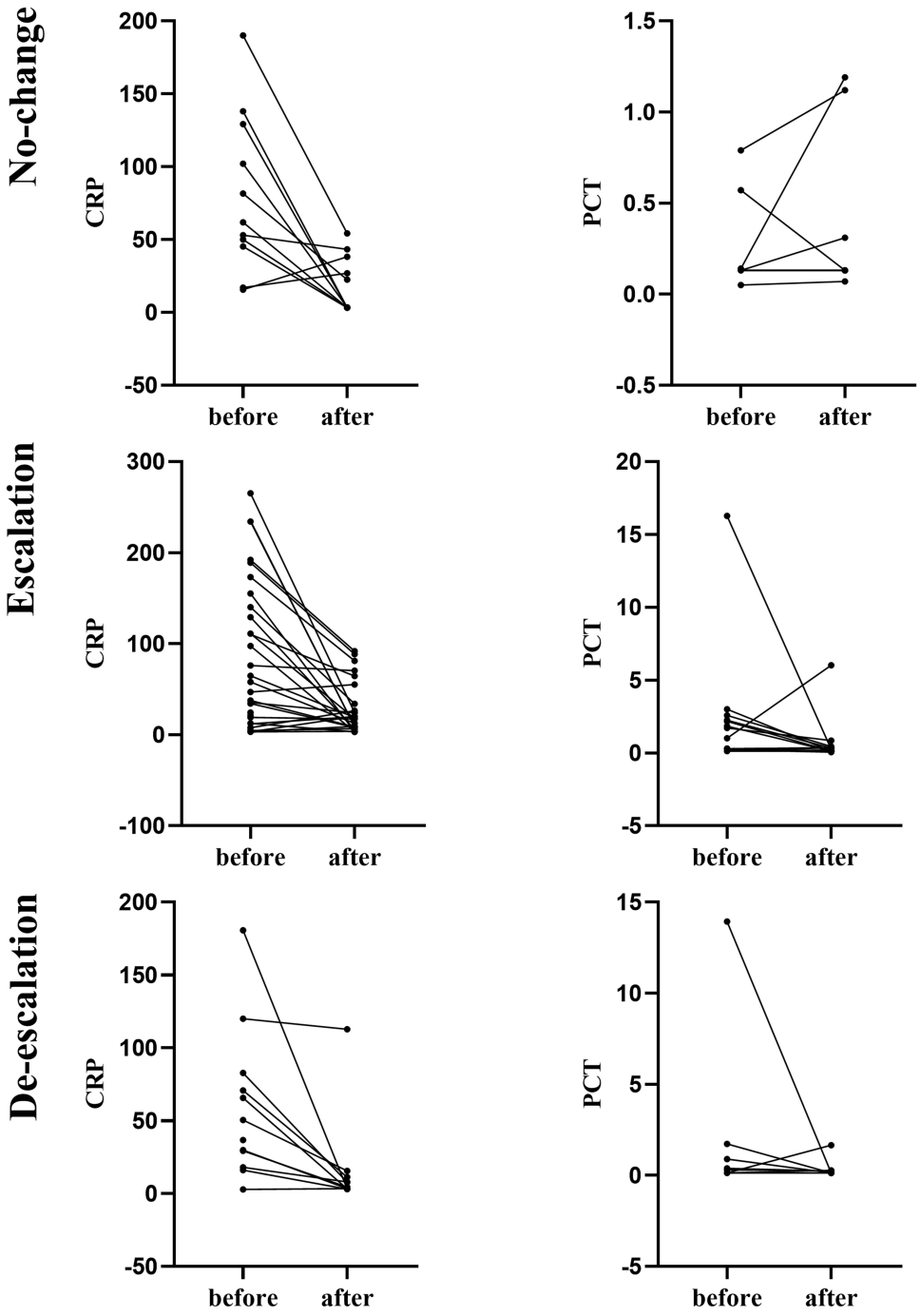

Figure 4 Comparison of indicators before and after adjusting treatment based on the mNGS results.
Abbreviations: mNGS, metagenomic next-generation sequencing; CRP, C-reactive protein; PCT, Procalcitonin.

AUC for the serum BDG assay was only 0.85. The optimal threshold BDG values for discriminating between PJP and PJC appeared to be 47.6 pg/mL, with a sensitivity of 68.75% and specificity of 90.00%. Our Study revealed the excellent ability of mNGS to discriminate PJP from colonization with a suggested cut-off of six reads. Consistent with the previous studies,[23,24] mNGS was more effective than conventional tests. A retrospective study showed that the diagnostic sensitivity of mNGS for PJP reached 100%,[12] which was significantly higher than that of GMS staining (25.0%) and BDG (67.4%). The specificity of mNGS (96.3%) was significantly higher than that of BDG (81.4%). A recent study has revealed that the diagnostic performance reaches its optimum level when the sequence number for mNGS was 14.[16]

However, the optimal cut-off value of mNGS in our study was six reads. This finding may be attributed to variations in detection methodologies, which may be related to different detection techniques. 1, 3-β-D glucan is a significant component of the cellular wall of fungi, and an increased concentration of serum BDG may suggest the presence of a potential fungal infection.[16] More and more research found that elevated serum BDG can assist diagnose PJP.[16,24] In addition, one study found that when serum BDG < the positive value, the negative prediction rate can reach 90%,[25] so effectively ruling out the possibility of PJP infection. In contrast to this study, we believed that serum BDG negative could not necessarily rule out infection. Our study found that when serum BDG exceeded 100. 5 pg/mL, the negative predictive value was only 47.5%. The performance of Polymerase Chain Reaction (PCR) and GMS were not conducted at our hospital due to the constraints imposed by experimental technology. Similarly, PJP can cause serum BDG elevation, but the elevation of serum BDG can also occur in patients with other fungal infections.[16] In this study, we found that patients with elevated serum BDG who received long-term corticosteroid therapy or immunosuppressive therapy should be aggressively treated with mNGS. In addition, patients with immunosuppressed status or suspected PJP are also recommended to be actively tested for NGS. In summary, mNGS was an excellent tool in distinguishing between PJP infection and colonization.

Another notable benefit of mNGS is that it can identify a wide range of pathogens, over the capabilities of existing microbiological testing methods. This feature allows for a comprehensive diagnostic approach, since a single mNGS test can effectively detect many pathogens in patients with co-infections. PJP patients frequently experience co-infections with various pathogens as a result of their compromised immune. mNGS is widely known for its capability to detect pathogens by direct sequencing of the extracted DNA from specimens, having a revolutionary impact on microbiological diagnosis.[26] Our study revealed that a significant proportion of PJP patients (88.16%) exhibited coinfection with numerous pathogens. And the positive detection rate was significantly higher than that of traditional culture. The most common pathogens co-infected with *P. jirovecii* by mNGS were *Cytomegalovirus* and *Epstein-Barr virus*. In accordance with prior literature reviews,[23,27] we should also be wary of co-infections such as viruses when diagnosing PJP. All PJP patients in our study underwent sputum, BALF, or blood cultures. One study concluded that conventional tests and mNGS exhibited comparable efficacy in the diagnosis of fungal infections.[12] The observed difference might possibly be attributed to the limited culture samples used in our study, which just included blood, sputum, and BALF. In contrast, the conventional detection techniques performed in other studies were more comprehensive, involving PCR and assay methodologies. Furthermore, the focus of our study was on individuals who have been diagnosed with PJP, a group of individuals thought to be susceptible to viral co-infections. Thus, the positive detection rate of mNGS in our study was significantly higher than that of culture compared with other study. The transfer time of mNGS from receiving specimens to reports is approximately 24 hours, and the shortest can be within 6 hours,[28] which is significantly lower than the time required for conventional microbial culture. Consequently, patients with severe infectious diseases may increasingly depend on mNGS. And it is still difficult to interpret the report of mNGS. For example, the identification of low-reads pathogens poses a significant challenge for mNGS in distinguishing between infection and colonization.

The guidance of mNGS for the treatment of PJP patients mainly depends on whether to add TMP/SMZ or to adjust the antibiotic therapy of the co-pathogens. In our study, 42 patients received TMP/SMZ in PJP group before the mNGS results were available, and 31 patients added TMP/SMZ according to mNGS results. More importantly, the other three patients were not treated with TMP/SMZ due to sulfonamides allergy and other factors, but were treated with caspofungin. Many laboratory parameters improved after the adjustment of therapy, especially CRP. This suggested that mNGS was effective in identifying co-infecting agents and guiding the treatment of pneumonia in complex infections, consistent with previous studies.[13] The results of mNGS provide opportunities for targeted therapies, thereby enhancing the problems of antibiotic overuse and drug resistance, and improving the prognosis of patients.[19]

We also had some limitations. Firstly, since this was a retrospective study from a single center, there was an unavoidable bias in sample selection. We need more perspective study to explore the efficacy of NGS for the diagnosis of PJP. Secondly, only limited RNA virus tests were performed. Therefore, RNA virus infection may not yet be identified. Finally, GMS and PCR techniques were not carried out in this research center, and the diagnosis of PJP were made by two professors. Thus, there needs to be uniform criteria due to subjective bias.

Conclusion

mNGS is a promising and valuable technique with good performance for diagnosing PJP, the detection of co-pathogens, and antibiotic treatment.

Ethical Approval Statement

The study was conducted according to the good clinical practice guidelines and the Declaration of Helsinki. This study was approved by the research ethics committee of Wuhan Union Hospital (2023-0177), and the need for obtaining written informed consent was waived due to the retrospective nature of this study and the fact that the data did not contain personal information about the patients. Patient data was used only for this study and was kept strictly confidential.

Acknowledgment

We express appreciation to the study participants for their valuable efforts, as well as to the Microbiology Laboratory of Wuhan Union Hospital for their generous support.

Author Contributions

Meng-Qi Huang and Ting-Ting Zheng contributed equally to this work and share first authorship. 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.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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