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Research Article

Metagenomics Next-Generation Sequencing Provides a Reliable Method for the Early Diagnosis of Pneumocystis Jirovecii Pneumonia after Kidney Transplantation: A Single-Center Retrospective Cohort Study

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Abstract

Objectives: Pneumocystis jirovecii pneumonia (PJP) is one of the most common pulmonary infections after kidney transplantation. The pneumocystis jirovecii (PJ) cannot be detected by conventional culture, and there are limitations in terms of lung tissue biopsy, sputum collection and sample smear staining. If PJP patients cannot be treated in the early stage in a timely and effective manner, the long-term survival rate will debase, so early diagnosis of PJP is key to successful treatment. **Material and Methods**: From January 2018 to January 2023, 110 pulmonary infection patients were enrolled in this study at the First Affiliated Hospital of Xi'an Jiaotong University. 46 patients were confirmed PJP through mNGS (metagenomic next-generation sequencing) and conventional detection method. The percentage of PJ-positive, consistency, detection efficiency and other pathogen species were compared between the two test methods. Besides, the clinical characteristics of the PJP group (n=46) and non-PJP group (n=64) were analysed retrospectively. **Results**: 46 of the 110 patients with pulmonary infection were diagnosed with PJP, and the average time of onset after surgery was 7.21 ± 2.55 months, the incidence of PJP was 2.3% (46/1977). 42 patients were eventually cured, and 4 patients died. 33 patients had mixed pulmonary infections, PJ and human cytomegalovirus (CMV) were the most common pathogen combinations, and 13 patients had monotypic pulmonary infections. In contrast, 16 patients were PJ-positive according to conventional pathogen detection, for a detection rate of only 34.78% (16/46), and the difference between the two detection methods was statistically significant ($\chi 2 = 92.0$, $P < 0.01$). In addition, our study revealed that patients who were treated with tacrolimus, had insufficient use of sulfamethoxazole-trimethoprim (SMZ-TMP), had a history of CMV infection and had acute rejection (AR) were more likely to develop PJP ($P < 0.05$). **Conclusions**: Compared with conventional detection method, mNGS has more advantages in the early diagnosis of PJP. Precision medicine can be adopted to reduce the cost and improve the cure rate based on mNGS results. More importantly, we should focus on kidney transplant recipients with high risk factors for developing PJP.

Keywords: Kidney transplantation; Pneumocystis jirovecii pneumonia (PJP); Pathogen; mNGS; Conventional detection method

Introduction

Kidney transplant recipients are immunocompromised due to long-term immunosuppression and are prone to infectious diseases, especially pulmonary infections, which are among the most common causes of death in kidney recipients [1-3]. Pneumocystis jirovecii pneumonia (PJP) is common in organ transplant recipients and AIDS patients [4]. Three to six months after kidney transplantation, it has a high incidence and has characteristics such as insidious onset, various manifestations, rapid progression and difficult identification quickly and accurately [5]. In the past, there was a lack of specific and efficient diagnosis, delayed treatment, and mortality as high as 90%-100%, which seriously endangered patients' lives [6]. Therefore, early diagnosis of PJP is key for successful treatment.

mNGS has been applied in medical research and clinical diagnosis, such as for the diagnosis of infection types, determination of resistance genes and prevention and control of infectious diseases [7]. This method can rapidly, efficiently, and accurately obtain entire pathogen genomic information by high-throughput sequencing of DNA or RNA in patient samples [8,9]. In our study, the clinical data of 46 kidney transplant recipients with PJP were retrospectively analyzed to investigate the clinical value of mNGS for the early diagnosis of PJP and to provide a reference for diagnosis and treatment.

Material and Methods

Ethics Statement

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (No. XJTU1AF2023LSK-2022-178), and informed consent was obtained from all subjects and/or their legal guardian(s) before enrollment. No executed prisoners were used for any part of this study.

Study Population

This was a single-center hospital-based retrospective cohort study conducted at the First Affiliated Hospital of Xi'an Jiaotong University from January 2018 to January 2023. During this period, we completed a total of 1977 kidney transplant procedures, which involved 1201 male patients and 776 female patients with an average age of 48.2±13.9 years (6-66 years), including 1741 cases of deceased donation (DD) kidney transplantation and 236 cases of living kidney transplantation, the relationship between living donors and recipients were all lineal relative including parents and children (n=211), brothers (n=10), sisters (n=8), and marriage bonds (n=7). A total of 110 patients were diagnosed with pulmonary infection, for an incidence rate of 5.56% (110/1977). The data of demographics, onset time, transplant type, induction and maintenance of immunosuppressive agents, clinical signs and symptoms, CT presentation, laboratory test results at admission and after treatment, etiology, therapy, complications, hospitalization days, concomitant illness, and the treatment outcomes were recorded.

Diagnostic for PJP

The diagnostic criteria for PJP [10-12]: **(1)** Patients had a history of immunosuppression; **(2)** HIV-negative patients; **(3)** PJ was detected in sputum, lung tissue, or BALF (bronchoalveolar lavage fluid); **(4)** Fever, dry cough, and progressive dyspnea were the most common clinical symptoms of PJP, and some patients may not have fever; **(5)** Patients had typical chest CT manifestations, (Figure 1). Exclusion criteria: **(1)** Unable to cooperate to complete the mNGS examination; **(2)** Incomplete clinical data; **(3)** Follow-up was not completed on time; **(4)** PJ colonization.

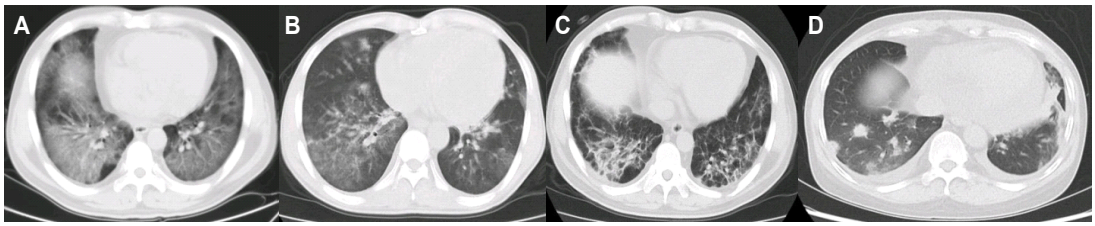

Figure 1. Four common chest CT manifestations of PJP in kidney transplant recipients, A. Ground glass type; B. Patchy-diffuse type; C. Interstitial type; D. Cystic degeneration type.

Detection Methods

In our study, mNGS and conventional pathogen detection were performed immediately after admission (Figure 2). BALF, as the source of specimen with high evidence level and the most practical clinical practice, is widely recommended by major international guidelines, so we chose it for mNGS. Patients fasted 3-4 h before surgery, 2% lidocaine local mucosal anesthesia of nasal cavity and airway, fiberoptic bronchoscopy was inserted into the trachea, embedded in the corresponding lesion position, injected with 37°C normal saline, and recovered to the lavage bottle by negative pressure suction device. Generally, the recovery volume should reach 40%-60% of the injected fluid, the fluid should be immediately sent to the laboratory, and the lavage fluid should be checked and analyzed within 2 hours. All BALF samples were used directly for mNGS to extract nucleic acids, construct a library, sequence and compare the results, and determine the pathogenic microorganisms in the sample according to the sequence information. Low-quality reads and reads less than 14 bp in length were removed (Figure 3A, 3B).

The conventional detection methods used were as follows: **(1)** Blood culture, sputum culture, smear, antigen detection, and lung tissue pathology (Figure 3C); **(2)** BALF staining (Periodic acid silver methenamine staining, Giemsa staining); **(3)** PCR (polymerase chain reaction); **(4)** Serological examination can also be used for auxiliary diagnosis, including 1,3-β- D-glucan (BDG), Galactomannan (GM Test), lactate dehydrogenase (LDH), procalcitonin (PCT) and interleukin 6 (IL-6) et al.

In this study, based on the results of several previous studies [13-15], clinical manifestations and imaging evidence, we concluded that the combined test of BDG (threshold: 88.6 pg/ml) and BALF mNGS (threshold:14 Reads) could better distinguish PJ infection and colonization.

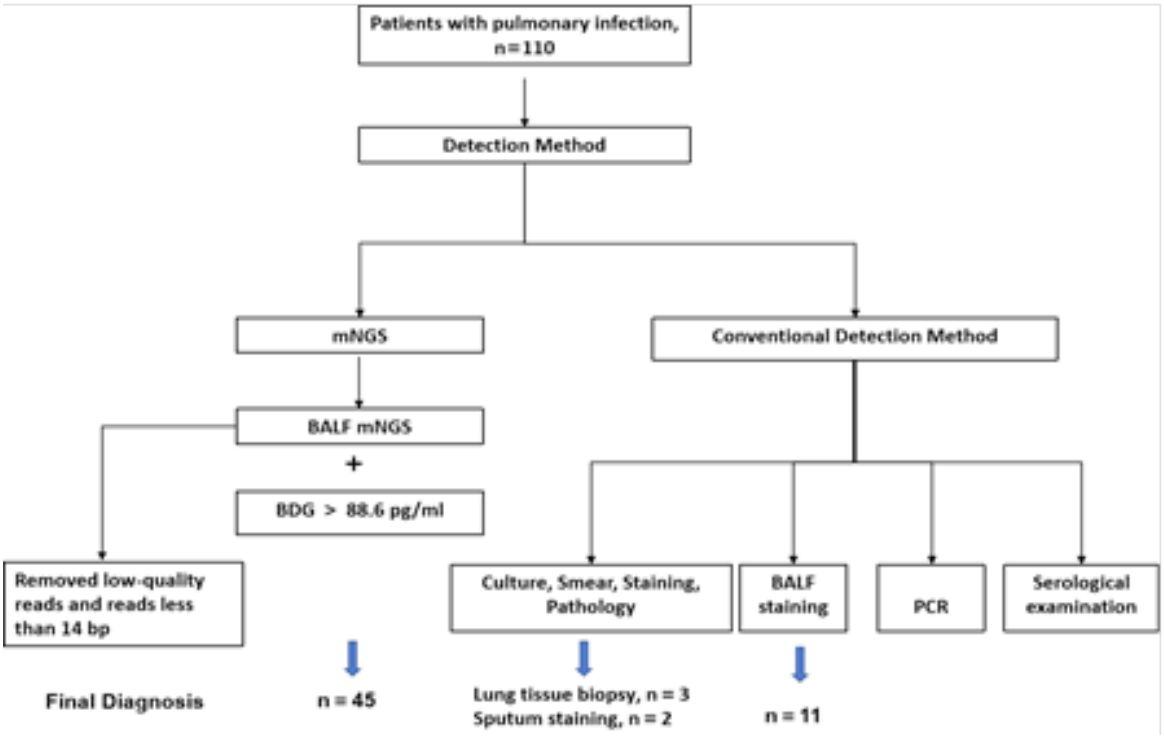

Figure 2. Processing procedure of mNGS and conventional detection method.

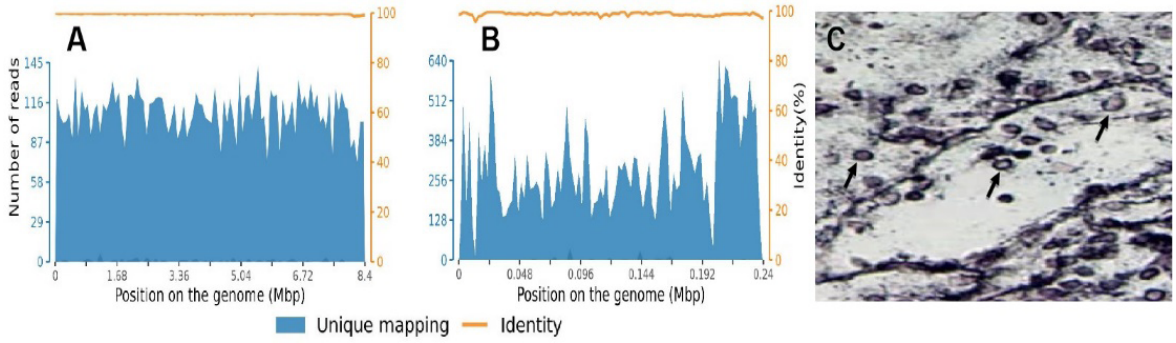

Figure 3: A. The total length of the genome covered by PJ was 518473 bp, with a coverage of 6.18% and an average depth of 1.06X.

B. The total length of CMV coverage onto the genome was 202287 bp, with 85.84% coverage and an average depth of 10.86X.

C. Microscopic detection of PJ trophozoites after staining of sputum smears, HE×100.

Statistical Methods

SPSS 26.0 software was used for statistical processing. The quantitative data are expressed as the mean and standard deviation ( ). Independent sample t tests were applied to test whether the data conformed to a normal distribution, or the rank sum test was applied to test whether the data did not conform to a normal distribution. Enumeration data are expressed as case numbers, and the chi-square test ($\chi 2$) or Fisher's exact probability test was performed. All the statistical analyses in this paper were performed bilaterally, and the difference between the two tests was considered to be statistically significant at $P < 0.05$.

Results

Comparison of Clinical Data between the PJP and Non-PJP Groups

We divided the patients into PJP and non-PJP groups based on whether the kidney transplant recipients were diagnosed with PJP. The differences in clinical data, such as sex, age, onset time, transplant type, perioperative induction protocol, and outcome, were not statistically significant ($P > 0.05$). Overall, our study revealed that patients who were treated with tacrolimus, had insufficient use of SMZ-TMP (dosage or usage time), had a history of CMV infection, or had AR were more likely to develop PJP ($P < 0.05$). (Table 1).

Characteristic	PJP group（n=46）	Non-PJP group（n=64）	T or χ^2	P value
Sex (M/F)	30/16	44/20	0.15	0.7
Age (y)	50.16±11.21	48.67±12.69	0.85	0.47
Onset time (month)	7.21±2.55	6.92±2.77	0.78	0.38
Transplant type			0.14	0.7
DD	40	54		
Living donor	6	10		
Immunosuppressive protocol			0.02	0.9
Tacrolimus	34	48		
Ciclosporin	12	16		
SMZ-TMP - Use qualified rate	43.48%（20/46）	76.56%（49/64）	12.53	0.001
Complications after kidney transplantation*				

Urinary system infection	13.04%（6/46）	12.5%（8/64）	0.007	0.93
CMV infection	34.78%（16/46）	7.81%（5/64）	12.6	0.001
BKV infection	26.09%（12/46）	23.43%（15/64）	0.101	0.75
AR	19.56%（9/46）	6.25%（4/64）	0.04	0.034
delayed graft function (DGF)	17.39% (8/46)	17.19% (11/64)	0.001	0.98
Outcome (recovery rate)[#]	8.70% (4/46)	7.81% (5/64)	—	1

*Complications after kidney transplantation refer to complications occurring from the perioperative period to the time of pulmonary infection diagnosis, [#]Fisher's exact probability test.

Table1: Demographic and clinical data from patients in the PJP group and non-PJP group.

Analysis of the Results of mNGS and Conventional Detection Method

There was 47 positive patients according to mNGS detection of BALF samples, 46 patients had confirmed PJP, namely BDG > 88.6 pg/ml and BALF mNGS > 14 reads, 1 patient BDG was 50 pg/ml and mNGS was 8 reads, so this patient was diagnosed with PJ colonization. The bacterial pathogen types identified by mNGS were more dispersed than those identified by other methods, and there was no obvious aggregation. The common pathogens were Klebsiella pneumoniae, Enterococcus faecium, Pseudomonas aeruginosa, Bacillus coli, E. coli, Mycobacterium abscessus, Staphylococcus epidermidis, Streptococcus pneumoniae, creber acid production, Blastomyces albicans, or Candida sp. Unlike the distribution of bacterial pathogens, the viruses were relatively more concentrated, with 16 cases of combined CMV infection.

There were 16 cases of positive PJ results according to conventional detection methods, including 11 cases of BALF staining, 2 cases of sputum staining, and 3 cases of lung tissue biopsy, 30 cases were negative, with a sensitivity of 34.78%, PJP cannot be completely ruled out even with a negative respiratory sample. Moreover, 16 patients had mNGS values greater than 14 reads. The difference in sensitivity between the two detection methods was statistically significant ($\chi 2 = 92.0$, $P < 0.05$) (Figure 4).

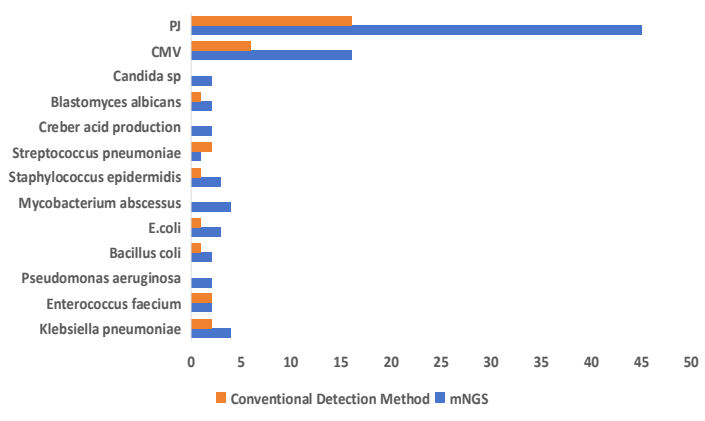

Figure 4: Analysis of the results of mNGS and conventional detection method. (Some rare organisms or pathogens of little clinical significance are not listed.).

Comparison of the Results between mNGS and Conventional Detection Method

Patients often have pulmonary infections combined with one or more pathogens after kidney transplantation, and once two or more pathogens are detected, the pulmonary infection is considered mixed. A total of 33 patients with mixed infections were detected by mNGS, PJ and CMV mixed infections were the most common (16/46, 34.78%), and 13 patients had monotypic infections, which was also in accordance with the distribution of mNGS pathogens. 9 patients with mixed infections and 7 patients with monotypic infections

were detected by conventional methods. Compared with conventional methods, mNGS has more advantages in terms of pathogen type, detection time, sensitivity, specificity, mixed infection diagnosis and benefit (medicine, costs, side effects, efficacy), especially for the diagnosis of PJ and CMV infection (Table 2).

Diagnostic method		Diagnostic results	Sensitivity	Specificity
mNGS (reads)	＞14	46	1	0.9787
	＜14	1		
Conventional detection method	+	16	0.3478	0.5313
	-	30		
P			＜0.01	＜0.01

Table 2: Comparison of the results between mNGS and conventional detection method.

Patient Outcomes

In the PJP group, 42 patients were cured, 4 patients died, for a cure rate of 91.3% and a length of stay of 16.72 ± 5.80 days. Moreover, no secondary infections, relatively serious complications and graft function was stable during the follow-up period from 1 to 5 years. It is impressive that mortality was not statistically significant in PJP patients compared with other types of pneumonia, which further demonstrates the value of mNGS in the early diagnosis of PJP.

Discussion

Kidney transplantation is currently recognized as the most effective treatment for end-stage kidney disease. However, PJP after kidney transplantation is one of the most serious complications that seriously influences the long-term survival of patients [16]. PJ is an important opportunistic fungal infection in an immunocompromised population that may develop through airborne transmission or reactivation of previous infections and has significant morbidity and mortality in solid-organ transplantation (SOT) [17, 18]. Before universal prophylaxis, the incidence of PJP within 6 months after transplantation was nearly 24%; however, with the implementation of universal and standardized prophylax programs in most transplant centers, the incidence of PJP decreased significantly after transplantation [19]. It is frustrating that PJ could not be obtained by conventional culture, and there are errors caused by sampling, staining, pathology and image reading; thus, the early diagnosis rate of PJP was low in the past [20]. Most patients with PJP have cough with little sputum, it is difficult to obtain sufficient and satisfactory samples by direct cough or sputum induction by inhalation, and the sensitivity of sputum induction is only 30%-55%, which cannot be applied as a routine treatment [21]. In addition, lung tissue biopsy or lung puncture have limitations because of the invasive nature of the operation [22]. Moreover, there is a lack of definitive treatment without reliable pathogenic evidence and only passive empirical treatment, which has the shortcomings of a broad spectrum and large dose and leads to high cost, side effects, dysbiosis and poor efficacy. Therefore, a simple, rapid, highly sensitive and specific, comprehensive detection method is crucial.

mNGS does not require any assumptions and is less affected by antibiotic exposure; in contrast to traditional microbial culture methods, mNGS does not require purified culture or extraction of nucleic acids directly from clinical samples, sequences or analyses [23]. Pathogenic microorganisms can be detected in clinical samples rapidly and objectively via sequence alignment. mNGS has the advantages of high sensitivity, specificity and rapid detection, especially for patients with critical conditions or undetermined infections, and the percentage of positive mNGS results is more than 3 times greater than that of traditional methods for the diagnosis of viruses and bacteria in organ transplant recipients [24]. Some studies have shown that in the diagnosis of PJP, mNGS sensitivity is 100% compared with staining (25.0%) and BDG (67.4%) [25]. Besides, mNGS also has shortcomings, such as high hardware requirements, high cost, and difficulties in filtering bacteria, especially for samples with a large number of pathogens, which require an assessment by combining test results with epidemiological and clinical manifestations.

Although mNGS provides a more efficient diagnostic tool, it is worrying that mNGS cannot fully distinguish between infection and colonization. In order to avoid clinical overtreatment and delay treatment, it is important to identify colonization and infection [26]. When mNGS reads is low, the diagnosis of PJP is somewhat questioned, and determining the threshold of mNGS can provide clinicians with more accurate information. At present, many studies have shown that BDG can assist in the diagnosis of PJP, and the value can distinguish PJ colonization and infection. The

higher the value, the greater the possibility of infection, but the cut-off value of the two is not conclusive [13]. The combination of BDG and BALF mNGS can effectively distinguish between PJ infection and colonization, and help to guide clinical diagnosis and treatment [15,27]. In this study, one patient had a BDG = 50 pg/ml and BALF mNGS = 6 Reads, which was identified as a PJ colonization.

In our study, PJP accounted for 41.81% (46/110) of pulmonary infections after kidney transplantation and was one of the most common pulmonary infections after kidney transplantation. Therefore, the efficacy of treatment for PJP is associated with the overall outcome of pulmonary infections after kidney transplantation. In recent years, with the prolonged survival time of transplanted kidneys, the development of immunosuppressive drugs and the innovation of diagnostic techniques, the incidence of PJP has tended to increase. The incidence of PJP in this study was 2.33% (46/1977), which is consistent with the incidence of PJP after kidney transplantation ranging from 0.3% to 2.6% in the literature [28]. Only 16 patients were confirmed to be PJ-positive by conventional pathogen detection, which is significantly different from the mNGS results and is one of the crucial reasons for the high mortality rate of PJP in the past. Although the mNGS result was negative for one patient, the patient could be diagnosed via lung tissue biopsy, clinical symptoms, abnormal chest CT images, and evidence of the efficacy of empirical anti-PJP treatment, which may be associated with treating for more than 1 week, missing the optimal sampling time and resulting in a low viral load; moreover, complete pathogen detection within 3 days of treatment is suggested to reduce the influence of antibiotics.

According to the mNGS results, PJP often results in mixed infection, and treatment of other pathogens is critical in addition to treatment for PJ. mNGS detected a total of 33 patients with mixed infections; among these patients, mixed PJ and CMV infections were the most common (18/46, 39.13%), and 13 patients had monotypic infections, which is also consistent with the distribution of mNGS pathogens. Duan identified mixed infection as an independent risk factor for poor prognosis in patients with PJP [29], and PJ with CMV infection aggravated the patient's condition [30, 31]. Therefore, once PJP infection occurs together with other pathogens, especially CMV infection, enough attention needs to be given. Among the four patients who died in the PJP group, 3 had mixed infections of PJP with CMV.

The clinical data, such as sex, age, onset time, transplant type, perioperative induction protocol, and outcome, were not significantly different between the PJP group and the non-PJP group ($P > 0.05$). In addition, our study showed that patients with insufficient use of SMZ-TMP were more likely to develop PJP ($P < 0.05$). SMZ-TMP is the drug of choice for treating PJP, and no drug has been shown to have better results than SMZ-TMP [32]. Notably, SMZ-TMP can cause adverse reactions, such as hepatic and kidney function impairment, allergic rash, and myelosuppression [33]. Due to these side effects and poor patient compliance, the SMZ-TMP dosage was insufficient for more patients in the PJP group. There is no clear consensus on the recommended duration of PJP prophylaxis, especially for patients at high risk of PJP [34]. We suggest that it is necessary to extend the treatment duration to prevent PJP in patients with risk factors. Furthermore, further studies are needed to determine the optimal duration of PJP prophylaxis. More patients in the PJP group had a history of acute rejection because patients with acute rejection required higher doses of ATG therapy, had lower immunity and were more prone to PJP ($P < 0.05$). PJP is similar to CMV pneumonia, both are interstitial pneumonia and are closely related to human immune mechanisms [35]. Several studies have confirmed that the two are closely related, that CMV is an independent risk factor for PJP occurrence and that CMV infection can accelerate the occurrence of PJP [36, 37]. The proportion of CMV infection was significantly greater in the PJP group than in the non-PJP group ($P < 0.05$).

This retrospective study aimed to evaluate whether mNGS has more advantages than conventional pathogen detection for the early diagnosis of PJP.

Precision medicine can be adopted to reduce the cost and improve the cure rate based on mNGS results. Although mNGS has more advantages than does conventional pathogen detection, it cannot replace this method completely, and combined use of these methods can improve pathogen diagnosis. The pathogenic characteristics of PJP after kidney transplantation were further investigated in our study, which provided instructions for the prevention and treatment of infection. Our data suggest that our strategies for the diagnosis, prevention, and treatment of PJP after kidney transplantation are effective and that the therapeutic effect of PJP is comparable to that of non-PJP. For key populations, targeted prevention should be given to reduce the incidence of PJP and improve the effect of transplantation.

However, the study has many limitations, Infection and colonization were still controversial, the number of patients was limited, and the mNGS detection of samples was limited to BALF, which may cause pathogen omissions and need to be further investigated in the clinic. Furthermore, the aim of our study was to confirm the importance of mNGS in the early diagnosis of PJP, but there has been little discussion on the use of anti-infective therapy, immunosuppressants, and adjuvant therapy.

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Conflict of Interest Statement

The authors declare no financial or commercial conflicts of interest.

Data Availability Statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

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