

Metagenomic Next-Generation Sequencing in Diagnosis of a Case of *Pneumocystis jirovecii* Pneumonia in a Kidney Transplant Recipient and Literature Review

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Background: Despite the increasing incidences of *Pneumocystis jirovecii* pneumonia (PCP) in renal transplant recipients, diagnosis of PCP remains challenging due to its nonspecific clinical presentation and the inadequate performance of conventional diagnostic methods. There is a need for novel diagnostic methods.

Case Presentation: A 27-year-old woman developed acute pneumonia 4 months after renal transplantation. Blood tests revealed a low CD4 count, a normal 1,3-beta-D-glucan level and other changes typical of inflammatory responses. Chest imaging showed bilateral diffuse infiltrates. Microscopic examination of stained sputum and bronchoalveolar lavage fluid (BALF) smear specimens did not find *Pneumocystis* organisms. There was also no evidence for other pathogens known to cause pneumonia in various antibody and culture tests. Direct metagenomic next-generation sequencing (mNGS) analysis of a BALF specimen identified a large number of *P. jirovecii* reads, allowing to confirm the diagnosis of PCP. Following treatment with trimethoprim-sulfamethoxazole for two weeks, the patient was cured and discharged.

Conclusion: This case report supports the value of mNGS in diagnosing PCP, highlights the inadequate sensitivity of conventional diagnostic methods for PCP, and calls for the need to add PCP prophylaxis to the current Diagnosis and Treatment Guideline of Invasive Fungal Infections in Solid Organ Transplant Recipients in China.

Keywords: *Pneumocystis jirovecii* pneumonia, renal transplant, diagnosis, metagenomic next-generation sequencing

Introduction

Pneumocystis pneumonia (PCP) is a major opportunistic infection in immunocompromised patients. With the introduction of antiretroviral therapy and prophylaxis of PCP, the morbidity and mortality of PCP in HIV-infected patients has decreased significantly, but the incidence of PCP in non-HIV infected patients is increasing gradually,¹⁻⁴ especially in renal transplant recipients.⁵ Following the first report of a cluster of 5 cases in Switzerland in 1991, at least 393 cases of PCP in renal transplant recipients have been reported in approximately 50 studies across the world (Table S1).

To date, the causative agent of PCP in humans, *Pneumocystis jirovecii*, still cannot be reliably grown in vitro, and thus clinical diagnosis of PCP depends largely on

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microscopic detection of *P. jirovecii* organisms in respiratory specimens following chemical or immunofluorescence staining.^{6,7} However, these methods suffer from low sensitivity and specificity. Detection of serum 1,3-beta-D-glucan (BDG) appears to be sensitive but lack specificity for *P. jirovecii*.⁸ Development of various polymerase chain reaction (PCR)-based diagnostic assays has significantly improved the sensitivity and specificity;^{9,10} several commercially available *P. jirovecii* PCR kits have been approved for clinical use in some countries, primarily in Europe, but not in other countries, such as the United States and China.

Recently, metagenomic next-generation sequencing (mNGS) has rapidly emerged as a promising single, universal (or hypothesis-free), culture-independent pathogen detection tool that can be performed directly on clinical specimens.^{11,12} mNGS determines sequences of all nucleic acids in a sample (regardless of their origins) and then assigns sequence reads to their reference genomes to determine which microbes are present and in what proportions.¹³ In contrast to the current molecular assays including PCR and microarray which target only one or a limited number of known pathogens using specific primers or probes, mNGS targets all DNA or RNA present in a sample, allowing to detect the entire microbiome (including atypical or novel microbes) as well as the human host genome or transcriptome in patient samples. This method has been successfully used as a diagnostic tool for various infectious diseases including PCP.^{14–17} Its application to diagnose PCP in renal transplant recipients has been attempted in one study,¹⁸ and its value awaits further evaluation. Here we report a case of PCP diagnosed by mNGS following negative results in multiple conventional diagnostic tests. In

addition, we provide a literature review of diagnostic methods for PCP in renal transplant recipients.

Case Report

A 27-year-old woman was admitted to our hospital (Nov 2, 2018), complaining of two weeks of cough, intermittent low fever and dyspnea during daily activity. Four months prior to the admission, she underwent a renal transplantation because of kidney failure caused by chronic glomerulonephritis. Following successful transplantation, she took immunosuppressive medication, including tacrolimus (2.5 mg twice a day), mycophenolate mofetil (750 mg twice a day) and methylprednisolone (12 mg daily) for four months. During this period, she did not receive influenza immunization or prophylaxis for opportunistic infections including *Pneumocystis* and *cytomegalovirus* (CMV) infections as per the Diagnosis and Treatment Guideline of Invasive Fungal Infections in Solid Organ Transplant Recipients in China (2016 edition).¹⁹ Two weeks prior to admission to our hospital, she presented to a local community hospital with complaints of fever, cough and dyspnea during daily activity. Chest X-ray showed bilateral patchy, cloudy opacities in the lower lungs with some adjacent pleural thickening and adhesion (Figure 1A). After empiric treatment with oral moxifloxacin for 7 days, there was no apparent improvement, with fever remaining high (38°C) and dyspnea aggravated.

Upon admission to our hospital, physical examination found a normal body temperature of 36.7°C, blood pressure of 143/93 mm Hg, pulse of 116 beats per minute, and respiration rate of 24 per minute. Moist rales appeared bilaterally, with no lower-extremity edema.

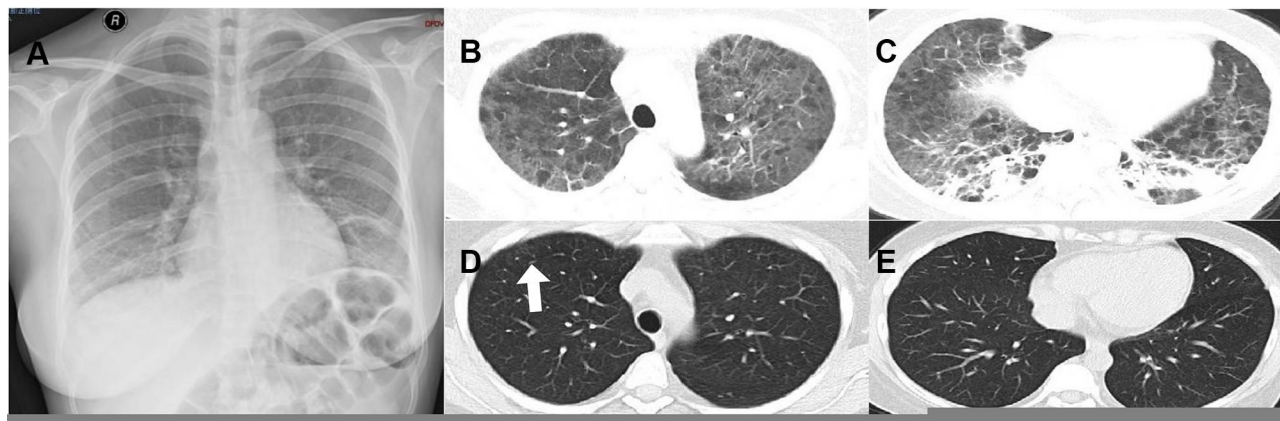


Figure 1 Chest images. (A) Chest X-ray showing bilateral patchy, cloudy opacities in the lower lungs with some adjacent pleural thickening and adhesion before treatment. (B and C) Chest HRCT showing diffused stripes as well as reticular and ground-glass opacities with honeycomb structures, primarily in the lower lungs before treatment. (D and E) Chest HRCT scan showing a faint patchy opacity in the lateral segment (white arrow).

Due to hypoxemia (PO₂: 63 mmHg, PCO₂: 29 mmHg, SO₂: 90%), she was given oxygen therapy with mask hydrogen inhalation shortly after admission. High-resolution computed tomography (HRCT) demonstrated diffused stripes and reticular and ground-glass opacity with honeycomb structures, primarily in the lower lungs (Figure 1B and C). Other laboratory tests revealed abnormal values for almost all parameters tested elevated levels of several biomarkers for inflammatory responses and decreased counts of lymphocyte and CD4 cells, as detailed in Table 1. Electrocardiogram was normal while echocardiogram showed left ventricular hypertrophy with decreased diastolic dysfunction. Serological assays showed positive results for IgM antibodies to *Parainfluenza virus*, but negative results for antibodies to respiratory syncytial virus, influenza virus (types A and B), cytomegalovirus (CMV), *Adenovirus*, *Legionella pneumophila*, *Mycoplasma pneumonia*, *Chlamydia pneumoniae*, *Q-rickettsia*, and *Cryptococcus neoformans*. Serum galactomannan and BDG levels were both normal (Table 1). Diff-Quick staining of sputum smear did not find *Pneumocystis* organisms. Blood culture was negative. *Mycobacterium tuberculosis* was negative based on blood test with T-SPOT.TB (Oxford

Immunotec Ltd. UK) as well as acid-fast staining of sputum smear.

Considering the patient was at the high-risk stage to develop opportunistic infections, she was firstly given intravenous antibacterial (meropenem 1g thrice a day) and antiviral (ganciclovir 125g twice a day) treatment on the first day of hospitalization. After 3 days of treatment, she still had fever (38°C) and dyspnea. The antimicrobial treatment was changed to intravenous caspofungin (50mg daily), oral trimethoprim-sulfamethoxazole (TMP-SMX, 320/1600mg quadruple a day), and intravenous methylprednisolone (40mg daily), which resulted in improvement of general conditions and abatement of fever and dyspnea in 3 days. However, chest HRCT showed worsening of the opacity in the lower lungs.

On the 13th day of hospitalization, she underwent endobronchial ultrasound transbronchial lung biopsy combined with bronchoalveolar lavage fluid (BALF) collection under laryngeal mask airway instead of a regular bronchoscopy examination due to respiratory failure. Subsequent microbiological tests of BALF were negative for *M. tuberculosis* and *P. jirovecii* by the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, US) and Diff-Quick staining, respectively. In addition, BALF culture revealed

Table 1 Main Laboratory Test Results Before and After Treatment

Laboratory Parameters	Normal Range	Pre-Treatment [*]	Post-Treatment ^{*a}
White blood cell count/L	3.50–9.50 × 10 ⁹	12.65 × 10 ⁹	6.55 × 10 ⁹
Hemoglobin, g/L	115.0–150.0	165.0	149.0
Platelet count/L	101–320 × 10 ⁹	248 × 10 ⁹	151 × 10 ⁹
Neutrophil %	40.0–75.0	86.7	54.7
Lymphocyte %	20.0–50.0	7.4	18.3
Alanine aminotransferase, U/L	7.0–40.0	14.0	96.0
Aspartate aminotransferase, U/L	13.0–35.0	9.0	30.0
NT-proBNP, ng/L	0–97	214	n/a
Serum albumin, g/L	40.0–55.0	43.0	67.0
Lactate dehydrogenase, U/L	120–250	301	361
Creatinine, umol/L	41.0–73.0	189	169
Urea nitrogen, mmol/L	2.60–7.50	13.3	17.8
Calcium, mmol/L	2.11–2.52	2.40	2.34
C-reactive protein, mg/L	<10	48.10	n/a
Procalcitonin, ng/mL	0.00–0.05	0.09	n/a
1,3-beta-D-glucan, pg/mL	<60	<10.0	n/a
Galactomannan	<0.5	<0.5	n/a
Total T lymphocyte count/μL	810–2860	739	n/a
CD4+ T cell count/μL	420–1440	256	n/a
CD8+ T cell count/μL	238–1250	456	n/a
CD4+/CD8+ ratio	0.80–2.74	0.56	n/a

Notes: ^{*}Abnormal values are indicated in bold face. ^aOn the 16th day of hospitalization.

Abbreviations: n/a, not available; NT-proBNP, N-terminal pro b-type natriuretic peptide.

no growth of bacteria or fungi; galactomannan level was in the normal range (OD index <0.5).

The consistently negative results in all etiological tests prompted us to submit 3 mL of BALF for mNGS by the Beijing Genomics Institute (Wuhan, Hubei Province, China) using the MGISEq 2000 platform (MGI Tec, Shenzhen, China). The results of mNGS were received <72h after shipping out the BALF sample. Based on the mNGS report (Table S2), there were 10 bacterial species (5 to 1406 reads per species), 1 fungal species (1,665,693 reads) and 1 viral species (12 reads). None of these species are well-established pathogenic organisms causing respiratory infections in humans except for the fungus *P. jirovecii*, which is the most dominant species, accounting for 99% of all microbial reads (6.8% of the total filtered reads including human DNA reads). The reads for *P. jirovecii* allowed assembly of the complete mitogenome of *P. jirovecii* (35,285 bp, GenBank accession number MT319762), which is 98–99% identical to 13 known *P. jirovecii* mitogenomes.^{20,21} In addition, these reads allowed assembly of the partial nuclear genome of *P. jirovecii*, which is ~90% complete compared to the *P. jirovecii* RU7 reference genome²² and which is included in a separate manuscript to be published elsewhere. Examination of the mitochondrial and nuclear genome assemblies showed no known nonsynonymous mutations in the dihydropteroate synthase (*dhps*), dihydrofolate reductase (*dhfr*) and cytochrome b (*cob*) genes.⁶

One week after transbronchial lung biopsy (5 days after receiving mNGS report), pathological examination of lung biopsy with hematoxylin-eosin (HE) stain (Figure S1a) reported that alveolar spaces were filled with abundant foamy or granular eosinophilic exudate as well as a few lymphocytes infiltrate; the alveolar septa became thickened due to edema and dilated capillaries. Gomori methenamine-silver staining of the lung biopsy displayed a plenty of dark brown oval or cup-shaped organisms in alveolar spaces, consistent with the morphology of *P. jirovecii* (Figure S1b).

After completion of the treatment regimen with caspofungin for 14 days and TMP-SMX for 16 days, her symptoms disappeared, general conditions returned to normal and laboratory findings improved (Table 1). The patient was discharged after 16 days of hospitalization. She continued to take oral TMP-SMX for another two weeks. At her 2-week follow-up visit, she had no symptoms and chest HRCT scan exhibited no abnormal finding except for a faint patchy opacity in the lateral segment (Figure 1D and E). At her

9-month follow up, she appeared healthy with no symptoms and chest HRCT was normal.

Discussion

Despite its high prevalence in immunocompromised individuals, diagnosis of PCP remains challenging due to its nonspecific clinical manifestations and the inadequate performance of current conventional diagnostic methods. This challenge is well reflected in the present case report. As a renal transplant recipient without prophylaxis for opportunistic infections, the patient in this report was apparently at high risk for PCP, as further supported by the low CD4 count (192/ μ L). However, the *P. jirovecii* organism was consistently missed in microscopic examination of stained sputum smear and BALF samples. In addition, serum BDG level was completely normal (Table 1). These findings point out the inadequate sensitivity of microscopic examination of stained samples and serum BDG assay for diagnosing PCP.

In literature review, we noted a total of 393 reported PCP cases in renal transplant recipients, of which 70% (275) were detected by microscopic examination, 42% (164) by PCR and 6% (23) by pathological examination (Table S1).^{18,23–67} At least 16 of these cases in 4 studies were tested for serum BDG levels, with 11 of them being normal. The observation of normal BDG levels in these PCP studies including our present case is in contrast to the high sensitivity of serum BDG test (91% to 96%) demonstrated in three meta-analyses.^{68–70} Given the high load of *P. jirovecii* organisms in BALF in our patient as revealed by mNGS, the finding of a very low level of serum BDG is unexpected but consistent with the report of Zhang et al¹⁸ in which one renal transplant patient with PCP showed a normal serum BDG level (<31 pg/mL) and a high load of *P. jirovecii* organisms in BALF (9092 reads). The reasons for this are unclear and may be due to the failure of the BDG test or the lack of circulating BGD in the blood.

The observation of the very low level of serum BDG, together with the consistently negative results in microscopic examination of sputum and BALF smear samples in this case had led us to think that the pneumonia was likely to be caused by a pathogen other than *P. jirovecii* (which is one reason we did not seek to perform PCR for *P. jirovecii*). To look for other potential pathogens, we performed mNGS to this case directly using a single BALF sample (3 mL) without depletion of human DNA or enrichment of *P. jirovecii* DNA. Unexpectedly, a large number of *P. jirovecii* reads (1,665,693) was obtained, accounting for

6.8% of the total filtered mNGS reads (including human DNA reads), which is substantially higher than the ratio (<0.4–1%) described previously in NGS sequencing of DNA directly extracted from lung tissue or BALF samples from patients heavily infected by *P. jirovecii*.^{20–22} This finding, combined with the medical history and clinical manifestations as well as the results of pathological examination of lung biopsy firmly established *P. jirovecii* as the causative agent of pneumonia in this case. The successful assembly of the complete mitogenome and partial nuclear genome of *P. jirovecii* from this case suggests a potential to directly sequence the complete or a large proportion of the *P. jirovecii* genome using BALF specimens from heavily infected patients.

To date, there have been 5 reports on successful use of mNGS to diagnose pulmonary infections involving *P. jirovecii* (Table 2).^{14–18} While the sample size is small (1 to 13 specimens each), these studies involve the use of different types of specimens (BALF, sputum, lung biopsy and blood) and different NGS platforms. The numbers of sequence reads for *P. jirovecii* are highly variable (2 to 303,572) among studies though all are less than the *P. jirovecii* reads identified from our case (1,665,693). Two of these studies reported that mNGS is more sensitive than conventional methods (non-PCR based).^{14,18} Theoretically, PCR should be not only more sensitive but also simpler, faster and less expensive than mNGS. However, PCR testing depends on hypothesis (presumptive diagnosis) and currently, there is no PCR assay available and approved for

clinical diagnosis of PCP in China. Under the current Chinese Guidelines for Diagnosis and Treatment of HIV/AIDS (2018),⁷¹ microscopic detection of *Pneumocystis* cysts or trophozoites in stained respiratory samples is the only way to confirm PCP diagnosis, without including PCR or other molecular methods. Compared to PCR as well as other conventional diagnostic methods, mNGS has the main advantages of hypothesis-free testing and unbiased samples, allowing identification of all potential known and new or unexpected pathogens.^{11–13} The whole process of mNGS in our case took less than 72h (from shipping out the BAL sample to receiving the mNGS report). These advantages enabled us to identify *P. jirovecii* as the cause of the disease in our patient. Another advantage of mNGS is its capacity to provide genome-wide information that may be useful for strain identification and drug resistance prediction. In our case, analysis of mNGS data revealed no nonsynonymous mutations in *dhps*, *dhfr* or *cob* gene.⁶ Nevertheless, the application of mNGS to clinical diagnosis is still challenging due to its fairly high cost, difficulty to obtain high-quality samples, demand for sophisticated bioinformatics analysis, and barriers in standardization of the entire process from sample collection to result interpretation. Clearly, all these issues warrant further investigation in the future.

The increasing incidence of PCP outbreaks in renal transplant recipients worldwide has underscored the necessity of prophylaxis in this patient population.⁷² The patient in this report was not given any prophylaxis according to the Diagnosis and Treatment Guideline of Invasive Fungal

Table 2 Reports of PCP Cases Detected by Metagenomics Next-Generation Sequencing (mNGS)

Reference No.	Authors	Year	No. of PCP Cases	Common Underlying Diseases	Sample Types	NGS Platform	No. of Reads for <i>P. jirovecii</i> , Media (Range)	Most Common Co-Infecting Pathogens
[14]	Wang et al	2019	13	Cancers, anemia	BALF and lung biopsy	Torrent Proton	122 (6–303,572)	CMV, <i>Aspergillus</i>
[15]	Irinyi et al	2019	3	n/a	BALF, sputum	Nanopore MinION	9 (2–14)	<i>Candida</i> , <i>Lecanora</i> , <i>Diplodia</i>
[16]	Camargo et al	2020	1	Leukemia	Blood	Illumina NextSeq 500	263	None
[17]	Li et al	2020	9	Immuno-deficiency	BALF	BGISEQ-50	246 (5–4857)	None
[18]	Zhang et al	2019	13	Cancers, pemphigus, anemia,	BALF, sputum, blood and lung biopsy	n/a	34 (5–787)	HHV, HSV1, <i>Candida albicans</i> ,

Abbreviations: n/a, not available; BALF, bronchoalveolar lavage fluid; HHV, human herpes virus; HSV1, human simple virus 1; CMV, cytomegalovirus.

Infections in Solid Organ Transplant Recipients in China (2016 edition).¹⁹ Even in the most recent edition (2019) of this guideline, PCP prophylaxis is still not recommended.⁷³ Our case report, together with the widespread adoption of PCP prophylaxis in solid organ transplant recipients in many countries,^{72,74,75} highlights the need to add PCP prophylaxis to the relevant guideline in China.

In summary, we presented a case of PCP in a Chinese patient who developed acute pneumonia after renal transplantation but showed negative results for *P. jirovecii* and other common known respiratory pathogens in multiple conventional diagnostic tests. Direct mNGS analysis of a single BALF specimen identified a large number of *P. jirovecii* reads, allowing to confirm the diagnosis of PCP. This case report supports the value of mNGS in diagnosing infectious diseases, highlights the inadequate sensitivity of conventional diagnostic methods for PCP, and calls for the need to add PCP prophylaxis to the current Diagnosis and Treatment Guideline of Invasive Fungal Infections in Solid Organ Transplant Recipients in China.

Statement of Ethics

This research complies with the guidelines for human studies and is in accordance with the Declaration of Helsinki. This work was approved by Institutional Review Board of the First Affiliated Hospital of Chongqing Medical University, China (No. 20172901). Written informed consent was obtained from the patient for the participation in this study and the publication of associated data including radiological and histological images. The authors confirmed that personal identity information of the patient data was unidentifiable from this report.

Funding

This research was supported in part by funds from the Chongqing Health and Family Planning Committee (2017ZDXM004) and the Chongqing Science and Technology Committee (cstc2017shmsA130031), and federal funds from the Intramural Research Program of the US National Institutes of Health Clinical Center.

Disclosure

The authors declare no conflicts of interest in this work.

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