Metagenomic next-generation sequencing for the diagnosis of *Pneumocystis jirovecii* pneumonia in solid organ transplant recipients

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To the Editor: Pneumocystis jirovecii (P. jirovecii) pneumonia (PJP) is an opportunistic infection that usually occurs in immunosuppressed patients. Solid organ transplant (SOT) recipients have a high incidence of PJP due to the use of immunosuppressive therapy. In recent years, metagenomic next-generation sequencing (mNGS) has been increasingly used in the diagnosis of infectious diseases. Herein, we report ten cases of PJP diagnosed by mNGS and compare the diagnostic value of mNGS with that of conventional laboratory methods.

This study was reviewed and approved by the Ethics Committee of Shulan (Hangzhou) Hospital (No. KY2021009). Informed consent was exempted because this was a retrospective study. We obtained patient data from the Medical Records and Statistics Room. We analyzed the data anonymously. The use of the raw data was permitted by the Ethics Committee of Shulan (Hangzhou) Hospital.

Ten patients with PJP who were hospitalized at the Shulan Hospital (Hangzhou) from July 2019 to September 2021 were retrospectively evaluated. All the enrolled patients were HIV-negative SOT recipients, of whom seven received kidney transplants and three received liver transplants. All patients received immunosuppressive therapy after surgery and had varying degrees and combinations of clinical symptoms, including fever, cough and expectoration, chest tightness, and dyspnea. Cases 1, 3, 4, 5, 7, and 10 had different degrees of hypoxemia, and all patients had varying degrees of ground-glass shadows on chest computed tomography (CT). The baseline characteristics of the patients are listed in Table 1.

Bronchoalveolar lavage fluid (BALF) from all patients was used for mNGS, and *P. jirovecii* was detected in all samples. Traditional staining of BALF smears using periodic acid-Schiff (PAS), periodic acid-methenamine silver (PAM), or Giemsa stains detected *P. jirovecii* in cases 1 to 5. In case 9, only a few suspicious fungal spores and hyphae were found by PAS staining. In case 7, *Cryptococcus* was found by PAS and PAM staining and mNGS. All BALF cultures were negative. In addition, mNGS detected other pathogens, including torque teno virus, human herpesvirus 5, human herpesvirus 7, and human gammaherpesvirus 4 [Table 1].

All patients received trimethoprim/sulfamethoxazole and other symptomatic treatments. Cases 4 and 10, who developed severe respiratory failure during hospitalization, were transferred to the intensive care unit for tracheal intubation and mechanical ventilation. However, their condition rapidly deteriorated, leading to septic shock, multiple organ failure, and eventually death. After a period of treatment, the clinical symptoms of the remaining patients significantly improved, and chest CT scans showed significant absorption of pulmonary exudates.

PJP is a life-threatening disease that mainly occurs in immunocompromised individuals. The causative pathogen *P. jirovecii* was considered to be a protozoan until 1988, when DNA analysis showed that its genomic structure was more closely related to that of fungi.[1] As *P. jirovecii* cannot be cultured, the gold standard for the diagnosis is microscopic visualization of the organism. Traditional diagnostic tests include non-immunofluorescent and immunofluorescent staining. However, microscopic methods are not sensitive, and their performance depends on the quality and type of samples and the technique used by the observer. In addition, a low pathogen burden, as in HIV-negative patients and those taking drugs for PJP prophylaxis, may lead to false-negative microscopy results.[2] Some biochemical markers, such as serum $(1,3)$–β-D-glucan (BDG) and lactate

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Table 1: Baseline characteristics of patients and mNGS results.

Case No.	Sex	Age (years)	Transplanted organ	Laboratory tests (smear and culture)	(1,3)-β-D-glucan (pg/mL)*	mNGS results and sequencing reads (n)	Detection platform	Symptoms	Hypoxemia
1	Male	63	Kidney	PAS-, PAM-, Giemsa-stained smear: Pneumocystis jirovecii; BALF culture: negative	<0.5	Pneumocystis jirovecii (103,226)	Practice Medicine	Fever, cough and expectoration, chest tightness, dyspnea	Yes
2	Male	45	Liver	PAS-, PAM-stained smear: Pneumocystis jirovecii; Giemsa-stained smear: negative; BALF culture: negative	<0.5	Pneumocystis jirovecii (1985); Torque teno virus (6)	Medcaredx	Fever, cough	No
3	Female	64	Liver	PAS-, PAM-, Giemsa-stained smear: Pneumocystis jirovecii; BALF culture: negative	<0.5	Pneumocystis jirovecii (22,621)	TClab	Fever, cough and expectoration, chest tightness, dyspnea	Yes
4	Male	50	Kidney	PAM-stained smear: Pneumocystis jirovecii; PAS-, Giemsa-stained smear: negative; BALF culture: negative	Day 4: 6.7; Day 11: 24.6; Day 14: 88.8; Day 21: 138.6	Pneumocystis jirovecii (336,010); Haemophilus parahaemolyticus (1052); Haemophilus sputorum (18); Enterobacter bormaechei (37); Schizophyllum commune (30); Human betaherpesvirus 5 (21,252); Torque teno virus (63); Human betaherpesvirus 7 (7); Torque teno virus 7 (3)	BGI	Fever, severe dyspnea	Yes
5	Female	47	Kidney	PAS-, PAM-, Giemsa-stained smear: Pneumocystis jirovecii; BALF culture: negative	24.5	Pneumocystis jirovecii (106,811); Human betaherpesvirus 5 (2360); Human gammaherpesvirus 4 (92)	Simcere	Fever, chest tightness, dyspnea	Yes
6	Male	56	Liver	PAS-, PAM-, Giemsa-stained smear: negative; BALF culture: negative	<0.5	Pneumocystis jirovecii (3106); Torque teno virus (36); Human herpesvirus 7 (10)	Practice Medicine	Fever, cough	No
7	Female	35	Kidney	PAS-, PAM-stained smear: Cryptococcus; Giemsa-stained smear: negative; BALF culture: negative	<0.5	Pneumocystis jirovecii (7128); Cryptococcus neoformans (139); Human betaherpesvirus 5 (3)	BGI	Chest tightness, dyspnea	Yes
8	Male	60	Kidney	PAS-, PAM-, Giemsa-stained smear: negative; BALF culture: negative	<0.5	Pneumocystis jirovecii (225); Human betaherpesvirus 5 (2238)	BGI	Fever, cough and expectoration	No
9	Male	53	Kidney	PAS-stained smear: a few suspicious fungal spores and hyphae; PAM-, Giemsa-stained smear: negative; BALF culture: negative	<0.5	Pneumocystis jirovecii (1535); Candida albicans (24); Haemophilus sputorum (5); Actinomyces israelii (13); Human betaherpesvirus 5 (4); Human gammaherpesvirus 4 (3)	BGI	Fever	No
10	Female	58	Kidney	PAS-, PAM-, Giemsa-stained smear: negative; BALF culture: negative	Day 3: 46.1; Day 20: 368.9	Pneumocystis jirovecii (5214); Human betaherpesvirus 5 (7); Human betaherpesvirus 7 (1); Staphylococcus aureus (5); Candida parapsilosis (3)	TClab	Fever, severe dyspnea	Yes

*(1,3)-β-D-glucan (normal reference range: 0-10.0 pg/mL). Practice Medicine (Practice Medicine Lab, Nanjing, Jiangsu Province, China); Medcaredx (Medcaredx Biotech, Shanghai, China); TClab (Tongchuang Lab, Hangzhou, Zhejiang Province, China); BGI (Beijing Genomics Institution, Nanjing, Jiangsu Province, China); Simcere (Simcere Pharmaceutical Group Limited, Nanjing, Jiangsu Province, China). mNGS: Metagenomic next-generation sequencing; BALF: Bronchoalveolar lavage fluid; PAS: Periodic acid-Schiff; PAM: Periodic acid-methenamine silver.

dehydrogenase, can also be used as auxiliary diagnostic tools, but their specificities are not high.[2]

NGS, also known as high-throughput or massively parallel sequencing, allows independent sequencing of thousands to billions of DNA fragments at the same time. NGS, including mNGS, has various applications in clinical microbiology and allows unbiased detection of pathogens.[3] Our research revealed that mNGS had a higher detection rate and detection speed for PJP than those of traditional detection methods. *P. jirovecii* was detected in the BALF of all ten patients using mNGS, while microscopic examination detected *P. jirovecii* in only five patients. Moreover, mNGS had a turnaround time of less than three days. Serum BDG was positive in only three of the ten patients, and its levels gradually increased in cases 4 and 10 as the disease progressed [Table 1]. This finding indicates that the serum BDG level may depend on the pathogen load, and false-negative results may be obtained when the pathogen load is low.

Currently, the interpretation of mNGS results is still challenging. As there is no universally recognized diagnostic threshold for infection, mNGS cannot distinguish between pathogen infection and colonization, nor can mNGS distinguish between live and quiescent microorganisms or extra-cellular DNA from dead microorganisms.[4] The determination of the positive threshold does not depend on a single indicator and should include, but not limited to, the number of sequencing reads of a specific microorganism, the value of reads per million, and genome coverage of the detected species.[5] Laboratories should set diagnostic thresholds for each testing platform and for different pathogens according to their own mNGS process. At present, mNGS microbial test reports issued by any domestic laboratory cannot be directly used for clinical diagnosis. In clinical practice, doctors need to comprehensively consider patient's symptoms, lung imaging findings, laboratory test results, and clinical characteristics of each identified microorganism to judge the presence of infection. The detection platforms used in our study all provided reports of detected microbial sequencing reads (numbers of microbial DNA sequences detected at the genus/species level), but none set a diagnostic threshold. In addition, Practice Medicine (Practice Medicine Lab, Nanjing, Jiangsu Province, China) provides the proportion of pathogens of the same type, and TClab (Tongchuang Lab, Hangzhou, Zhejiang Province, China) provides the relative abundance of a certain microbial genus or species among the detected microorganisms in a specimen. In general, the higher the number of sequence reads of a certain microorganism is, the greater the possibility that it is the pathogen is; however, differences in the types of pathogenic microorganisms and their pathogenic characteristics must also be considered.[5] In this study, the clinical diagnosis of PJP was made in all ten cases based on the detection of *P. jirovecii* sequences and on the patient's clinical data.

Although different mNGS platforms differ in sensitivity, which may lead to certain differences in the number of *P. jirovecii* sequencing reads, some correlations can be observed with clinical data of patients. In our study, cases 1, 3, 4, 5, 7, and 10 ranked the top six in the number of sequencing reads of *P. jirovecii,* and all had varying degrees of hypoxemia, with cases 4 and 10 presented with severe respiratory failure. Meanwhile, cases 2, 6, 8, and 9, with relatively low numbers of *P. jirovecii* sequencing reads, did not have hypoxemia. These findings suggest that the number of mNGS-detected sequencing reads of *P. jirovecii* may indicate the severity of the patient's condition. As disease severity is also related to the patient's age, underlying disease, and other factors, more researches are needed in the future to explore this hypothesis.

Another advantage of mNGS over conventional diagnostic methods is that mNGS can identify not only all potential known pathogens but also unexpected, novel pathogens or fastidious pathogens, and the results of mNGS are not affected by the prior use of antibiotics.[4] In this study, several other potential pathogens were identified, even though all ten patients had previously used antibiotics. Therefore, mNGS might be particularly beneficial for SOT patients, who are more likely to be infected by multiple pathogens owing to long-term use of immunosuppressive agents.

In conclusion, this study explored the application value of mNGS in the diagnosis of PJP and found that the sensitivity and detection speed of mNGS were better than those of traditional detection methods. However, our study has several limitations, including a small sample size and the use of more than one testing platform. Therefore, more research is needed in the future. In addition, the definition of the threshold of infection is urgently needed for certain applications of mNGS technology in clinical practice.

Conflicts of interest

None.

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