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Application of Metagenomic Next-Generation Sequencing to Diagnose *Pneumocystis jirovecii* Pneumonia in Kidney Transplantation Recipients

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Background: *Pneumocystis jirovecii* pneumonia (PJP) is one of the common opportunistic infections diagnosed in kidney transplantation recipients. It is difficult to identify early by use of classic tools such as Grocott-Gomori stains and polymerase chain reaction (PCR). Metagenomic next-generation sequencing (mNGS) is accurate, unbiased, and sensitive, and is promising in PJP diagnosis.

Material/Methods: Data on kidney transplantation patients diagnosed with PJP were retrospectively analyzed. The sensitivity and specificity of different tools such as mNGS, laboratory tests, and Grocott-Gomori stains for PJP diagnosis were compared. All recipients were treated with trimethoprim-sulfamethoxazole (TMP-SMX).

Results: There were a total of 12 kidney transplantation recipients diagnosed with PJP based on mNGS in our center from January 01, 2020 to October 27, 2020. Highly variable numbers of sequence reads for *P. jiroveci* (19 to 1041285) showed diagnostic significance. Bronchoalveolar lavage fluid (BALF) samples were tested by Grocott-Gomori staining, with only 6 of 11 (54.5%) positive. Other routine laboratory tests like routine blood tests, blood biochemistry, procalcitonin (PCT), immune function, (1,3)-β-d-glucan (BG), serum galactomannan (GM), and C-reactive protein (CRP) showed even lower efficacy. TMP-SMX appeared to be the ideal therapy for kidney transplantation recipients with PJP.

Conclusions: mNGS has utility in the diagnosis of PJP and mixed infections in kidney transplantation recipients, and TMP-SMX could be the ideal therapeutic drug for kidney transplantation recipients suffering from PJP.

Keywords: Kidney Transplantation • Metagenomics • Pneumocystis Infections

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Background

Infection is one of the common complications after kidney transplantation [1-4]. Long-term use of immunosuppressants weakens both humoral and cellular immune functions. It, along with multiple changes in the postoperative physiological structures, results in pulmonary infections and urinary tract infection [1,5]. Compared with the general population, infection in kidney transplantation recipients is far more complicated and they are prone to opportunistic infections, mixed infections, rare bacterial infections, and severe infections, which makes diagnosis more difficult. Delayed diagnosis leads to delayed treatment and to poor prognosis [1,6-8]. Therefore, correct diagnosis and timely treatment are extremely important for kidney transplantation recipients with infection.

Although staining, culture, antigen detection, PCR, and other methods have been widely used in clinical practice, it is still quite difficult to quickly obtain accurate pathogen results due to the complexity of kidney recipients' infections [9,10]. Therefore, we can only diagnose it by the recipients' symptoms, signs, imaging examination, and laboratory examinations, and then perform empirical anti-infection treatment, which is always broad-spectrum. It comes along with many disadvantages, such as the abuse of antibiotics, the formation of multi-drug-resistant bacteria, misdiagnosis, missed diagnosis, delays in diagnosis, increased treatment costs, and even death [8,11,12]. Faster and more accurate diagnostic technology is urgently needed.

Metagenomic next-generation sequencing (mNGS) is a new diagnostic technology that can detect all pathogens in recipients' samples quickly, accurately, and without bias, which is of great help in the identification of complex and rare pathogens [10,13,14]. With progress in the related technologies, mNGS has gradually become popular in clinical practice, especially for use in patients with severe infections, immunodeficiency, and long-term use of immunosuppressants after organ transplantation [9,13,15-17].

Pneumocystis jirovecii pneumonia (PJP) is a common opportunistic infection in kidney transplantation recipients [18]. These patients usually have symptoms of fever, dry cough, and anhelation, and their chest computed tomography (CT) scans suggest bilateral diffuse infiltrates [19-21]. Trimethoprim-sulfamethoxazole TMP-SMX is currently the drug of choice for both prophylaxis and treatment [22]. If not treated in time, these patients will quickly develop respiratory failure and die, and the mortality rate is up to 10% despite adequate therapy in kidney transplantation recipients [20,23]. The criterion standard to diagnose PJP remains the histological and microscopic identification of fungal asci and trophic forms by using stains like Grocott-Gomori in samples including tissue, bronchoalveolar lavage fluid (BALF), and sputum. Its sensitivity is still not ideal, which may cause delays in treating the patient's condition [24, 25]. PCR testing is fast and sensitive, but it relies on certain assumptions and has not been approved for the clinical diagnosis of PJP in some countries, such as the United States and China. Therefore, we assessed the use of mNGS, a new technology, for early diagnosis of PJP in kidney transplantation recipients.

Material and Methods

Patients and Collection of Samples

From January 01, 2020 to October 27, 2020, there were 21 kidney transplantation recipients with symptoms of fever, dry cough, or anhelation in our center. Their CT scans suggested interstitial lung disease. Among these patients, we performed mNGS testing on patients with PO2 below 70 mmHg, and 12 of them were diagnosed as having PJP based on mNGS (1 with a sample of peripheral blood, 9 with BALF, and 2 with both). For 11 patients who underwent bronchoscopy, we performed Grocott-Gomori staining, fungus immunofluorescence staining, and other tests with their BALF. In addition, we also performed routine blood tests, blood biochemistry, PCT, immune function, (1,3)-β-d-glucan (BG), serum galactomannan (GM), procalcitonin (PCT), and C-reactive protein (CRP) on peripheral blood (PB) samples.

BALF and PB samples were obtained (about 3 mL) and placed in the sterile sputum container, stored at -20°C, then sent to REALBIO TECHNOLOGY (Shanghai, China) for detection.

mNGS Procedure for Samples

The mNGS procedure for BALF and PB samples included sample collection, library construction, and sequencing. According to the manufacturer's protocols, the DNA was extracted by using a TIANamp Micro DNA extraction kit and the RNA was extracted using a QIAamp Viral RNA Mini Kit RNA extraction kit. Using NanoDrop (Thermo Scientific) and Qubit®2.0 (Invitrogen, USA), the DNA/RNA concentration was measured, and the molecular size was estimated by agarose gel electrophoresis. Then, the library was constructed by using the Hieff NGS® 384 Dual Index Primers Kit for Illumina®. The quality of the library was estimated using Agilent 2100 (Agilent, USA) to estimate the concentration. Finally, the libraries constructed from all the samples were pooled and sequenced on the Illumina NextSeq platform (Illumina, San Diego, CA).

For quality control and bioinformatic analysis, we processed the raw reads of sequencing to remove low-quality reads and adapter contamination to produce clean reads. Then, we

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Table 1. Baseline characteristics.

Recipient	Age (yr)	Gender	BMI (kg/m²)	RD	Dialysis form	Dialysis time	Transplant time	Transplant form	Hypertension	Diabetes	LVEF (%)	Immunosuppressant
1	64	Male	22.5	Unknown	PD	4 (yr)	1 (yr)	DCD	+	–	60	Tacrolimus +MMF +GC
2	36	Male	20.4	Unknown	HD	0.5 (yr)	5 (yr)	Living-related	+	–	n/a	Tacrolimus +MMF +GC
3	34	Male	17.9	Unknown	HD	4 (yr)	5 (month)	DCD	+	–	61	Tacrolimus +MMF +GC
4	62	Male	21.6	Unknown	PD	4 (yr)	2 (yr)	DCD	+	+	69	CsA +MPA +GC
5	40	Male	19.8	Polycystic kidney	HD	5 (yr)	1 (yr)	DCD	+	–	n/a	Tacrolimus +MMF +GC
6	54	Male	25.3	Diabetic nephropathy	HD	8 (yr)	5 (month)	DCD	+	+	59	Tacrolimus +MMF +GC
7	45	Female	17.5	Unknown	PD	0.5 (yr)	9 (yr)	DCD	+	–	85	Tacrolimus +MMF +GC
8	30	Male	20.5	Unknown	HD	10 (month)	1 (yr)	Living-related	+	–	62	Tacrolimus +MMF +GC
9	43	Male	21.0	Unknown	HD	1 (month)	9 (yr)	Living-related	+	–	64	Tacrolimus +MMF +GC
10	35	Female	17.3	HSP	None	0	1 (yr)	Living-related	+	–	n/a	Tacrolimus +MMF +GC
11	61	Male	19.9	Unknown	HD	9 (yr)	5 (month)	DCD	+	–	70	Tacrolimus +MMF +GC
12	28	Male	23.2	Unknown	None	0	1 (yr)	Living-related	+	–	n/a	Tacrolimus +MMF +GC

BMI – body mass index; RD – renal disease; HSP – Henoch-Schönlein purpura nephritis; PD – peritoneal dialysis; HD – hemodialysis; DCD – cardiac death donors; LVEF – left ventricular ejection fractions; CsA – ciclosporin; MMF – mycophenolate mofetil; MPA – mycophenolic acid; GC – glucocorticoid; n/a – not available; '+' – positive; '–' – negative.

mapped the clean reads to the human-source database to filter the human-source sequence. The remaining reads were aligned to the reference database (NCBI database and GenBank) to identify microbial species.

Treatment

In the past 5 years, our center has performed approximately 450 kidney transplants each year. Usually, the recipients were given basiliximab or anti-thymocyte globulin (ATG) for immunosuppression induction (7 recipients with ATG and 5 recipients with basiliximab in our study). Then, the postoperative maintenance regimens include ciclosporin (CsA)/tacrolimus, mycophenolate mofetil (MMF)/mycophenolic acid (MPA), and glucocorticoid. To prevent PJP infections, the recipients need to take TMP-SMX for 1 year after surgery. In our study, 3 recipients within 1 year after surgery did not take the prophylaxis due to their poor compliance or allograft function.

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Table 2. Reads of each pathogen in the mNGS.

Recipients	Sample	Bacterium	Fungus	Virus					Others*
		Streptococus	Pneumocytis	HSV1	EBV	CMV	HSV7	Human parvovirus B19	
1	BALF	–	155790	–	–	–	–	–	–
	PB	–	1325	40	7	5	–	–	–
2	BALF	–	1041285	–	–	–	–	–	–
3	BALF	–	607	172	96	467	–	–	–
4	BALF	16539	25370	–	3188	186	–	–	–
5	BALF	–	35754	–	–	55	15	–	–
6	BALF	–	238	–	21	6	–	–	–
7	BALF	–	98273	–	47	47	–	–	–
8	PB	–	19	–	–	–	–	–	–
9	BALF	–	257620	–	–	–	–	–	–
	PB	–	301	–	–	9	–	–	–
10	BALF	–	1033	–	–	–	–	3228	–
11	BALF	–	58223	–	–	93	–	–	–
12	BALF	–	4307	–	–	–	–	–	–

PB – peripheral blood; BALF – bronchoalveolar lavage fluid; HSV1 – human alpha herpesvirus 1; EBV – human gamma herpesvirus 4; CMV – human beta herpesvirus 5; HSV7 – human herpesvirus 7; * others – parasite, mycobacterium tuberculosis, mycoplasma, chlamydia, rickettsia; '–' – negative.

For those patients diagnosed with PJP by mNGS, we used TMP-SMX as anti-infective treatment, and regularly checked their serum creatinine.

Results

The baseline characteristics are shown in **Table 1**. Most recipients (83%) were male, and 70% chose hemodialysis as their form of dialysis before kidney transplantation. In addition, all of them had a history of hypertension.

The results of mNGS are shown in **Table 2**. Most recipients had very high reads of pneumocystis. From the results of the 2 recipients with samples of both peripheral blood and BALF, the reads in BALF were much higher than in the peripheral blood, which may suggest that it could be more sensitive to perform mNGS with BALF than with peripheral blood. In addition, we detected some viruses such as EBV, CMV, and HSV1, so mNGS is also a great reference for the diagnosis of mixed infections. Based on the results of mNGS and laboratory tests, Recipient 4 and Recipient 10 were quickly diagnosed as having mixed infections. The pathogens of Recipient 4 were pneumocystis and streptococcus. For Recipient 10, it was pneumocystis and human parvovirus B19.

Eleven patients with BALF samples had Grocott-Gomori staining of BALF performed at the same time to find fungal asci, but only 6 patients had positive results, so the positive rate of Grocott-Gomori stains was only 54.5% in our study (**Table 3**). We performed a series of laboratory tests on these 12 patients (**Table 3**), and their BG were all positive. In addition, for 11 recipients with samples of BALF, we performed cultures for *Cryptococcus neoformans* capsular polysaccharide antigen assays, and fungal immunofluorescence stains, and the results were all negative. In addition, most recipients had relatively high C-reactive protein (CRP) and neutrophils (Neu)% in peripheral blood, with relatively low numbers of helper T cells and cytotoxic T lymphocytes, but it had low clinical significance because of low specificity.

Twelve patients diagnosed with PJP by mNGS were administered TMP-SMX as anti-infective treatment, while 2 co-infected patients were given other anti-infective drugs based on mNGS,

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and another 10 patients were given TMP-SMX only. Among them, 11 patients gradually recovered and were discharged, and 1 patient (Recipient 1) was transferred to the ICU for further treatment because of uncontrollable pulmonary infections (he was co-infected with human parvovirus during hospitalization). It is worth mentioning that for Recipient 10, with mixed infections, we used TMP-SMX combined with human gammaglobulin (20 g daily for 5 days); her hemoglobin increased progressively and her pulmonary infection recovered gradually.

Since the use of TMP-SMX may cause damage to renal function, we regularly checked the serum creatinine levels (**Table 3**). Comparing their serum creatinine at the time of admission and discharge, we found that 3 patients had relatively significant increases (>10%) in serum creatinine.

Table 3. Laboratory tests.

Recipient	Grocott-Gomori stains (BALF)	Cryptococcus neoformans capsular polysaccharide antigen (PB)	BG (PB, pg/ml)	GM (PB)	Culture (BALF)	Culture (PB)
1	+	–	235	0.99	–	–
2	+	–	559	0.55	–	–
3	+	–	773	1.39	–	–
4	–	–	598	<0.25	–	–
5	–	–	623	0.29	–	–
6	–	–	324	0.17	–	–
7	+	–	507	<0.25	–	–
8	n/a	–	768	0.13	n/a	–
9	+	–	115	0.24	–	–
10	+	–	729	0.17	–	–
11	+	–	1000	<0.25	–	–
12	+	–	390	0.18	–	–

Recipient	Cryptococcus neoformans capsular polysaccharide antigen (BALF)	Fungal immunofluorescence stains (BALF)	PCT (ng/ml)	CRP (mg/l)	WBC ($\times 10^9$/L)	Neut (%)
1	–	–	0.24	44.3	8.5	96.4
2	–	–	0.82	23.3	4.6	86.6
3	–	–	n/a	33.7	2.7	72.6
4	–	–	1.53	52	9.2	92.2
5	–	–	0.16	61.8	10.2	81.4
6	–	–	0.05	1	7.3	77.7
7	–	–	0.23	32.2	10.4	87.5
8	n/a	n/a	0.15	85	16.6	83.9
9	–	–	0.27	116.1	5.7	70.6
10	–	–	0.15	13.9	3	66.1
12	–	–	0.15	84.2	5.2	84.4
12	–	–	0.12	72.2	12.3	73.3

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Table 3 continued. Laboratory tests.

Recipient	Eo (%)	Th (CD4+) /ul	Th (%)	Tc (CD8+)/ul	Tc (%)	Th/Tc	SCr1 (umol/L)	SCr2 (umol/L)
1	0	12	13.9	40	45.7	0.3	314	281
2	0	109	28.9	209	55.1	0.52	296	297
3	0	183	26.9	205	30	0.89	374	299
4	0	77	28.4	107	39.6	0.72	193	140
5	0.6	84	18.4	151	32.9	0.56	166	155
6	0.1	113	24.7	114	24.8	0.99	181	145
7	1.2	220	53.4	113	27.4	1.95	165	135
8	1	705	48.7	488	33.7	1.44	128	183
9	0.5	55	24.9	100	45.4	0.55	411	317
10	5	234	36.4	372	57.9	0.63	150	188
12	1.5	296	54.9	130	24.2	2.27	267	330
12	0.6	1197	46.9	611	23.9	1.96	184	178

PB – peripheral blood; BALF – bronchoalveolar lavage fluid; BG – β-d-glucan assays; GM – serum galactomannan assays; PCT – procalcitonin; CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; WBC – white blood cell; Neut – neutrophil; Eo – eosinophilia; Th – helper T cell; Tc – cytotoxic T lymphocyte; SCr1 – serum creatinine before hospitalization; SCr2 – serum creatinine after hospitalization; n/a – not available; '+' – positive; '–' – negative.

Discussion

In summary, typical symptoms, typical chest CT images, and positive BG results could be hints for the diagnosis of PJP, but these often lack specificity; interstitial pneumonia caused by other pathogens such as Cryptococcus can also have the above manifestations. At the same time, the sensitivity of Grocott-Gomori stains is inadequate [25], which may cause missed diagnosis, and many patients may not able to tolerate bronchoscopy or alveolar lavage. In addition, since dry cough is a typical symptom, sputum is not obtained in many patients for Grocott-Gomori staining. In addition, PCR testing is fast and sensitive, but it relies on certain assumptions and has not been approved for the clinical diagnosis of PJP in some countries, such as the United States and China. Therefore, mNGS has great significance in the diagnosis of PJP and mixed infections in kidney transplantation recipients. It does not require making assumptions about pathogens, and is sensitive to bacteria, viruses, fungi, and parasites, with no bias [13,14,26,27].

The sequence reads for *P. jirovecii* were highly variable (19 to 1 041 285) in our study, which agrees with other reports (2 to 303 572) [25]. Therefore, small reads could also have diagnostic significance. For patients who cannot tolerate bronchoscopy or alveolar lavage, the sample of peripheral blood is also useful. Still, in our experience, BALF samples are more sensitive. mNGS can help clinicians make rapid diagnosis, provide early treatment and timely intervention, and speed the clinical decision-making of antibiotic treatment programs, thereby promoting early recovery of the recipients and reducing hospitalization time and economic expenditure. In addition, it can greatly ease the abuse of antibiotics, which will have extremely important value for our entire society [12,14].

In the process of using mNGS to assist clinical diagnosis, there are some shortcomings. For example, the cost of mNGS is still relatively high [9,14]. However, the use of mNGS is helpful for early diagnosis of pathogens, thereby avoiding a series of unnecessary examinations and treatments, and the patient's course of disease and hospital stay will be greatly reduced. Taken together, the patient's cost may be reduced. In addition, the routine use of TMP-SMX after transplantation, coupled with the low popularity of mNGS, resulted in the small sample size of this study. However, as more and more researchers and clinicians realize the utility of this technology, we may be able to conduct a series of studies with larger sample size.

We have also observed that the timely use of TMP-SMX after the diagnosis of PJP had very good effects; the mortality rate in our center is about 8%, which is lower than the 10% reported in other studies [23]]. However, TMP-SMX can damage renal

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function; therefore, it is very important to select the appropriate dose and course of treatment, and to monitor renal function.

Conclusions

Our clinical experience in using mNGS shows that mNGS has utility for the diagnosis of PJP and mixed infections in kidney transplantation recipients.

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Statement of Ethics

All the DCD kidneys were procured from voluntary donation after citizens' death by the Organ Procurement Organization (OPO) in the presence of the Red Cross, and the transplantation application was approved by the Organ Transplant Ethics Committee. The authors were accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. The study was approved by the First Affiliated Hospital of Zhejiang University Ethics Committee Board (NO: IIT20200601A) and individual consent for this retrospective analysis was waived.

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