Application of metagenomic next-generation sequencing of bronchial needle brushing specimen in precise diagnosis of infectious peripheral pulmonary lesions

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Abstract

Background: Reports on the application of metagenomic next-generation sequencing (mNGS) to diagnose infectious peripheral pulmonary lesions (PPLs) remain scarce. No research has ever explored which specimen is preferred for mNGS. Methods: We applied mNGS to detect the presence of pathogen in matched transbronchial lung biopsy (TBLB), bronchoalveolar lavage fluid (BALF) and bronchial needle brushing (BB) specimens from 39 patients suspected of having infectious PPLs. We explored the differences in the composition and diagnostic efficacy of the three specimens. Results: We found that mNGS of TBLB, BALF, BB and TBLB + BALF + BB was more sensitive than conventional cultures for bacterial infections, fungal infections and general infections, the difference in sensitivity of mNGS between TBLB, BALF, and BB was not statistically significant. The sensitivity of mNGS of TBLB + BALF + BB for the diagnosis of fungal infections or general infections was higher than mNGS of TBLB and not significantly higher than mNGS of BALF or BB. We found that there was no statistically significant difference in the relative abundance of pathogen amongst the three types of specimens and in relative abundance of all of the six kinds of common oropharyngeal microbiota between TBLB and BB specimens. Conclutions: The study indicated that mNGS of TBLB, BALF or BB could yield a higher sensitivity for pathogen identification. mNGS of the BB samples might be an alternative choice for patients with infectious PPLs but couldn't tolerate more invasive TBLB procedures.

1. Introduction

Infectious diseases remain most common causes of death among all patient populations worldwide [1]. Pulmonary infections lead to more deaths each year than any other infectious disease categories [2]. Delayed identification of etiologic pathogens in pulmonary infections is the key cause of treatment failure and death. Current microbiological tests such as culture-based methods often fail to identify the etiologic pathogens in most cases of pulmonary infection, due largely to the limitations in terms of sensitivity [3, 4], speed and spectrums of available assay targets [5]. In these situations, failing to identify the etiology microorganisms often promotes the initiation of empiric antibiotic therapy, which may lack activity against an underlying microorganism, leading to treatment failure, disease progression, and consequent adverse outcomes [5, 6].

Swift identification of causative microorganisms and tailoring of the antimicrobial regimen is highly desirable, which would improve the prognosis of pulmonary infections due to optimization of antimicrobial treatment. Recent rapid advances in genomic sequencing techniques and bioinformatics has made it possible for metagenomic next-generation sequencing (mNGS) to be used in clinical diagnostics. [7 ˜ 9]. mNGS is an unbiased approach that can theoretically detect all microorganisms in a clinical sample [10], which offers potential alternatives for detecting etiologic microorganisms and distinguish them from background commensal microorganisms, which might pave the way for personalized medicine [11]. Several reports have attempted to detect pathogens using mNGS in infectious diseases, such as central nervous system infections [12 ˜ 14], digestive infections [15, 16], bloodstream infections [7, 18] and pulmonary infections [19 ˜ 23].

Published information on the use of mNGS in diagnosing pulmonary infections is sparse, and the types of samples tested by mNGS were mainly limited to percutaneous lung biopsy samples [19] and bronchoalveolar lavage fluid (BALF) samples [20 ˜ 23].

Accurate sampling of peripheral pulmonary lesions (PPLs), which were defined as lesions surrounded by normal lung parenchyma presents challenges and were unlikely to be visualized by bronchoscopy, is the key step. CT-guided percutaneous needle biopsy has a high diagnostic yield for PPIs, but is more invasive and with a relatively high incidence of complications, such as bleeding or pneumothorax [24, 25]. Bronchoscopy remains an appropriate initial investigation for PPLs, due to the lower complication rate [26]. Most previous studies have focused on the use of bronchoscopy in diagnosing malignancy. There is little published information on the use of bronchoscopy in diagnosing infectious PPLs.

The present study consists of two parts: on the one hand, we retrospectively evaluate the performance of mNGS for samples from infectious PPLs obtained using ultrathin bronchoscopy in conjunction with virtual bronchoscopic navigation (VBN) and rapid on-site cytological evaluation (ROSE), and compare diagnostic accuracy of mNGS with conventional cultures; on the other hand, we evaluate the comparative diagnostic performance and microbial composition from different sampling including transbronchial lung biopsy (TBLB), bronchial needle brushing (BB) and BALF with mNGS.

2. Materials and methods

2.1 Patient selection

All subjects gave written informed consent. Combining VBN, ultrathin bronchoscope, TBLB + ROSE + mNGS for the diagnosis infective PPLs is our routine process, and this is a retrospective study, therefore, no formal ethical approval was needed. We retrospectively enrolled consecutive patients who were clinically and radiologically suspected of having infectious PPLs and underwent ultrathin bronchoscopy assisted by the VBN system and mNGS for the diagnosis of PPLs in our hospital between July 2018 and July 2019. Patients who did not have TBLB, BB and BALF samples at the same time for mNGS were excluded.

2.2 Specimen collection and processing

All bronchoscopies were performed by the same experienced bronchoscopist. Each patient was locally anesthetized with 2% lidocaine, then an ultrathin bronchoscope (BF-typ XP260F; Olympus: external diameter, 2.8 mm; channel diameter, 1.2 mm) was navigated to the target bronchus as far as possible using the VBN system (Direct Path 1.0). TBLB, BB and bronchoalveolar lavage (BAL) of peripheral lesions were performed in sequence in all subjects. On each patient, TBLB was performed at first, meanwhile ROSE were performed during the examination to determine whether the sample was sufficient for diagnosis. Then BB was performed using a protective needle brush and BALF specimens were obtained after brushing samples. The specimens obtained with TBLB and BAL were separately sent to the clinical microbiology laboratory for culture and sequencing company for mNGS, the TBLB specimens were also sent to the pathological laboratory for pathological examination. The protective needle tips were cut with sterile scissors and placed in a 2 ml sterile microcentrifuge tubes containing 1 ml of sterile saline and sent to the sequencing company for mNGS.

2.3 ROSE

Biopsy specimens were expressed onto labeled glass slides and smearing was performed by an on-site cytotechnologist. Rapid staining was performed using Diff-Quik stain. Cytologic glass slides were then evaluated under light microscopy by the cytotechnologists for immediate interpretation of whether the sample was sufficient for a provisional diagnosis as well as for all later laboratory requirements. A quick feedback was sent back to the bronchoscopist by cytotechnologists. The bronchoscopist terminated or modified the sampling process based on the information. If sufficient specimens were obtained and diagnostic objective had been met from imprint cytology, sampling was stopped, conversely, but otherwise, sampling was continued with the appropriate adjustment.

2.4 mNGS

Sample Processing and Nucleic Acid Extraction : Lung biopsy specimen was collected and cut into small pieces. Samples of 0.5–3 ml BALF and soaking solution of brush tips were collected from patients following standard procedures, respectively. DNA was extracted using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH) according to the manufacturer's recommendation. **Construction of DNA libraries** : Single-stranded DNA circle (ssDNA circle) library was constructed after DNA-fragmentation, end-repair, adapter-ligation, DNA denaturation into single strands, DNA circularization. DNA nanoballs (DNBs) were generated from the ssDNA circle using rolling circle amplification (RCA). Finally, qualified DNBs were loaded on the flow cell and sequenced on BGISEQ-50 platform. **Sequencing and bioinformatic analysis** : High-quality sequencing data were generated by removing low-quality, and short (length $<$ 35bp) reads, followed by a computational substraction of human host sequences mapped to the human reference genome (hg19) using Burrows-Wheeler Alignment. After removal of low-complexity reads, the remaining data were classified aligning to four Microbial Genome Databases simultaneously, consisting of viruses, bacteria, fungi, and parasites. The databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). It contains 4,061 viral taxa whole genome sequence, 2,473 bacterial genomes or scaffolds, 199 fungi related to human infection, and 135 parasites associated with human diseases.

2.5 Statistical analysis

The sensitivity and specificity of mNGS for the diagnosis of pulmonary infection was calculated with the final diagnosis as the gold standard. SPSS Statistics 25.0 statistical software was used for statistical analysis, and GraphPad Prism 8 was used to create statistical charts. The chi-square test was used to compare the rates between groups. Variance analysis was used to compare measurement data sets between groups. $P < 0.05$ was considered statistically significant.

3. Results

Between July 2018 and July 2019, 12 male and 27 female patients with a median age of 38 years (15 to 77 years) were enrolled in the present study. 74% (29/39) patients were immune-impaired. 33 of the 39 patients were eventually diagnosed with lung infection, and 6 of the lung lesions were considered for non-infectious lesions. (9 cases) 23% of the cases were multiple infections. In the end, 43 infections were diagnosed in 33 patients with pulmonary infection, including 14 bacterial infections, 21 fungal pneumonia, 7 viral pneumonia and 1 mycoplasmal pneumonia. All of the patients in this study received empiric broad spectrum antibiotics prior to sample collection.

Performance of conventional culture for the identification of pathogens.

Cultures of BALF were positive in 7 of 43 pulmonary infections. Pathogenic microorganisms that were positive in culture of BALF include Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Corynebacterium and fungi. Pathogenic microorganisms detected only by mNGS, but not by culture, include Nocardia, Mycobacterium tuberculosis, Pneumocystis jirovecii, Haemophilus parainfluenzae, Streptococcus_pneumoniae, Pyramidobacter piscolens and Prevotella. The culture confirmed the bacterial pathogenic microorganisms in 2 out of 3 cases of P. aeruginosa pneumonia, 2 out of 2 cases of Klebsiella pneumoniae pneumonia, 1 case of E. coli pneumonia, and 1 case of Corynebacterium pneumonia. In one case, sputum smear showed positive acid-fast bacilli, mNGS detected Mycobacterium tuberculosis, and culture of BALF was negative. The sensitivity and specificity of microbial culture for diagnosis of pulmonary infection were 16.3% and 60.0%, respectively. The sensitivity of microbial culture for diagnosis of bacterial infections and fungal infections were 42.9% and 4.8%, respectively (Table 1). The positivity rates of mNGS and culture tests for bacterial infections, fungal infections, general infectious groups are illustrated in Figure 1. As expected, regardless of bacterial infections, fungal infections, or general infections, mGNS is more sensitive than conventional cultures.

Performance of mNGS from three types of samples for the identification of pathogens.

(Table 1 and Figure 2) For bacterial infections, the difference in sensitivity of mNGS between TBLB, BALF,

BB, and the combined mNGS of the three types of specimens was not statistically significant. For fungal infections and general infections, the three (mNGS of TBLB, mNGS of BALF and mNGS of BB) were compared in pairs, the difference in their sensitivity was not statistically significant. While the sensitivity of the combined mNGS was significantly higher than that of the mNGS of TBLB. mNGS of TBLB had the highest specificity, followed by mNGS of BB, while mNGS of BALF had the lowest specificity. However, this study did not make a statistical comparison of specificity differences because there were fewer non-infected cases, and the results of statistical analysis of specificity would be less reliable. If both sensitivity and specificity are taken into consideration, mNGS of BB has advantages over mNGS of TBLB and mNGS of BALF.

The performance of mNGS and standard procedures for detecting Aspergillosis

8 of 21 fungal pneumonia are Aspergillus pneumonia. Of the 8 cases, 7 cases were positive for mNGS, 5 cases were positive for Galactomannan antigen detection (GM test). 2 cases were positive for pathology, and only 1 case was positive for routine culture. One case was positive for ROSE, typical septate hyphae with sharp-angled bifurcation could be found with ROSE (Fig. 3A). Although the sensitivity of microbial culture was very low, the case missed by mNGS happened to be detected by microbial culture, and GM test also was positive (Table 2).

The performance of mNGS and standard procedures for detecting Cryptococcus

mNGS seemed to be inferior to serum antigen testing for detecting Cryptococcus. Serum cryptococcal capsular polysaccharide antigen was positive for all of patients with Cyptococcal pneumonia, while one case was missed and unidentifiable by mNGS. ROSE showed granulomatous inflammation in all cases. Cryptococcus was detected by ROSE in three of these four patients (Fig. 3B ˜ D, Table 3).

Characteristics of pathogens and common oropharyngeal microbiota in different types of specimens.

Figure 4 and Table 4 show the comparison of the relative abundance of pathogenic microorganisms (which refers to the ratio of the number of reads of pathogenic microorganisms detected to the number of reads of the same type of microorganism detected throughout the samples.) at the species level with mNGS obtained from TBLB, BALF and BB samples. The statistical analysis showed that the relative abundance of pathogenic bacteria (Fig. 4A), pathogenic fungi (Fig. 4B) and pathogenic viruses (Fig. 4C) was not statistically different among the three specimens.

We compared six kinds of common oropharyngeal microbiota (Prevotella, Neisseria, Streptococcus, Veillonella, Fusobacterium, and Rothia) richness (which refers to total number of different genera out of the six genera identified in each sample) and the relative abundance at the genus level against the three types of samples (TBLB, BALF and BB samples). Surprisingly, we found no statistically significant differences between TBLB and BB specimens with respect to abundanceof all of these six kinds of common oropharyngeal microbiota (Table 5 and Fig.5). The relative abundance of Prevotella in BALF specimens was higher than that of TBLB specimens and the relative abundance of Veillonella in BALF specimens was higher than that of BB specimens (Table 5 and Fig5). The total number of different genera in TBLB, BALF and BB specimens was 2.90 [2.39 ˜ 3.41], 4.28 [3.79 ˜ 4.78] and 4.13 [3.56 ˜ 4.70], respectively. Community richness in the TBLB specimens significantly decreased than that of BALF specimens and BB specimens. (Fig. 6).

4. Discussions

Currently, culture-based techniques that routinely employed to isolate pulmonary pathogens often using selective culture media designed for specific groups of microorganisms. The culture conditions are biased towards known, previously encountered microorganisms, and some novel and rare microorganisms might be missed. So the detection rate of microorganisms in conventional culture is low. Miao et al.'s study reported the sensitivity of culture for diagnosing infectious disease was 35.2% [22]. Jain et. al reported that no bacterial pathogen was ever isolated by culture in up to 75% of pneumonia cases [4], which was similar to the culture-negative rate seen in another study [3]. The present study found that the culture-positive rate

for the diagnosis of lung infections was even lower, as low as 16.3%. The possible reasons for the lower culture-positive rate are as follows, first of all, all of the patients in the present study received empiric broad spectrum antibiotics prior to sample collection. Detection of microorganisms from routine culture is limited due to the early administration of broad-spectrum or prophylactic antimicrobial drugs that could have been sufficient to affect culture results but not to eradicate infection [23, 27]. Secondly, 74% patients were immune-impaired in the present study, microorganisms infecting the immune-impaired host can be fastidious to grow or non-cultivable [3, 28, 29].

The mNGS allows for unbiased detection of virtually any pathogen present in a given sample through direct sequencing of the sample's extracted DNA [30, 31]. We conducted a literature review and found that there are currently several studies on the use of mNGS for the diagnosis of pulmonary infections. Although mNGS had different sensitivities for detecting pathogens in these studies, and the gold standards used to calculate sensitivity vary, some were based traditional methods [19 ˜ 21] and some were based on the final diagnosis [22, 23] as the gold standard. However, a similar conclusion is reached that mNGS is more sensitive and more advantageous than traditional methods in identifying pathogenic microorganisms. Li et al. applied mNGS to detect the microbial pathogens in CT-guided puncture lung biopsy tissues, they reported that the sensitivity and specificity were 100.0% and 76.5% for bacteria, 57.1% and 61.5% for fungi when compared to culture [19]. Langelier et al.'s study applied mNGS of BALF to detect microbial pathogens in hematopoietic cell transplant patients with acute respiratory illnesses, which reported the sensitivity of mNGS for detecting respiratory microbes (human metapneumovirus, respiratory syncytial virus, Stenotrophomonas maltophilia, human herpesvirus 6 and cytomegalovirus) was 100% when compared to standard testing [20]. Zhang et al.'s study reported 13 cases of Pneumocystis pneumonia (PCP) identified through mNGS of BALF or sputum or blood. Pneumocystis jirovecii was detected by mNGS in all samples and by conventional methods in 5 out of 13 samples, respectively. They concluded that mNGS showed satisfying Pneumocystis pneumonia detection rate compared to conventional methods [21]. Pan et al.'s study explored the application of mNGS of BALF in the microbiologies diagnosis of community acquired pneumonia in immune-impaired patients. They reported standard procedures identified pathogens in 6 out of 13 patients, while mNGS detected pathogens in 12 out of 13 patients [32]. Miao et al.'s study reported the sensitivity of mNGS for diagnosing infectious disease was 50.7%. However, in their study, the study was conducted on patients with various types of infectious diseases, not just lung infections and specimens were not limited to respiratory specimens [22]. In the present study, the gold standard used to calculate sensitivity and specificity was the final diagnosis. We found that the sensitivity of mNGS of BALF to detect pathogenic microorganisms was significantly higher than that of traditional culture of BALF, regardless of bacterial pneumonia (85.7% versus 42.9%), fungal pneumonia (71.4 % versus 4.8%), or generalized pneumonia (81.4% versus 16.3%).

It is worth noting that this study demonstrates that the advantages of mNGS in the field of fungus testing are more prominent. Culture indentified pathogens in only one fungal pneumonia patients (1/21), while mNGS detected pathogens in 19 fungal pneumonia patients (19/21). The two patients missed by mNGS were a patient with cryptococcal pneumonia and a patient with Aspergillus pneumonia. The culture and GM test of the patients with Aspergillus pneumonia missed by mNGS happen to be positive. Although the culture-positive rate is low, it is very necessary to combine culture, GM test and mNGS for the diagnosis of fungal pneumonia, so as to avoid missed diagnosis to the greatest extent. It should be emphasized that mNGS is not omnipotent. For cryptococcal pneumonia, mNGS does not have an advantage. As shown in this study, the capsular polysaccharide antigen was positive in all four cases of cryptococcal pneumonia, but one case was missed by mNGS. ROSE also played an important role in the diagnosis of cryptococcal pneumonia. We saw granulomas in TBLB specimens of all 4 patients by ROSE for the first time, and Cryptococcus was found in TBLB specimens in 3 of them by ROSE. Therefore, our recommendation for the diagnosis of cryptococcal pneumonia is that further detection of capsular polysaccharide antigen is of great significance, while mNGS is not necessary, if cryptococcal pneumonia is highly suspected based on the patient's exposure history, clinical manifestations, imaging findings and ROSE results.

In terms of detecting bacteria, mNGS still has advantages over culture, although this advantage is not as prominent in fungal detection. Pseudomonas aeruginosa, Klebsiella pneumoniae and Escherichia coli

are easily detected by culture. However, some bacterial pathogens detected by mNGS, including Nocardia, Mycobacterium tuberculosis, Pneumocystis jirovecii, Haemophilus parainfluenzae, Pyramidobacter piscolens and Prevotella, were not detected by culture. These bacteria are either fastidious microbes (such as anaerobe) or require long incubation times (such as Mycobacterium tuberculosis). In P4, two obligate anaerobic bacteria (Pyramidobacter piscolens and Prevotella) were identified, in P25, Pyramidobacter piscolens were identified. Pyramidobacter piscolens and Prevotella were usually isolated from the oral cavity of patients with dental pulp disease, periodontal infection or alveolar abscess and healthy people. They may be a potential pathogen of pulp disease and periodontal disease [33, 34]. Study also reported that Prevotella induces severe bacteremic pneumococcal pneumonia in mice [35].

In the present study, 74% patients were immune-impaired and 23% of patients with pulmonary infection were confirmed to be mixed infections by mNGS. Conventional culture is powerless in identifying mixed infections, while mNGS exhibited its remarkable advantages in detecting pathogens of mixed pulmonary infections in immune-impaired patients. Therefore, mNGS might be more likely to benefit immune-impaired patients who are susceptible population of various pathogens.

Using mNGS, we further explored the characteristics of flora composition in the three respiratory specimens (including the relative abundance of pathogens and the relative abundance and richness of common oropharyngeal microbiota). Surprisingly, we found that TBLB and BB samples were similar in flora composition except for the richness of common oropharyngeal microbiota, while the relative abundance and richness of common oropharyngeal microbiota in BALF were higher than TBLB.

In our study, in comparison to BALB and BALF, BB gave a higher number of true positive, in comparison to BALF, BB gave much lesser number of false positive and false negative cases, showing its superiority in diagnosing infective PPLs. So regardless of the diagnostic efficacy, or the relative abundance of the pathogenic microorganisms and the contamination of common oropharyngeal microbiota, BB is no worse than TBLB. In the present study, BB yielded a better diagnostic performance most likely because it allowed cells and microorganisms to be collected from a larger area. This suggests that in future, high throughput sequencing of the BB samples might be an alternative choice for patients with infectious PPLs but couldn't tolerate more invasive TBLB procedures, such as patients with hematological diseases who cannot tolerate TBLB because of thrombocytopenia or poor platelet function.

The current study has several limitations: the first limitation is a small sample size, the number of patients who had mNGS in all three samples is limited due to the relatively expensive cost of mNGS sequencing. The second limitation is using a retrospective design. The third limitation is that this study did not make a statistical comparison of specificity differences. Because ROSE was performed before the mNGS specimens were taken, if the clinical information, CT results and ROSE performance were all indicative of infectious lesions, then the sample was further sampled for mNGS, so the number of negative cases was small. In this case, the calculated specificity is not reliable, so the specificity is not compared between groups.

Conclusions:

Although there are some limitations, this is the first study to evaluate the usefulness of combining VBN, ultrathin bronchoscope, ROSE and mNGS to diagnosis infective PPLs. To our knowledge, no other study to date has explored the difference in diagnostic efficacy, the relative abundance of the pathogenic microorganisms and the contamination of common oropharyngeal microbiota amongst the mNGS of BALB, mNGS of BALF and mNGS of BB. We concluded that mNGS from TBLB, BALF or BB is more sensitive than conventional cultures for bacterial infections, fungal infections and general infections, the difference in sensitivity of mNGS between TBLB, BALF, and BB was not statistically significant. mNGS of TBLB had the highest specificity, followed by mNGS of BB, while mNGS of BALF had the lowest specificity, although we did not prove whether the difference between the three is statistically significant. we found that TBLB and BB samples were similar in flora composition except for the richness of common oropharyngeal microbiota, while the relative abundance and richness of common oropharyngeal microbiota in BALF were higher than TBLB. We also found mNGS exhibited its remarkable advantages in detecting pathogens of mixed pulmonary

infections in immune-impaired patients. The advantages of mNGS in the field of fungus testing are more prominent than that in the field of bacteria testing. However, mNGS is not omnipotent, for some kinds of microbes, such as cryptococcal pneumonia, mNGS does not have an advantage.

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Tables

Table 1. Performance of mNGS and conventional microbial culture

Tests Patients	mNGS	Serum cryptococcal capsular polysaccharide antigen	ROSE showed granuloma and multinucleated giant cells	Cryptococcus was detected by ROSE
Patient 1	(-)	(+)	(+)	(+)
Patient 7	(+)	(+)	(+)	(-)
Patient 20	(+)	(+)	(+)	(+)
Patient 24	(+)	(+)	(+)	(+)

BALF: bronchoalveolar lavage fluid; mNGS: metagenomic next-generation sequencing; TBLB: transbronchial lung biopsy; BB: bronchial needle brushing; a: P¿0.05, compared to a combination of mNGS from TBLB+BALF+BB; b: P ¡0.05, compared to microbial culture with BALF; c: P ¡0.05, compared to a combination of mNGS from TBLB+BALF+BB.

Table 2. Performance of various methods for diagnosis of Aspergillosis

Tests Patients	mNGS	GM test ([?] 0. 65, positive)	Pathological examination	Culture	ROSE
Patient 3	(+)	(-)	(-)	(-)	granulomatous inflammation

Tests Patients	mNGS	GM test ([?] 0. 65, positive)	Pathological examination	Culture	ROSE
Patient 9	(+)	(+)	(-)	(-)	hyphae of Aspergillus were detected, visible necrosis
Patient 11	(+)	(+)	(+)	(-)	granulomatous inflammation and suppurative inflammation, visible necrosis
Patient 18	(+)	(+)	(-)	(-)	suppurative inflammation and visible necrosis
Patient 22	(-)	(+)	(-)	(+)	granulomatous inflammation and suppurative inflammation, visible necrosis
Patient 26	(+)	(-)	(+)	(-)	visible necrosis
Patient 28	(+)	(-)	(-)	(-)	inflammation
Patient 32	(+)	(+)	(-)	(-)	inflammation

mNGS: metagenomic next-generation sequencing; GM test: Galactomannan antigen detection; ROSE: rapid on-site cytological evaluation; (+): positive; (-): negative.

Table 3. Performance of mNGS and standard procedures for detecting Cryptococcus

Tests Patients	mNGS	Serum cryptococcal capsular polysaccharide antigen	ROSE showed granuloma and multinucleated giant cells	Cryptococcus was detected by ROSE
Patient 1	(-)	(+)	(+)	(+)
Patient 7	(+)	(+)	(+)	(-)
Patient 20	(+)	(+)	(+)	(+)
Patient 24	(+)	(+)	(+)	(+)

mNGS: metagenomic next-generation sequencing; ROSE: on-site cytological evaluation; (+): positive; (-): negative

Table 4 Relative abundance of pathogenic microorganisms

Sample types Bacterias	Relative abundance of bacterias, Mean (95% confidence interval) (%)	Relative abundance of bacterias, Mean (95% confidence interval) (%)	Relative abundance of bacterias, Mean (95% confidence interval) (%)
	TBLB	BALF	BB

Sample types Bacterias	Relative abundance of bacterias, Mean (95% confidence interval) (%)	Relative abundance of bacterias, Mean (95% confidence interval) (%)	Relative abundance of bacterias, Mean (95% confidence interval) (%)
Prevotella	2.48 [0.52 ˜ 4.43]	7.82 [3.71 ˜ 11.92] [a]	4.89 [1.40 ˜ 8.38]
Neisseria	2.17 [0.44 ˜ 3.90]	5.59 [1.70 ˜9.49]	2.98 [0.74 ˜ 5.23]
Streptococcus	5.45 [0.66 ˜ 10.24]	10.58 [4.96 ˜ 16.19]	7.58 [1.98 ˜ 13.17]
Veillonella	1.11 [0.28 ˜ 1.95]	2.91 [0.94 ˜ 4.89] [b]	1.07 [0.29 ˜ 1.84]
Fusobacterium	0.11 [0.01 ˜ 0.23]	0.18 [0.47 ˜ 0.31]	0.21 [0.79 ˜ 0.34]
Rothia	0.55 [0.08 ˜ 1.01]	1.99 [0.90 ˜ 3.09]	1.23 [0.19 ˜ 2.64]

BALF: bronchoalveolar lavage fluid; mNGS: metagenomic next-generation sequencing; TBLB: transbronchial lung biopsy; BB: bronchial needle brushing.

Table 5 Relative abundance of six kinds of common oropharyngeal microbiota

Sample types Bacterias	Relative abundance of bacterias, Mean (95% confidence interval) (%)	Relative abundance of bacterias, Mean (95% confidence interval) (%)	Relative abundance of bacterias, Mean (95% confidence interval) (%)
	TBLB	BALF	BB
Prevotella	2.48 [0.52 ˜ 4.43]	7.82 [3.71 ˜ 11.92] [a]	4.89 [1.40 ˜ 8.38]
Neisseria	2.17 [0.44 ˜ 3.90]	5.59 [1.70 ˜9.49]	2.98 [0.74 ˜ 5.23]
Streptococcus	5.45 [0.66 ˜ 10.24]	10.58 [4.96 ˜ 16.19]	7.58 [1.98 ˜ 13.17]
Veillonella	1.11 [0.28 ˜ 1.95]	2.91 [0.94 ˜ 4.89] [b]	1.07 [0.29 ˜ 1.84]
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Rothia	0.55 [0.08 ˜ 1.01]	1.99 [0.90 ˜ 3.09]	1.23 [0.19 ˜ 2.64]

TBLB: transbronchial lung biopsy; BALF: bronchoalveolar lavage fluid; BB: bronchial needle brushing. a: P ¡0.05, compared with TBLB; b:P ¡0.05, compared with BB

Figure legends

Fig. 1 Positivity rate comparison between metagenomic next-generation sequencing (mNGS) and microbial culture for bacterial infections (n = 14), fungal infections (n = 21), general infectious (n = 43). The number of positive samples (y-axis) for mNGS is plotted against the mNGS and microbial culture for bacterial infections (n = 14), fungal infections (n = 21), general infectious groups (x-axis).

Fig.2 Positivity rate comparison between metagenomic next-generation sequencing (mNGS) of transbronchial lung biopsy (TBLB), bronchoalveolar lavage fluid (BALF) and bronchial needle brushing (BB) samples, and mNGS of TBLB + BALF + BB for bacterial infections (n = 14), fungal infections (n = 21), general infectious (n = 43). The number of positive samples (y-axis) for mNGS is plotted against the TBLB, BALF, BB and TBLB + BALF + BB groups (x-axis).

Fig. 3 A: typical septate hyphae with sharp-angled bifurcation could be found with rapid on-site cytological evaluation (ROSE), the red arrow refers to sharp-angled bifurcation, and the green arrow refers to the septate of the hyphae. (Diff-Quik [DQ] stain, 1000 × magnification); B ˜ D: Cryptococcus was detected by ROSE in three patients, the blue arrow refers to Cryptococcus (DQ stain, 1000 × magnification).

Fig. 4 Comparison of relative abundance of bacterial pathogens, fungal pathogens and viral pathogens in patients with bacterial (A), fungal (B) and viral (C) infections, respectively against the three types of specimens (transbronchial lung biopsy (TBLB), bronchoalveolar lavage fluid (BALF) and bronchial needle brushing (BB) specimens).

Fig. 5 Comparison of relative abundance of Prevotella (A), Neisseria (B), Veillonella (C), Streptococcus (D), Fusobacterium (e) and Rothia (F) in all of the researched objects against the three types of specimens (transbronchial lung biopsy (TBLB), bronchoalveolar lavage fluid (BALF) and bronchial needle brushing (BB) specimens).

Fig. 6 common oropharyngeal microbiota richness in TBLB, BALF and BB specimens.