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Metagenomic next-generation sequencing diagnosis of peripheral pulmonary infectious lesions through virtual navigation, radial EBUS, ultrathin bronchoscopy, and ROSE

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Abstract

Objective: To evaluate the efficacy of combined rapid on-site evaluation of cytology (ROSE), ultrathin bronchoscopy, virtual bronchoscopic navigation, radial endobronchial ultrasound (EBUS), and metagenomic next-generation sequencing (mNGS) for diagnosis of peripheral pulmonary infectious lesions.

Methods: Specimens from patients with peripheral lung infection were obtained by transbronchial lung biopsy (TBLB) and bronchoalveolar lavage (BAL), and mNGS was used to detect pathogenic microorganisms. The sensitivity and specificity of mNGS were compared between TBLB tissue and BAL fluid.

Results: The most common pathogens of pulmonary infectious lesions in this study were *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. The specificity of mNGS was higher in TBLB tissue than in BAL fluid, but mNGS of BAL fluid had higher sensitivity.

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Conclusions: The combination of ROSE, ultrathin bronchoscopy, virtual bronchoscopic navigation, radial EBUS, and mNGS technology yielded high efficacy for the diagnosis of peripheral pulmonary infectious lesions. TBLB and BAL specimens have respective advantages in specificity and sensitivity for mNGS analysis.

Keywords
mNGS, ROSE, transbronchial lung biopsy, bronchoalveolar lavage, ultrathin bronchoscopy, virtual bronchoscopic navigation, radial EBUS, cytodiagnosis

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Introduction

Metagenomic next-generation sequencing (mNGS) has been widely used in fundamental research, such as genomics, genetics, and biomedicine.[1] mNGS allows simultaneous and independent sequencing of thousands to billions of DNA fragments, has been recently used for pathogen detection.[2] Using proper reference databases, mNGS can detect pathogenic microbes and marker genes, such as antibiotic resistance genes, potentially increasing the precision of infectious disease diagnosis.[3] The application of mNGS in diagnosis of respiratory infection has been reported in several studies, but data are limited in terms of the sensitivity and specificity of mNGS for diagnosis of peripheral pulmonary infectious lesions.[4] This study investigated the efficacy of combined rapid on-site evaluation of cytology (ROSE), ultrathin bronchoscopy, virtual bronchoscopic navigation (DirectPath), radial endobronchial ultrasound (EBUS), and mNGS in diagnosis of peripheral pulmonary infectious lesions.

Methods

Patients

Patients with peripheral pulmonary infectious lesions, who were treated at the respiratory endoscopy center of Tianjin Medical University General Hospital from July 2018 to January 2019, were enrolled in this study. These patients were not responsive to treatment with a variety of antibiotics. All patients were fully informed of the tests and risks, and provided written informed consent to undergo biopsy surgery. This study was approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China). Echocardiography, routine blood analysis, C-reactive protein analysis, blood coagulation test, blood biochemistry analysis, hepatitis B detection, human immunodeficiency virus detection, syphilis detection, procalcitonin quantitation analysis, serum 1,3-β-D-glucan test, galactomannan test, sputum smear microscopy, sputum acid-fast staining, and sputum culturing analysis were performed before biopsy surgery.

Specimen collection

Bronchoscopy (routine bronchoscopy [BF-F260, 5.5-mm outer diameter of the tip (Olympus, Tokyo, Japan)] and ultrathin bronchoscopy [BF-P-260F, 4-mm outer diameter of the tip (Olympus)]), including transbronchial lung biopsy (TBLB) and bronchoalveolar lavage (BAL), was performed to collect specimens. For patients

with peripheral lesions from which it was difficult to obtain specimens, ultrathin bronchoscopy plus radial EBUS was performed at or near the lesion with virtual bronchial imaging and virtual navigation. The operating procedure was accurate and minimally invasive, and no patients were sedated. A total of 6 to 10 lung tissue specimens, 4 to 6 g each, and alveolar lavage fluid, 60 to 80 mL each, were obtained during combined ROSE. Small quantities of lung tissue specimens were used to make round smears of approximately 1 cm in diameter on a sterile slide for sterile cytology analysis. The slides were then immediately placed in Diff Quik AB staining liquor (Zhuhai Besso Biological Company, Guangdong, China) and rapidly stained in solution A (30 seconds) and solution B (40 seconds). Lung tissue specimens were sent for pathology analysis. The bronchopulmonary segment was rinsed four times with 60 to 80 mL of sterile saline at 37°C. A column of 5 to 15 mL BAL fluid was collected for DNA extraction and stored at −80°C.

mNGS and analysis

TBLB tissue samples from patients were collected and cut into small pieces in accordance with standard clinical procedures in our hospital. A 1.5-mL microcentrifuge tube with 0.7 mL lysis buffer, tissue sample pieces, and 1 g of 0.5-mm glass bead were attached to a horizontal platform on a vortex mixer and agitated vigorously at 2800 to $3200 \times g$ for 30 minutes. A 0.3-mL volume of sample was separated into a new 1.5-mL microcentrifuge tube and DNA was extracted using the TIANamp Micro DNA Kit (DP316, Tiangen Biotech, Beijing, China), in accordance with the manufacturer's recommendation. A 0.5-mL volume of BAL fluid was collected in a 1.5-mL microcentrifuge tube with 1 g of 0.5-mm glass beads. The sample was mixed and DNA was extracted in the manner described above for TBLB tissue samples. DNA libraries were constructed through DNA fragmentation, end-repair, adapter-ligation, and polymerase chain reaction amplification. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to perform quality control of the DNA libraries. Quality qualified libraries were sequenced by using the BGISEQ-50 platform.[5] High-quality sequencing data were generated by removing low-quality and short reads (length <35 bp), followed by computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows-Wheeler Alignment.[6] The remaining data by removal of low-complexity reads were classified by simultaneous alignment with four Microbial Genome Databases, consisting of viruses, bacteria, fungi, and parasites. The classification reference databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). At the time of this analysis, RefSeq contained 4,061 whole genome sequences of viral taxa, 2,473 bacterial genomes or scaffolds, 199 genomes or scaffolds of fungi related to human infection, and 135 genomes or scaffolds of parasites associated with human diseases. Based on the results of controls and calibrators, data-analysis algorithms were used to exclude microorganisms that were not significantly related to infection. Microorganisms with clinical significance were reported with sequencing reads of the microorganisms detected at genus/species levels.

Statistical analysis

Data were statistically analyzed by SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). The t-test and χ^2 test were used for data analysis. P values < 0.05 were considered to be statistically significant. Two-by-two

contingency tables were derived to determine sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). All statistics are reported as absolute values with 95% confidence intervals (95% CIs). Comparative analyses were conducted by using the McNemar test.

Results

Patients

A total of 69 patients were enrolled in this study, namely 39 men and 30 women. The median age of the patients was 48.8 years (IQR: 19 to 59 years).

Sputum culturing

A total of 63 pathogenic bacteria were detected by culturing methods from 88 sputum specimens, with a detection rate of 71.59%. Among these isolates, 35 were Gram-negative bacteria, comprising 7 *Acinetobacter baumannii*, 10 *Klebsiella pneumoniae,* 12 *Pseudomonas aeruginosa*, 4 *Escherichia coli*, 1 *Acinetobacter mobilis*, and 1 *Serratia marcescens*. A total of 20 isolates were Gram-positive bacteria, comprising 12 *Streptococcus viridans*, 5 *Staphylococcus aureus*, and 3 *Enterococcus faecium*. Pathogenic fungi were detected in the remaining eight samples, comprising four *Candida albicans* and four other fungi.

Lung biopsy print cytology

Sixteen patients exhibited purulent infection, and 24 patients exhibited general inflammatory changes. Seven patients exhibited foamy macrophage aggregation, distal airway epithelial cell hyperplasia, degeneration, necrosis, and denaturation, which were suggestive of viral infection. Three patients exhibited monocytes as the main infiltrating inflammatory cell type, which suggested infection with mycoplasmas and *Chlamydia spp*. Eight patients exhibited granulomatous inflammation. Eleven patients exhibited foam-like macrophages in clusters, which is a typical feature of Mason's bodies; these patients also showed alveolar septal thickening, which was consistent with this cellular organization.

Distribution and relative abundance of pathogenic microorganisms in TBLB tissue and BAL fluid from patients, determined by mNGS

In lung tissue from six patients, mNGS did not detect any pathogens. In these samples, the relative abundance of commensal prokaryotic microorganisms was 77.27%, and the remaining 22.73% of pathogens were viruses. The most common pathogens detected were *Pseudomonas spp*., *K. pneumoniae*, and *A. baumannii*. In BAL fluid specimens, the relative abundances of pathogenic microorganisms and viruses were 66.67% and 33.33%, respectively. No eukaryotic microorganisms were detected (Table 1). mNGS of TBLB tissues and BAL fluid detected oral microbes and bacteria, although these results were not statistically significant because a subset of the oral microbes were not regarded as pathogens.

Combined comprehensive diagnostic results of final clinical outcome, laboratory examination, pathological, ROSE, and mNGS

The combined diagnostic results of the 69 patients are shown in Table 2. Twelve patients (17.39%) exhibited *P. aeruginosa* pneumonia and twelve patients (17.39%) exhibited *K. pneumoniae* pneumonia; nine patients (13.04%) exhibited *A. baumannii* pneumonia and nine patients (13.04%) exhibited secondary organizing pneumonia. Of the remaining 27 patients, 26 exhibited

Table 1. Distribution and composition ratio of pathogens detected by metagenomic next-generation sequencing of transbronchial lung biopsy tissue and bronchoalveolar lavage fluid.

Pathogen	Transbronchial lung biopsy (n = 69)		Bronchoalveolar lavage fluid (n = 69)	
	Number of strains	Composition ratio	Number of strains	Composition ratio
Bacteria	**50**	**77.76**	**44**	**73.33**
Enterococcus faecium	4	6.06	0	0.00
Staphylococcus aureus	2	3.03	2	3.33
Pseudomonas aeruginosa	12	18.17	10	16.67
Acinetobacter baumannii	9	13.64	10	16.67
Klebsiella pneumoniae	12	18.17	11	18.33
Haemophilus influenzae	3	4.55	3	5.00
Pasteurella bettyae	1	1.52	1	1.67
Mycobacterium tuberculosis	3	4.55	3	5.00
Mycoplasma pneumonia	3	4.55	3	5.00
Nocardia cyriacigeorgica	1	1.52	1	1.67
Virus	**16**	**24.24**	**16**	**26.67**
Human betaherpesvirus 5	3	4.54	3	5.00
Human adenovirus 7	6	9.09	6	10.00
Human adenovirus B1	6	9.09	6	10.00
Human mastadenovirus B	1	1.52	1	1.67
Total	**66**	**100.00**	**60**	**100.00**

All values are number of strains (percentage).

either tuberculosis, pulmonary nocardiosis, or one of nine additional types of pneumonia; one patient (1.45%) could not be diagnosed by this combined diagnostic approach.

Sensitivity, specificity, PPV, and NPV of mNGS for pathogenic microorganisms in TBLB tissue and BAL fluid

The sensitivity and specificity of mNGS of TBLB tissue were 88.89% and 80.00%, while PPV and NPV were 94.12% and 66.67%. The sensitivity and specificity of mNGS of BAL fluid were 61.11% and 80.00%, while PPV and NPV were 91.67% and 76.36%. The sensitivity of pathogen detection was better in TBLB tissue than in BAL fluid ($P < 0.05$). The specificity of mNGS was better in BAL fluid than in TBLB tissue ($P < 0.05$). The results of mNGS of TBLB tissue were more accurate, while mNGS of BAL fluid detected more pathogenic microorganisms.

Discussion

Owing to the unclear identities of causative pathogens, targeted anti-infective treatment has been limited. Poor infection control methods and delayed treatment have resulted in undesirable patient outcomes. Traditional molecular analysis for pathogen detection have mainly relied on polymerase chain reaction with a limited number of targets; therefore, new or rare pathogens have not been detected. mNGS technology has become available in clinical practice for diagnosis of infectious disease.[7,8] Studies have increasingly demonstrated the superiority of mNGS in the diagnosis of unknown etiology, particularly regarding detection of

Table 2. Combined comprehensive diagnostic results of final clinical outcome, laboratory examination, pathological, ROSE, and mNGS.

Diagnostic results	Patients (n = 69)
Pseudomonas aeruginosa pneumonia	12 (17.39)
Klebsiella pneumoniae pneumonia	12 (17.39)
Acinetobacter baumannii pneumonia	9 (13.04)
Secondary organizing pneumonia	9 (13.04)
Adenoviral pneumonia	6 (8.70)
Enterococcus faecium pneumonia	4 (5.80)
Mycoplasma pneumoniae pneumonia	3 (4.35)
Tuberculosis	3 (4.35)
Haemophilus influenzae pneumonia	3 (4.35)
Staphylococcus aureus pneumonia	2 (2.90)
Pasteurella pneumonia	1 (1.45)
Pulmonary nocardiosis	1 (1.45)
Human mastadenovirus pneumonia	1 (1.45)
Acute fibrogenic organizing pneumonia	1 (1.45)
Acute interstitial pneumonia	1 (1.45)
Unable to diagnose	1 (1.45)

Numbers of patients are shown as n (%);
ROSE, rapid on-site evaluation of cytology; mNGS, metagenomic next-generation sequencing.

viruses and antibiotic-resistant bacteria. ROSE is a rapid cytological interpretation technique that can be used for tissue sample acquisition.[9] When tissue is taken from the targeted site, a portion of the specimen is smeared on a slide for cytological analysis. This analysis involves satisfactory evaluation of tissue, real-time guidance of interventional methods, formation of a preliminary diagnosis or narrowing of the differential diagnosis, and optimization of target site specimens for further treatment, as well as the clinical information and cytological background needed for disease analysis and outcome interpretation.[10]

Traditional histopathology methods, owing to the small amount of target tissue available, are limited in their usefulness for the diagnosis of pulmonary infectious diseases. Asano showed that the diagnostic accuracy of virtual endoscopy combined with ultrathin bronchoscopy for peripheral lesions with a diameter $\leq$30 mm was 67.1%.[11] Radial EBUS involves safe and stable procedures, and has been widely used in peripheral pulmonary infectious lesions. Metagenomic sequencing is performed on pathogens in patient samples by using a second-generation sequencing "sample-sequence" protocol. mNGS can detect unknown pathogens and a variety of mixed infectious pathogens by one-stop precision sequencing; this simplifies the detection process, improving pathogen detection sensitivity, shortening detection time, and enabling in-depth identification and classification of pathogens and their abilities to resist current treatment methods. The analysis of drug genes and virulence factors has an incomparable advantage over traditional detection methods. mNGS is expected to change the understanding of clinical microbiology and is a convenient and efficient technique for studying the epidemiology of infectious diseases.[12] Thus far, virtual bronchoscopic navigation (DirectPath), radial EBUS, ultrathin bronchoscopy, and ROSE combined with mNGS technology can enable rapid and accurate diagnosis.

In this study, pulmonary infectious lesions were mainly caused by *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*, consistent with the distribution of pathogens in hospital-acquired pneumonia.[13] ROSE results for some patients showed foam-like macrophages aggregated into groups, as well as fibroblasts and some fibrocytes; no major pathogenic microorganisms were detected. The combination of ROSE with patient history, laboratory tests, and mNGS can allow identification of patients with secondary organizing pneumonia. ROSE combined with mNGS technology improved the accuracy of infection diagnosis, provided better information for

treatment, and both reduced the cost of treatment and improved patient outcomes through reduced use of antibiotics. mNGS has the potential for clinical application in identifying difficult and rare pathogens.

mNGS of TBLB tissue has higher specificity, whereas mNGS of BAL fluid has higher sensitivity. Notably, mNGS of BAL fluid detected a wider range of pathogenic microorganisms in patients and could narrow the spectrum of suspected pathogens. In contrast, mNGS of TBLB tissue provided precise diagnostic results. Specimens that are difficult to obtain should be diagnosed by BAL. Therefore, both TBLB and BAL have specific advantages in terms of the collection of samples for mNGS. Because microorganisms detected by mNGS might be commensal or pathogenic, comprehensive diagnosis should consider the location and clinical symptoms of the infection, as well as the type of infection and potential prognosis.

The results of this study demonstrated that combined ROSE, ultrathin bronchoscopy, virtual bronchoscopic navigation, radial EBUS, and mNGS could detect pathogenic microorganisms at early stages of peripheral lung infectious lesions, thereby contributing to early diagnosis, transmission control, and precise treatment.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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