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Original Article

Metagenomic next-generation sequencing for the detection of pathogenic microorganisms in patients with pulmonary infection

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Abstract: Objective: To explore the clinical value of metagenomic next-generation sequencing (mNGS) in diagnosing pulmonary infectious diseases. Methods: A retrospective analysis was performed on 82 patients with pulmonary infection who were admitted to the Eighth Affiliated Hospital of Guangxi Medical University & Guigang City People's Hospital from January 2020 to December 2021. The pathogens were detected by mNGS and conventional methods (culture and PCR). Then, the type and number of detected pathogens, as well as the specificity and sensitivity, were compared between the two methods. In addition, the positive rates of bacteria, fungi, tubercle bacillus, and mixed infection in bronchoalveolar lavage fluid, sputum, pleural effusion, and blood detected by mNGS, and the advantage in required test time were evaluated. Results: More types and numbers of pathogens were detected by mNGS with a higher sensitivity but a lower specificity, as compared to the conventional detection methods (all $P<0.05$). The positive rates and integrity rates of bacteria, fungi, and tubercle bacillus detected by mNGS were higher than those by conventional methods (all $P<0.05$). Moreover, there was no difference in the overall sensitivity of mNGS among different sample types, but the sensitivities of mNGS in bronchoalveolar lavage fluid and sputum samples were significantly higher than those of conventional methods (both $P<0.05$). The average test time for mNGS was shorter than that of conventional methods. Conclusion: mNGS can detect more types and numbers of pathogenic microorganisms, improve the detection sensitivity, and reduce the detection time in patients with pulmonary infection.

Keywords: Metagenomic next-generation sequencing (mNGS), pulmonary infection, alveolar wash, diagnostic efficiency, pathogen

Introduction

Infectious diseases are public health problems, and they have high morbidity and mortality worldwide. Pulmonary infection, as the most common infectious disease, is clinically characterized by acute onset, severe condition, rapid development, difficult treatment, and high mortality [1-4]. With the increase of the elderly population, environmental pollution and high incidence of basic diseases (hypertension, diabetes, stroke and cardiovascular disease), the current number of patients with pulmonary infection is increasing year by year, and the affected group is becoming younger [5-8].

Pathogen diagnosis is a key step in anti-infective treatment. How to quickly and accurately identify pathogens is of great significance in guiding clinical treatment. The current gold standard for diagnosing infectious diseases relies on blood culture, which, however, cannot meet clinical needs in a timely and effective manner due to the long cycle, susceptibility to contamination, and low sensitivity [9, 10]. Other diagnostic methods, such as pathogen morphological detection, immunology, or biochemical detection, have become first-line methods because of their relatively simple operation, low cost as well as high sensitivity and specificity [11, 12]. Nevertheless, the pathogen spectrum of the above detection methods is relatively narrow, and a reading accuracy by clinicians is highly required, so there are some missed diagnoses [13]. Next-generation sequencing (NGS), also known as second-gen-

eration sequencing, can perform parallel sequencing of millions to billions of DNA molecules at one time, so it is also called high-throughput sequencing. It can directly conduct high-throughput sequencing of nucleic acids in clinical samples and determine the type of pathogenic microorganisms in the samples by comparing with the database. In this way, NGS can accurately identify bacteria. NGS has preliminarily obtained favorable effect in clinical practice, but results of studies on its sensitivity and specificity in detecting pathogens of pulmonary infection are controversial, and there are few studies about the detection efficiency of different specimen types [14]. Therefore, this study applied both metagenomic next-generation sequencing (mNGS) and conventional methods to detect the pathogens of pulmonary infection, in order to obtain more research targets for improving the treatment of pulmonary infection.

Materials and methods

General data

This retrospective analysis included clinical data of 82 patients with pulmonary infection admitted to the Eighth Affiliated Hospital of Guangxi Medical University & Guigang City People's Hospital from January 2020 to December 2021. All patients received bronchoalveolar lavage fluid (BALF)-mNGS. This study was approved by the Ethics Committee of Guigang City People's Hospital.

Inclusion criteria: 1) Patients were diagnosed with pneumonia according to the *Guidelines for the diagnosis of pulmonary infections* by American Thoracic Association [15]. 2) Patients' chest CT scan showed new-onset pneumonia images including patchy changes, local lung tissue or interstitial consolidation, with or without pleural effusion. 3) Patients were over 18 years old. 4) Patients received BALF-mNGS during hospitalization. 5) Patients' samples met the BALF-mNGS requirements. 6) Patients also received detection by conventional methods (chest CT, blood cell analysis, CRP, procalcitonin and other inflammatory indicators).

Exclusion criteria: 1) Patients had incomplete clinical data. 2) Patients refused to receive BALF-mNGS.

Collection of baseline data

The general data of patients and the results from various detection methods were collected from the inpatient system of the Eighth Affiliated Hospital of Guangxi Medical University & Guigang City People's Hospital.

Sample collection and mNGS detection

The day before fiberoptic bronchoscopy, patients were asked to use a broad-spectrum antibacterial agent (Yikou gargle, containing chlorhexidine acetate: 0.58 g/L-0.70 g/L) to rinse the mouth, so as to inhibit the growth of oral bacteria and to prevent the bacteria flowing into the airway with saliva and infecting the specimens. The front end of Olympus P260 fiberoptic bronchoscope (Olympus, Beijing, China) was placed at the lesioned bronchial subsegment suggested by previous imaging. After accurate positioning, alveolar lavage was performed with normal saline (volume: 100 mL, temperature: 37°C). Then, the alveolar lavage fluid was collected, and about 30 mL was retained for detection. For conventional culture methods, blood agar (Art. No. FS-5663P, Shanghai Fusheng Industrial Co., Ltd., China), MacConkey agar (Art. No. CM0007B, Shanghai Ai Yan Biotechnology Co., Ltd., China), chocolate agar plate (Art. No. YP02115, Shanghai Yuanmu Biological Co., Ltd., China) and Sabouraud agar (Art. No. LA4590, Beijing Solebo Technology Co., Ltd., China) were used for culturing bacteria and fungi at 35°C. Sputum culture was performed with ordinary agar after liquefying clean sputum. The above cultures lasted for 5 days at most. Organisms were identified using an automated system (Vitek 2 Automated System, bioMerieux, Marcy-L 'Etoile, France). Bacteria were identified by colony morphology and Gram staining, while filamentous fungi were identified by smear results.

Evaluation of detection results

The samples for mNGS were sent to a gene company (Fuda Test Group, China) for second-generation sequencing of microorganism nucleic acids, and the results were evaluated and identified with the microbial sequences in the library. Other conventional methods to determine the pathogens were as follows. 1) For bacteria, fungi and other microorganisms, culture was seen as the gold standard. 2) Virus

Table 1. Baseline data of the included patients

Category	Statistics
Sex (n)	
Male	46
Female	36
Age (years old, $\bar{x}$ ± sd)	18-72 (62.2±4.9)
Underlying disease (n, %)	
COPD	12 (14.63%)
Bronchiectasis	6 (7.32%)
Asthma	4 (4.88%)
Diabetes	11 (13.41%)
Hypertension	14 (17.07%)
Coronary heart disease	5 (6.10%)
Neoplastic disease	3 (3.66%)
Course of disease (day, $\bar{x}$ ± sd)	8.87±2.48
Hospitalization time (day, $\bar{x}$ ± sd)	14.5±3.6
Mortality	10/82 (12.19%)

Note: COPD: Chronic obstructive pulmonary disease.

was detected by a PCR test. Total RNA was extracted (TRIzol Reagent, Beijing Tiangen Biochemical Technology, China), and the cDNA was obtained by reverse transcription (HiScript II One Step QRT-PCR SYBR Green, Nanjing Novizan Biotechnology, China) according to the instructions. The mRNA expression of common respiratory viruses was detected by qPCR, with β-actin as the internal reference gene. All primers were synthesized by Shanghai BioSune, China.

Evaluation methods

To evaluate the positive rate, sensitivity, and specificity of mNGS, 41 samples of suspected lung infection, which were later confirmed to be non-infected, were obtained as a reference to analyze the detection efficiency of mNGS. Based on the extracted data, a 2×2 contingency table was established to calculate the sensitivity and specificity. It was considered positive if the mNGS test result was consistent with the culture result. Positive rate = (number of positive cases)/total number of cases * 100%.

Statistical methods

All data were analyzed using statistical software SPSS 23.0. Measured data that conformed to the normal distribution were expressed as mean ± standard deviation ($\bar{x}$ ± sd), and the comparisons between two groups were conducted using independent samples t test. Counted data were expressed as case number or percentage (n/%), and the comparisons of rates between groups was performed using Chi-square test, test level α=0.05. A difference of $P<0.05$ was considered significant.

Results

General data of the research subjects

A total of 82 patients (46 male and 36 female) were included in this study. The patients were 18-72 years old, with an average age of (62.2± 4.9) years old. Their underlying diseases included chronic obstructive pulmonary disease, bronchiectasis, diabetes, hypertension, coronary heart disease, and neoplastic diseases, with a course of disease of 1-2 weeks (8.87± 2.48 days). Their average hospitalization time was 14.5±3.6 days, and the overall mortality during hospitalization was 12.19%. See **Table 1**.

Diagnostic efficacy of mNGS and conventional detection methods

In the specimens from the 82 cases, 78 cases were diagnosed positive, and 4 cases were negative by mNGS, while 48 cases were diagnosed positive, and 34 cases were negative by conventional methods. Combined with the diagnostic results in 41 reference samples, the 2×2 contingency table showed that the sensitivity of mNGS was higher, but the specificity was lower than those of the conventional methods (both $P<0.05$). See **Table 2**.

Diagnostic value of mNGS for different pathogens

Among the 82 cases of pulmonary infection, the pathogens were identified in 78 cases. The results of mNGS showed 22 cases of bacteria, 21 cases of fungi, 16 cases of tubercle bacillus and 16 cases of mixed infection, while the conventional methods showed 16 cases of bacteria, 15 cases of fungi, 8 cases of tubercle bacillus and 9 cases of mixed infection. The total number of pathogens detected by mNGS was higher than that by the conventional methods ($P<0.05$). See **Table 3**. It was found that mNGS showed higher rates of positive bacteria, fungi, tubercle bacillus and mixed infection than the

Table 2. Comparison of diagnostic efficacy of mNGS and conventional methods

Detection method	Infection cases (n=82)	Non-infection cases (n=41)	Sensitivity	Specificity
Conventional methods			48/82 (58.54%)	34/41 (82.93%)
Positive (+)	48	7		
Negative (-)	34	34		
mNGS			78/82 (95.12%)	29/41 (70.73%)
Positive (+)	78	12		
Negative (-)	4	29		

Note: mNGS: metagenomic next-generation sequencing.

Table 3. Comparison of number of pathogens detected by the two methods

Detection method	Total number of cases	Number of specimens with pathogens	Number of specimens without pathogens
mNGS	78	75	3
Conventional methods	78	48	30
X^2	-	25.981	
P	-	0.000	

Table 4. Comparison of detection rates of different pathogens

Detection method	Bacteria (n=23)	Fungus (n=21)	Tubercle bacillus (n=18)	Mixed infection (n=16)
BALF-mNGS	22	21	16	16
Conventional methods	11	15	8	9
X^2	10.273	4.861	6.125	6.583
P	0.001	0.021	0.013	0.010

Notes: χ^2: Chi-square test was used to compare the detection rates between mNGS and the conventional methods. BALF: bronchoalveolar lavage fluid; mNGS: metagenomic next-generation sequencing.

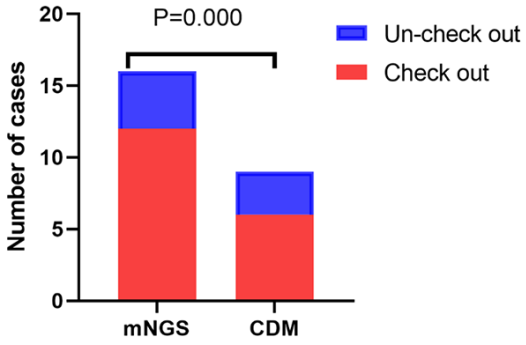

Figure 1. Comparison of pathogen integrity rates detected by different methods. Notes: CDM: Conventional detection methods; mNGS: metagenomic next-generation sequencing.

conventional methods (all P<0.05). See **Table 4**. Besides, among the 16 cases of mixed infection detected by mNGS, 12 cases were confirmed, while 3 cases were confirmed among the 9 cases of mixed infection detected by conventional methods, indicating that the pathogen integrity detected by mNGS was higher than that detected by conventional methods (75.00% vs. 33.33%, P<0.05). See **Figure 1**.

Positive rates of different microorganisms in different specimens detected by mNGS

In this study, mNGS showed no difference in the overall positive rates among different sample types, but the positive rates in BALF and sputum detected by mNGS were significantly higher than those detected by conventional detection methods (both P<0.001). See **Table 5**.

Comparison of required test time

The test time of all samples was 3-5 days by mNGS, with an average of (2.45±0.62) days, while that of the conventional methods was (3.85±0.95) days. The test time of mNGS was significantly shorter than that of the conventional methods. See **Figure 2**.

Table 5. Positive rate in different specimens detected by the two methods

Detection method	BALF (n=82)	Sputum (n=82)	Pleural effusion (n=82)	Blood (n=82)
mNGS	82	79	31	17
Conventional methods	45	56	21	9
χ^2			3.556	
P			0.615	

Notes: χ^2: Chi-square test was used to compare the positive rates of different samples detected by mNGS and the conventional methods. BALF: bronchoalveolar lavage fluid; mNGS: metagenomic next-generation sequencing.

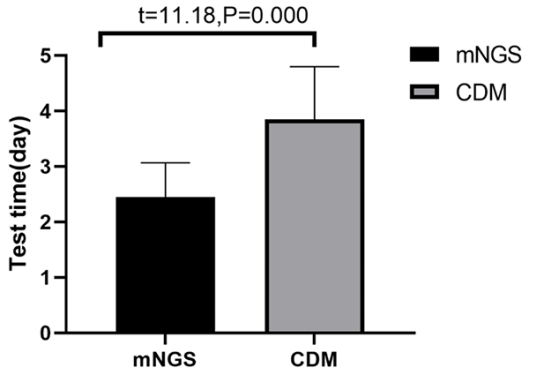

Figure 2. Comparison of test time between the two methods. Notes: CDM: Conventional detection methods; mNGS: metagenomic next-generation sequencing.

Discussion

Pulmonary infection is a common organ system infection that can occur at any age. A variety of pathogens can be the source of the infection, and the treatment principles for different infectious pathogens are also different [16, 17]. In addition, the latest epidemiological surveys have shown that there are changes in respiratory infections, such as changes in bacterial species, the emergence of new virus infections (e.g., COVID-19) and single pathogen infection to multiple mixed infections [18]. These changes bring challenges to clinical anti-infective treatment, and targeted treatment for pathogens is an important prerequisite for anti-infective treatment.

In recent years, mNGS has shown great advantages in identifying pathogens and has obtained promising clinical results. However, whether it can replace traditional etiological testing is still controversial [19-21]. A previous study showed that the detection sensitivity of mNGS was 94.2%, and this study showed a similar sensitivity (95.12%) in detecting the pathogens of pulmonary infection, which was higher than the sensitivity of conventional methods (58.54%). Meanwhile, the total number of pathogens detected by mNGS was also larger than that detected by conventional methods (96.15% vs. 61.54%), which indicates that mNGS has good application value in monitoring lung infection. The possible reason could be that mNGS can directly detect nucleic acid without culturing, which reduces potential contamination during specimen storage and transportation. Meanwhile, it has a high accuracy in detecting genetic material. Our results are supported by previous research conclusions [22]. However, the integrity rate of the pathogens from mNGS in this study was higher than 78.56% in another related study, which may be attributable to the mild symptoms of the infected patients in this group, the fact that most of the pathogens were common ones, and the sample size of this study [23]. Apart from that, this study showed that the specificity of mNGS was 70.73%, which is consistent with a specificity of 72.45% reported in previous study, but lower than the specificity of conventional methods (82.93%). The possiblemechanism is that mNGS detects a wide range of pathogens, and the database is large, which reduces its specificity. With the improved sensitivity, the specificity is reduced in mNGS, indicating that the clinical diagnostic value of mNGS is insufficient. Similar findings were reported by a previous study [24].

In this study, when comparing the diagnostic efficacy of mNGS for different pathogens, the results showed that the overall positive rates among different samples were not statistically significant, but the positive rates in BALF and sputum detected by mNGS were higher than those detected by conventional methods. A possible reason could be that BALF and sputum contain a lot of DNAs, and mNGS performance depends on the amount of DNA, thus the positive rate by mNGS was increased, which is similar to previous conclusions [25]. It is suggested that BALF and sputum samples

should be obtained for mNGS etiological diagnosis in clinical work.

We further analyzed the diagnostic efficiency of mNGS for different pathogens, and mNGS showed higher positive rates of bacteria, fungi, tubercle bacillus and mixed infection, higher pathogen integrity rate (75.00% vs. 33.33%) and shorter test time as compared to those of the conventional methods, which is similar to a previous report [26]. There are several possible reasons. First, mNGS does not require microbial isolation, so the loss of direct evidence of pathogens is reduced. Second, mNGS can analyze 99% of microorganisms in nature that have not been culture-proven yet in a short time, thereby improving the detection rate of various pathogens and the diagnostic efficiency in the clinic. Finally, the powerful analytical ability of mNGS is also superior to conventional methods in the integrity rate during the detection of mixed infection, thus, the clinical comprehensive anti-infection level can be improved [26].

This study has some limitations. 1) The cost of mNGS testing is expensive. 2) Whether there is an over-diagnosis in patients without combined underlying diseases or in single infection needs to be explored by further research. 3) This study is a retrospective study with a small number of cases, so there is a research bias. A larger-sample study is needed to improve the research conclusions.

In conclusion, mNGS can improve the specificity of detecting pathogens in patients with pulmonary infection, but the specificity is, meanwhile, reduced. The positive rates of different pathogens and different specimens are different, so, the results of mNGS should be judged according to the clinical manifestations and accurate analyses, to guide anti-infection treatment.

Disclosure of conflict of interest

None.

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