Chapter

Metagenomic Next-Generation Sequencing (mNGS) for the Diagnosis of Pulmonary Aspergillosis

Hao Tang, Shujun Bao and Caiming Zhong

Abstract

The diagnosis of pulmonary aspergillosis is a critical step in initiating prompt treatment and improving patients' prognosis. Currently, microbiological analysis of pulmonary aspergillosis involves fungal smear and culture, serum (1,3)-β-D-glucan (G) or galactomannan (GM) tests, and polymerase chain reaction (PCR). However, these methods have limitations. Recent studies have demonstrated that polymorphisms in pentraxin3 (PTX3), a soluble pattern recognition receptor, are associated with increased susceptibility to invasive aspergillosis. mNGS, a new microbial diagnostic method, has emerged as a promising alternative. It has high sensitivity in identifying pulmonary aspergillosis and can accurately distinguish species. Additionally, it outperforms other methods in detecting mixed infections and instructing the adjustment of antimicrobial treatments. As a result, mNGS has the potential to be adopted as the gold standard for the diagnosis of pulmonary aspergillosis.

Keywords: mNGS, fungi, pulmonary aspergillosis, diagnosis, other methods

1. Introduction

Aspergillus belongs to Ascomycetes fungi, including Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, and Aspergillus terreus, which are known to cause a wide range of diseases, such as allergic reactions, airway or lung infections, and extrapulmonary spread, especially skin infections. Lungs are the most common sites of Aspergillus infection in human. Meanwhile, pulmonary aspergillosis are often difficult to be diagnosed and have a high mortality rate. The clinical spectrum of pulmonary aspergillosis can present in various forms, including chronic pulmonary aspergillosis (CPA), aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), and invasive pulmonary aspergillosis (IPA). The histopathology of the lesion can be understood as a phenotype that reflects the interaction between decreased host defense mechanisms and increased fungal virulence. In the case of Aspergillus infection, tissue reactions to the fungus are diminished, but the reasons and degree of the decrease in defense mechanisms vary among cases. Pulmonary aspergillosis presents

Type	IPA	CPA	ABPA	Aspergilloma
Main patient population	Patients with immune deficiencies; individuals without obvious immune deficiencies can also occur.	Patients with mild immune suppression caused by pulmonary diseases.	Hypersensitive reaction to Aspergillus antigens, which is commonly observed in patients with long-term asthma or cystic fibrosis.	Patients with normal immune function but pre-existing lung cavities (such as tuberculous cavities, lung cysts, bronchiectasis, etc.).
Crucial diagnostic methods	Chest CT, direct microscopy, histopathology, culture, as well as serum and BALF GM test	Combination of chest CT and specific IgG antibody detection for Aspergillus	Specific IgE antibody detection for Aspergillus	Chest CT

Table 1.
The main patient population and crucial diagnostic methods of different types of pulmonary aspergillosis.

in various forms in the disease spectrum depending on the interaction between Aspergillus and the host, and there may be an overlap between different clinical phenotypes. Moreover, the key diagnostic methods for different types of pulmonary aspergillosis vary. The main patient population and crucial diagnostic methods of different types of pulmonary aspergillosis are shown in **Table 1**.

In recent years, several new rapid diagnostic methods for pulmonary aspergillosis have emerged, in addition to traditional pathological and cultural diagnostic methods. These include the detection of antigens, antibodies, novel molecular markers, and genes. Among them, the second-generation sequencing technology for genetic testing has gained attention since 2014 [1], which has high sensitivity and a short detection cycle [2]. It can detect various samples such as sputum, bronchoalveolar lavage fluid (BALF), blood, and cerebrospinal fluid, making it a promising microbial identification technology. Which means that mNGS can classify a wide range of pathogens in several infection sites, including the respiratory tract [3], blood [4], central nervous system [5], and focal sites [6]. It is especially useful in detecting special and rare pathogens, as well as analyzing drug-resistance genes and virulence factors related to pathogens. The popularization of mNGS has proven to be of great value in diagnosing pulmonary aspergillosis.

2. The diagnostic value of mNGS in pulmonary aspergillosis

2.1 Principles and methods of mNGS

In recent years, the clinical application of mNGS has become an important method for diagnosing pulmonary fungal infections, which is of significant clinical value. Unlike traditional methods, mNGS technology does not require the culture of clinical samples. Instead, it extracts nucleic acid from all microorganisms in samples, constructs a standard sequencing library, and conducts high-throughput sequencing. Finally, the bioinformatics analysis confirms genome sequence comparison of each species, allowing for identification of microorganisms and calculation of various

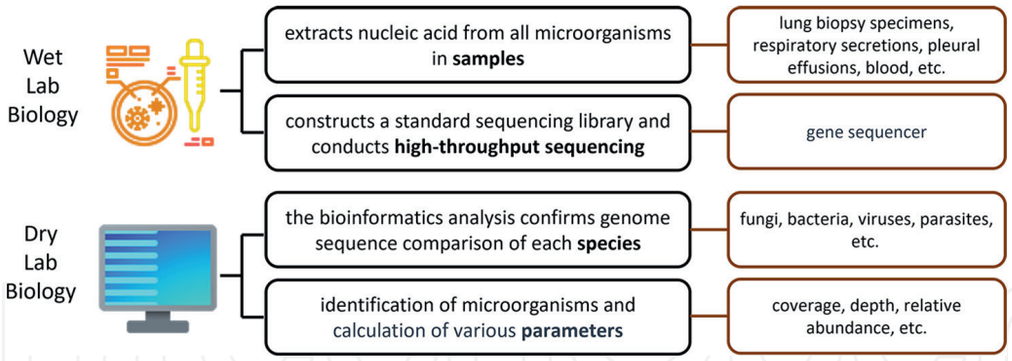

Figure 1.
Detection workflow of mNGS.

parameters. Please refer to **Figure 1**. mNGS has been utilized to identify the etiology of various clinical samples, significantly improving the diagnostic rate of pulmonary fungal infections. Several studies have demonstrated that mNGS is more sensitive than traditional tests, such as pathology, culture, serological tests, and PCR in fungal detection. Clinical studies have further confirmed the efficacy of mNGS in diagnosing common fungi and its ability to improve the sensitivity of pathogenic diagnosis of pneumonia fungi, making it an effective supplement to traditional microbiological detection methods for fungal infections.

2.2 Sample source for mNGS

Several studies have employed mNGS to detect microorganisms in lung biopsy specimens, respiratory secretions, and pleural effusions. For instance, mNGS was performed on formalin-fixed paraffin-embedded (FFPE) lung tissue samples from patients with granulomatous lesions and unclear diagnoses. Results showed that mNGS had a detection rate of 87.8% for total fungi and mycobacteria, which was 68.3% higher than histopathology. Moreover, pathogenic bacteria could be identified at the species level [7]. Other studies utilized mNGS to detect pathogenic microorganisms in computer tomography (CT)-guided lung biopsies of patients with lung diseases. Results showed that the specificity and positive predictive value of mNGS for detecting fungi were 100%, which was higher than histopathological methods [8].

Furthermore, the sensitivity of mNGS in detecting pathogenic bacteria and fungi in respiratory tract samples (such as nasopharyngeal swabs, sputum, and BALF) from patients with pulmonary infection was higher than traditional methods, such as respiratory virus culture and PCR analysis [9]. Other studies analyzed the diagnosis of pulmonary invasive fungal infections (IFIs) using mNGS and found that the fungal species detected by mNGS in BALF were higher than those detected by standard culture methods [10].

Some studies also tested patients with pulmonary infection combined with pleural effusion for mNGS, and the positive rate was higher than that of the culture method. Candida and Pneumospora were the most common fungal infections detected [11].

Bronchoscopic lung biopsy and BALF samples were also analyzed to investigate the differences in the detection of mNGS. Results showed that mNGS had a wider range of pathogen detection than traditional tests, especially for pulmonary fungal infection. The proportion of fungi detected and identified by mNGS was significantly higher than that by conventional tests [12]. Moreover, the sensitivity of mNGS in

diagnosing pulmonary fungal infection was significantly higher than conventional tests, such as pathology, GM test, and culture. However, there was no difference in sensitivity and specificity between lung biopsy-mNGS and BALF-mNGS [13]. In other studies, transbronchial lung biopsy (TBLB), BALF, and bronchoalveolar needle brush (BB) specimens were tested for mNGS to detect suspected pulmonary infectious diseases. Results showed that the sensitivity of mNGS in detecting fungi in the three specimens was higher than that of conventional culture, but there was no difference in the sensitivity of mNGS among different specimen types [14].

According to the above researches, we can conclude that compared to the sensitivity of mNGS in the lung biopsy specimens, there is no obvious difference in that in respiratory secretions. Therefore, BALF/BB can be alternative samples in the detection of mNGS.

2.3 Fungi that can be detected by mNGS

The incidence of pulmonary fungal infections in clinical settings is attributable to a diverse range of pathogens such as Aspergillus, Mucor, Pneumospora [15], Cryptococcus, among others. Conversely, Candida is a relatively infrequent cause of pulmonary infections.

Lung infections associated with other pathogenic fungi such as Histoplasma capsulatum, Talaromyces marneffei, and Exophiala dermatitidis are even less common. mNGS has shown significant value in identifying rare fungi. It has been reported that a 27-year-old Chinese male with chronic progressive lung disease was asymptomatic for over a year until the disease progressed to the epiglottis, causing progressive pharyngeal pain. Despite negative results from BALF and epiglottic tissue cultures, as well as epiglottic and pulmonary pathology, mNGS was able to detect Histoplasma capsulatum in both epiglottic organizations and BALF and determined the cause after itraconazole treatment was successful [16]. In another case, mNGS successfully identified Talaromyces marneffei infection in a non-human immunodeficiency virus (HIV) patient, which is the first reported case in North China [17]. In the third case, a 52-year-old man with cough, sputum, and hemoptysis showed multiple lesions on both sides according to chest CT. Although the pathogen could not be identified after three biopsies, the subsequent mNGS results and therapeutic response confirmed that the pathogenic pathogen was Exophiala dermatitidis, and the diagnosis was Exophiala dermatitidis pneumonia [18].

Additionally, pulmonary fungal infections were frequently combined with bacterial infections, and immune deficiency and the presence of pulmonary bulba were risk factors associated with fungal and bacterial co-infections [19]. Furthermore, mNGS has been found to be able to distinguish colonization and infection in certain fungi, the colonization group and infection group of Pneumocystis yersii were differentiated by BALF-mNGS, and the fungal load was significantly different between the two groups [20].

2.4 The clinical application of mNGS in pulmonary aspergillosis

There are limited clinical studies on the diagnosis of pulmonary aspergillosis using mNGS. Although the studies are mostly limited to case reports, and the number of cases included are small, the available studies have demonstrated the good diagnostic performance of mNGS.

mNGS plays a key role in the diagnosis of pulmonary aspergillosis, either alone or in combination with other diagnostic methods. See **Figure 2** for details.

Patients with aspergillus that can be detected by mNGS tend to have an incompetent immune function. One study reported that five patients' blood samples were positive for Aspergillus using mNGS, the five patients had various underlying diseases, including myelodysplastic syndrome (two cases), acute myeloid leukemia (two cases), and kidney transplantation (one case), which suggests that mNGS has guiding value in the diagnosis of IPA in immunocompromised patients [21]. In other studies, mNGS was used to detect plasma for the diagnosis of Corona Virus Disease 2019 (COVID-19)-associated pulmonary aspergillosis, suggesting that mNGS has good diagnostic performance for COVID-19-associated pulmonary aspergillosis when detecting plasma, and is highly specific [22]. Additionally, researchers detected mNGS in BALF samples and described three cases of IPA (respectively with chronic obstructive pulmonary diseases (COPD) and asthma history, and one case without underlying disease). Aspergillus fumigatus gene was found in all mNGS in BALF, indicating the diagnostic value of mNGS in non-neutropenic IPA patients [23]. Further, a study was conducted to analyze the accuracy of using mNGS to diagnose IPA in patients with different immune status. The study found that the consistency rate of IPA diagnosis in immunocompromised patients was significantly higher (82.1%) than in non-immunocompromised patients (52.9%). Therefore, mNGS detection combined with pulmonary CT imaging can be used for IPA diagnosis in patients with immunocompromised function. However, the diagnosis of IPA based on positive mNGS results in non-immunocompromised patients should be approached with caution [24].

Additionally, severe pneumonia caused by any type of pathogenic bacteria may be accompanied by Aspergillus infection. Therefore, some studies have used mNGS for early secondary infection screening, and secondary Aspergillus infection was found in a case of severe pneumonia caused by Legionella infection, leading to early precise treatment [25]. Another retrospective study found that mNGS performed well in the detection of common pathogens in Aspergillus infection, the most common bacteria were Klebsiella pneumoniae and Acinetobacter baumannii. Furthermore, 91.7% of pulmonary aspergillosis patients changed antibacterial therapy based on mNGS

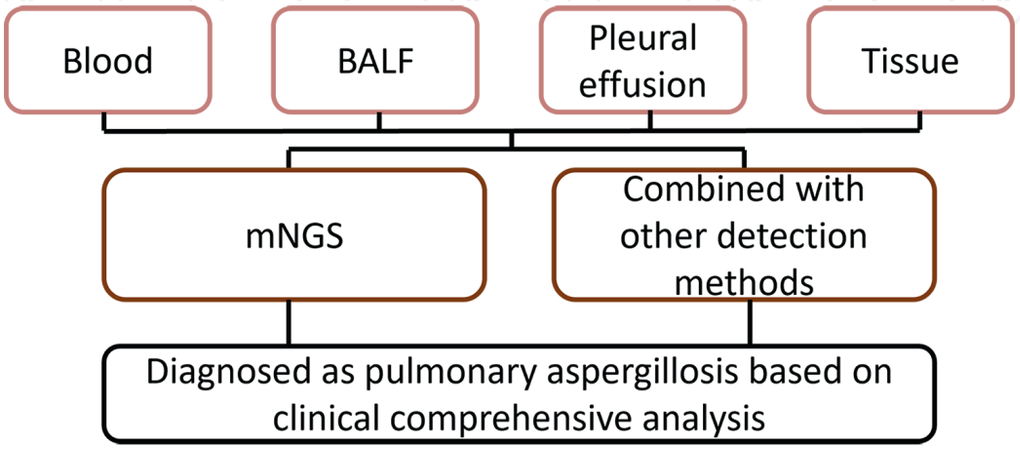

Figure 2.
The value of mNGS in the diagnosis of pulmonary aspergillosis.

Methods	Sensitivity (%)	Specificity (%)
Fungal smear	7.7	100
Culture	30.8	100
Serum(1,3)-β-D-glucan (G)	77.8–82.9	72.5–73.9
Galactomannan (GM)	57.7–77.8	90–92.9
PCR	64–86.7	84.2–99
mNGS	42.3–91.7	71.4–100

Table 2.
Comparing the sensitivity and specificity of different diagnostic methods of pulmonary aspergillosis.

results [26]. In summary, compared to other diagnostic methods, mNGS has higher sensitivity in the diagnosis of pulmonary aspergillosis. Please refer to **Table 2** for details.

mNGS can also be used as a supplement for routine microbial detection. A comparison of community-acquired pneumonia (CAP) patients diagnosed with IPA found that the sensitivity of GM to detect Aspergillus was the highest, followed by mNGS, culture, and smear. However, the specificity of mNGS, culture, and smear was 100%. The specificity of GM detection was 92.9% [27]. In patients with neutropenic, Aspergillus was detected by pathology and mNGS and diagnosed as IPA [28]. In patients with normal immune function, Aspergillus fumigatus was identified through endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) tissue with mNGS. Histological analysis of mediastinal biopsy and tissue fungal culture also indicated Aspergillus fumigatus infection, confirming the detection of mNGS [29]. In an infant patient initially suspected of having a lung tumor, mNGS identified Aspergillus fumigatus in BALF as the causative agent. The patient recovered quickly and was discharged after receiving appropriate antifungal therapy [30].

2.5 Precautions of mNGS report in the diagnosis of pulmonary aspergillosis

The interpretation of the mNGS report cannot disregard the value of a few or a dozen Aspergillus readings in the genus. This may occur when the thick cell wall of fungi makes nucleic acid extraction challenging. Additionally, after antifungal treatment, the number of fungi may decrease, resulting in low read lengths. In such cases, the sequenced samples may contain only free nucleic acid fragments that were lysed after the death of pathogenic bacteria. Moreover, the original pathogen load in the sample may have been low, leading to a low reading length. Consequently, some clinical studies fail to reflect the excellent diagnostic value of mNGS. In severe immune dysfunction patients suspected of pneumonia, the diagnostic accuracy of BALF's mNGS and conventional microbiological tests (CMTs) in detecting fungal infections was much lower. The low sensitivity to IPA was mainly responsible for this finding [31]. Furthermore, in rheumatic patients with suspected pneumonia and acute respiratory failure, the sensitivity of BALF sample culture combined with the GM test was superior to that of the mNGS test in detecting Aspergillus [32].

When low sequence numbers of microorganisms detected by mNGS are difficult to interpret clinically and cannot be verified by routine laboratory methods for clinical microorganisms, PCR detection may be used for confirmation. Combining mNGS with fungal PCR methods [33] can effectively detect fungi in clinical settings

and reduce the rate of missed diagnoses. A small amount of Aspergillus detected by BALF-mNGS can be confirmed by BALF-GM and/or Aspergillus-specific IgG antibody. While histopathology is an objective criterion for detecting fungus, it cannot identify the species and should not be considered the ultimate criterion. At present, the best diagnostic method for pulmonary aspergillosis is a combination of lesion morphology with culture or sequencing.

If pulmonary fungal infection is detected only by mNGS, it could lead to significant confusion in clinical diagnosis. Aspergillus is commonly present in the environment and can colonize the respiratory tract of healthy individuals. A positive culture or molecular test does not necessarily indicate an infection, as Aspergillus nucleic acid sequences have been found in nearly 40% of samples from non-pulmonary fungal-infected individuals. Consequently, microbial sequences detected by mNGS may originate from pathogenic microorganisms, normal flora, transient colonization, sample contamination, or even incorrect database comparison.

3. Other new rapid diagnostic methods for pulmonary aspergillosis

The new rapid diagnostic methods for pulmonary aspergillosis include antigen and antibody detection, novel molecular markers, and gene detection.

Antigen detection methods mainly include the G test and GM test. The G test detects (1,3)-β-D-glucan, a specific component in fungal cell wall, as a pan-fungal detection biomarker. GM is a foreign antigen released by Aspergillus mycelium when invading host tissue, and it is a specific polysaccharide in cell wall of Aspergillus. The GM test is used for early Aspergillus infection diagnosis through an enzymatic-linked immunosorbent assay. The blood-GM test is relatively simple and easy to operate, but its sensitivity is low in non-neutropenic patients such as COPD [34]. Additionally, it is affected by antibacterial drugs [35], intravenous immunoglobulin [36], and multiple myeloma [37]. The BALF-GM test has better diagnostic value than serum-GM test does [34]. European and American guidelines recommend that non-neutropenic patients choose the BALF-GM test to improve the detection rate [38, 39]. However, its sensitivity and specificity of the BALF-GM test depend on the population included in the study, the defined cut-off point, the detection platform, and the time node, and whether Aspergillus covering drug therapy is performed. There is no universal diagnostic threshold.

Antibody detection includes IgG and IgE detection. IgG antibody detection is the most sensitive microbial test for the diagnosis of CPA [40]. The increase of specific IgE of Aspergillus and total IgE levels can confirm the diagnosis of ABPA.

Furthermore, the level of plasma PTX3 in IPA patients was significantly increased, which could be used as a novel molecular marker for diagnosis [41]. Additionally, PCR is a sensitive and rapid gene detection method, but has certain false positive and high negative predictive value. Studies have confirmed that BALF-PCR detection has a high diagnostic value in the diagnosis of invasive aspergillosis with immunocompromised function [42].

4. Conclusions

The mNGS technique has considerable diagnostic value in pulmonary aspergillosis, however, it should be closely integrated with clinical comprehensive judgment to achieve accurate diagnosis.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Notes

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Nomenclature and abbreviations

mNGS	metagenomic next-generation sequencing
G	serum (1,3)-β-D-glucan
GM	galactomannan
PCR	polymerase chain reaction
PTX3	polymorphisms in pentraxin3
CPA	chronic pulmonary aspergillosis
ABPA	allergic bronchopulmonary aspergillosis
IPA	invasive pulmonary aspergillosis
BALF	bronchoalveolar lavage fluid
FFPE	formalin-fixed paraffin-embedded
CT	computer tomography
IFIs	invasive fungal infections
TBLB	transbronchial lung biopsy
BB	bronchoalveolar needle brush
HIV	human immunodeficiency virus
COVID-19	corona virus disease 2019
COPD	chronic obstructive pulmonary diseases
CAP	community-acquired pneumonia
EBUS-TBNA	endobronchial ultrasound-guided transbronchial needle aspiration
CMTs	conventional microbiological tests

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