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Original Article

Diagnostic value of nanopore sequencing technology in nontuberculous mycobacterial pulmonary disease

Wen-Hong Lin[1], Fei Tang[2], Sheng-Sheng Liu[1]

[1]Department of Tuberculosis, Anhui Chest Hospital, Hefei, Anhui, P. R. China; [2]Department of Endoscopy Center, Anhui Chest Hospital, Hefei, Anhui, P. R. China

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Abstract: Objective: To explore the diagnostic value of nanopore sequencing technology for detecting nontuberculous mycobacterial pulmonary disease (NTM-PD) in bronchial alveolar lavage fluid (BALF). Methods: A retrospective analysis was conducted on 83 patients with suspected NTM-PD admitted to Anhui Chest Hospital from January 2021 to November 2023. All patients underwent bronchoscopic examination, and BALF samples were collected for smear acid-fast staining, mycobacterial culture, and nanopore sequencing. The diagnostic efficiencies of these three methods were compared. Results: Among these patients, 27 were diagnosed with NTM-PD, 43 with pulmonary tuberculosis (PTB), and 13 with other lung diseases (OLD). The sensitivity, specificity, positive and negative predictive value of nanopore sequencing for diagnosing NTM-PD were 88.9%, 87.5%, 77.4%, and 94.2%, respectively. Nanopore sequencing demonstrated significantly higher sensitivity than smear and culture methods. The area under the receiver operating characteristic (ROC) curve (AUC) for nanopore sequencing was 0.882, significantly higher than that of smear (0.547) and culture (0.658), with *P* values less than 0.05. Conclusion: Nanopore sequencing technology has high diagnostic efficiency for NTM-PD and can directly identify bacterial species, but specificity issues should be considered in clinical application.

Keywords: Nanopore sequencing, nontuberculous mycobacteria, bronchial alveolar lavage fluid, diagnosis

Introduction

Nontuberculous mycobacteria (NTM) refer to mycobacteria other than the Mycobacterium tuberculosis complex and Mycobacterium leprae. NTMs are widely found in natural water sources, soil, and other environments. Approximately 200 species of Nontuberculous Mycobacteria (NTM) have been identified to date, with only a subset being pathogenic to humans as opportunistic pathogens. Based on their growth rates, NTM species are classified into two categories: slow-growing mycobacteria, which require up to 12 weeks for culture, and rapidly growing mycobacteria, which can be cultured within 7 days [1]. In recent years, there has been a global increase in the number of diseases caused by NTM [2, 3]. As in most countries, large-scale epidemiological studies on NTM infections are also lacking in China. However, the national tuberculosis epidemiological survey in China revealed that the isolation rate of NTM increased from 4.2% in 1985 to 22.9% in 2010 [4], indicating that NTM has become a significant public health issue. Indeed, NTM infections have been increasing in China in recent years and are a significant public health issue [5]. The most common site of NTM infection in humans is the lung organ, and the resulting pulmonary condition is referred to as NTM pulmonary disease (NTM-PD) [6]. Due to the clinical and radiological similarities between NTM-PD and pulmonary tuberculosis (PTB), differential diagnosis is challenging, and misdiagnosis as PTB or even drug-resistant PTB is common [7]. The clinical treatment strategies differ significantly between NTM and PTB, as NTMs exhibit high resistance to conventional anti-tuberculosis drugs, which can induce resistance in NTM strains. Furthermore, treatment strategies vary depending on the NTM species involved. Rapid diagnosis and accurate classification are thus crucial. Currently, mycobacterial culture remains the gold standard for diagnos-

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ing NTM infections but poses challenges due to its lengthy culture period, low positivity rate, and high biosafety requirements [8]. Studies have found that the probability of positive sputum cultures in NTM-PD patients is very low. Even with three consecutive cultures, the positive rate is still as low as 16.5% [9]. Therefore, it is particularly necessary to find rapid and accurate diagnostic methods. In 2014, Oxford Nanopore Technologies introduced third-generation nanopore sequencing, characterized by low operational costs, short testing cycles, long read lengths, and high accuracy [10]. Nanopore sequencing has been increasingly used to identify pathogenic microorganisms [11-13]. BALF allows for diagnosis in patients without sputum and reduces contamination in the diagnosis. However, studies on nanopore sequencing for NTM detection are limited. This study retrospectively analyzes the results of nanopore sequencing in BALF from suspected NTM-PD patients to assess its diagnostic value.

Materials and methods

Study subjects

A retrospective analysis was conducted on 87 patients with suspected NTM-PD, admitted to Anhui Chest Hospital from January 2021 to November 2023, based on imaging studies. All patients underwent bronchoscopy, and BALF samples were collected for smear acid-fast staining, mycobacterial culture, and nanopore sequencing. This study was approved by the Ethic Committee of Anhui Chest Hospital.

Diagnostic criteria for NTM-PD

NTM-PD was diagnosed based on respiratory and/or systemic symptoms, and imaging findings such as cavitary lesions, multifocal bronchiectasis, and multiple small nodular lesions, excluding other lung diseases. Diagnosis required one of the following criteria without external contamination of samples: 1. Positive cultures of the same pathogen from two separate sputum samples. 2. Positive molecular biology and/or culture for NTM in BALF. 3. Positive NTM culture in BALF along with a smear positive for acid-fast bacilli (2+) or higher. 4. Granulomatous inflammation or positive acid-fast bacilli smear in lung biopsy, with a positive NTM culture or molecular biology in BALF or sputum.

Inclusion and exclusion criteria

Inclusion Criteria: 1. Suspected NTM-PD based on respiratory and/or systemic symptoms and imaging findings suggestive of cavitary lesions, multifocal bronchiectasis, and multiple small nodular changes; 2. No contraindications to bronchoscopy and agreement to nanopore sequencing, smear, and culture tests.

Exclusion Criteria: 1. Unclear final diagnosis; 2. Laboratory-submitted sample failed quality control; 3. Incomplete clinical data.

BALF collection method

BALF was collected using an Olympus BF1T206 fiber bronchoscope in the endoscopy center, targeting bronchopulmonary segments indicated by chest CT findings. A 10-20 mL volume of 0.9% NaCl was instilled and aspirated using negative pressure into a sterile bottle.

BALF testing methods

(1) Nanopore Sequencing Technology: The detection procedure was carried out according to the method we previously reported [14]. Briefly, the process involved sample pretreatment, nucleic acid extraction, nucleic acid quality control, nanopore sequencing library construction, nanopore sequencing, and data analysis.

(2) Smear and Liquid Culture Methods: Standard procedures for smear acid-fast staining and mycobacterial culture were followed [15].

Statistical methods

Statistical analysis was performed using SPSS 25.0. Count data were expressed as numbers or percentages, and differences between groups were compared using the chi-squared test or Fisher's exact test. Pairwise comparisons among multiple groups were conducted using the Bonferroni method. ROC curves were generated using MedCalc 19.5.6 to evaluate the diagnostic value of different methods, and comparisons were made using the Delong method. A p-value of less than 0.05 was considered statistically significant.

Table 1. General information of 83 patients

Indicator	NTM-PD group (27 cases)	PTB group (43 cases)	OLD group (13 cases)	χ^2 value	*P* value
Gender					
Female	12	19	5	0.152	0.927
Male	15	24	8		
Age* (years)				0.252	> 0.999
15-59	22	34	10		
≥ 60	5	9	3		
History of smoking*				2.968	0.207
No	22	36	8		
Yes	5	7	5		
History of drinking*				3.372	0.149
No	26	38	10		
Yes	1	5	3		
Complication*				2.312	0.316
No	10	9	4		
Yes	17	34	9		

Note: * indicates the use of Fisher's exact test; NTM-PD: Nontuberculous Mycobacterial Pulmonary Disease; PTB: Pulmonary Tuberculosis; OLD: Other Lung Diseases.

Table 2. Symptoms and imaging characteristics of NTM-PD patients

Parameter	n	%
Clinical Symptoms		
Cough/Sputum	75	90.4
Hemoptysis	22	26.5
Chest Pain	7	8.4
Dyspnea	21	25.3
Fever	14	16.9
Night Sweats	12	14.5
Asymptomatic	6	7.2
Imaging Features		
Nodules	45	54.2
Spots	28	33.7
Cavities	36	43.4
Bronchiectasis	50	60.2
Enlarged mediastinal lymph nodes	11	13.2
Consolidation	8	9.6

NTM-PD: Nontuberculous Mycobacterial Pulmonary Disease.

Results

General information

A total of 83 patients suspected of having NTM-PD were included in the study, with ages ranging from 15 to 73 years. Among them, 47 were male and 36 were female. Based on the final clinical diagnosis, 27 cases were diagnosed as NTM-PD, with an age range of 18 to 73 years (15 males and 12 females). The PTB group consisted of 43 cases, aged 15 to 71 years (24 males and 19 females). The OLD group had 13 cases, aged 15 to 64 years (8 males and 5 females). The general information of the three groups of patients is shown in **Table 1**.

Clinical symptoms and imaging characteristics

The clinical symptoms of NTM-PD patients are nonspecific and often include cough, sputum production, hemoptysis, chest tightness, shortness of breath, and fever, as shown in **Table 2**. Imaging changes include pulmonary nodules, cavities, and bronchiectasis, as demonstrated in **Table 2** and **Figure 1**.

Comparison of diagnostic efficiency among three methods

Using clinical diagnosis as a reference, the sensitivities of nanopore sequencing, smear, and liquid culture for diagnosing NTM-PD were 88.9%, 11.1%, and 33.3%, respectively; specificities were 87.5%, 98.2%, and 98.2%; positive predictive values were 77.4%, 75.0%, and 90.0%; and negative predictive values were 94.2%, 69.6%, and 75.3%, as shown in **Table 3**. Further evaluation using ROC curves (**Figure 2**) showed AUC values of 0.882, 0.547, and 0.658

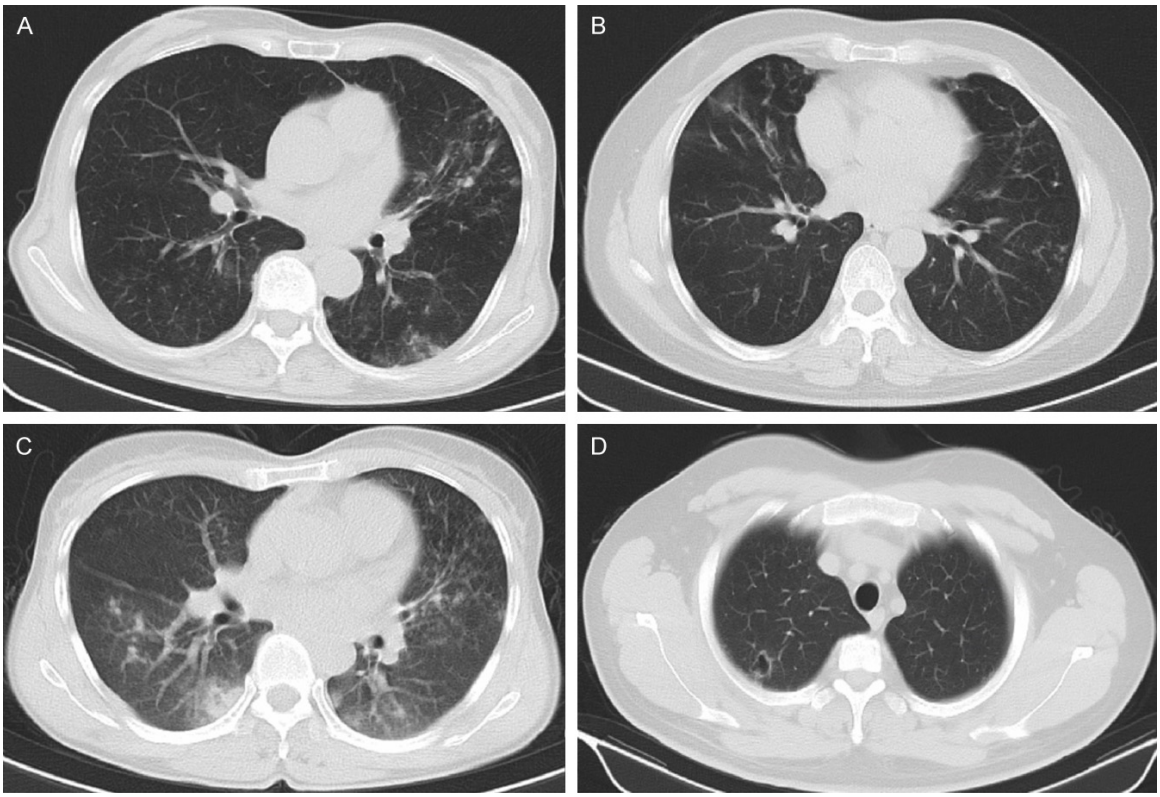

Figure 1. Representative chest CT images of NTM-PD patients. A. Female, 62 years old, infected with Mycobacterium intracellulare, showing nodules and bronchiectasis in the upper lobe of the left lung. B. Male, 47 years old, infected with Mycobacterium intracellulare, presenting with nodules and spot shadows in both lungs, with cylindrical bronchiectasis in some lesions. C. Female, 46 years old, infected with Mycobacterium abscessus, displaying nodules and spot shadows in both lungs. D. Male, 27 years old, infected with Mycobacterium kansasii, showing a thin-walled cavity in the upper lobe of the right lung. NTM-PD: Nontuberculous Mycobacterial Pulmonary Disease.

Table 3. Diagnostic efficiency of three methods for nontuberculous mycobacterial pulmonary disease

Method	Sensibility (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
Smear	11.1 (3/27)a	98.2 (55/56)	75.0 (3/4)	69.6 (55/79)a
Liquid Culture	33.3 (9/27)b	98.2 (55/56)	90.0 (9/10)	75.3 (55/73)b
Nanopore sequencing	88.9 (24/27)	87.5 (49/56)	77.4 (24/31)	94.2 (49/52)
χ^2 value	35.100	6.902*	0.936*	11.494
P value	< 0.001	0.020	0.717	0.003

Note: * indicates the use of Fisher's exact test; a, b indicate that the comparison between nanopore sequencing and smear, and culture, respectively, is significant at $P < 0.05$ (Bonferroni correction).

for nanopore sequencing, smear, and liquid culture, respectively. The AUC value of nanopore sequencing was significantly higher than that of smear ($Z = 7.323$, $P < 0.0001$) and culture ($Z = 3.188$, $P = 0.0014$).

NTM-PD strain identification analysis

Among the 27 NTM-PD patients, nanopore sequencing detected 24 cases, identifying Mycobacterium intracellulare in 8 cases, Mycobacterium abscessus in 11 cases, Mycobacterium kansasii in 2 cases, Mycobacterium colombiense in 1 case, and mixed infections in 2 cases. Culture identified 9 cases, including Mycobacterium intracellulare in 7 cases and Mycobacterium kansasii in 2 cases (**Table 4**). Six patients were positive by both methods, with discrepancies in strain identification in 2 cases. Using liquid culture as the gold stan-

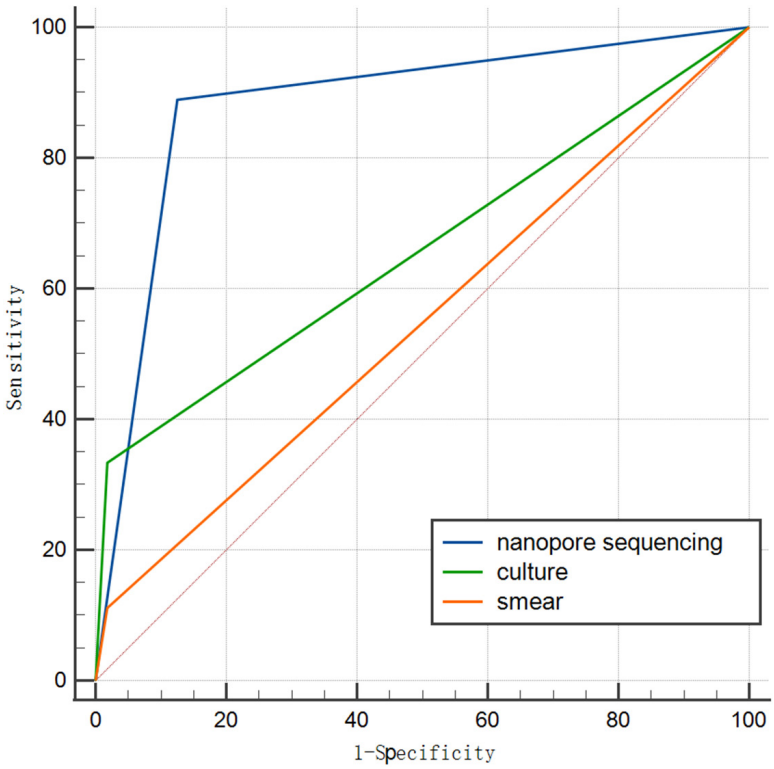

Figure 2. ROC curve analysis of three diagnostic methods.

Table 4. Strain identification results of nontuberculous mycobacteria (NTM) by two methods

Strain	Culture Method		Nanopore Sequencing Method	
	Cases	Proportion (%)	Cases	Proportion (%)
Mycobacterium intracellulare	7	77.8	8	33.3
Mycobacterium abscessus	0	0.0	11	45.8
Mycobacterium kansasii	2	22.2	2	8.3
Mycobacterium colombiense	0	0.0	1	4.2
M. turtle + M. abscessus	0	0.0	1	4.2
M. intracellulare + M. abscessus	0	0.0	1	4.2

dard, the accuracy of nanopore sequencing was 83.3% (5/6).

Discussion

NTM are widely present in natural environments such as water, soil, and dust, and can infect both humans and some animals. Existing data show that the incidence and prevalence of NTM diseases are increasing in several countries and regions, even surpassing those of tuberculosis (TB) in some areas [16, 17]. The large-scale epidemiological data on NTM diseases in China is currently lacking, similar to most other countries. However, surveys of tuberculosis epidemiology in China show that the NTM isolation rate increased from 4.3% in 1979 to 11.1% in 2000, and to 22.9% in 2010, reflecting a significant upward trend in NTM diseases in China [4]. NTM-PD is the most common NTM disease, accounting for about 70%-80% of cases [18]. Its clinical manifestations are similar to pulmonary TB, and both present with positive sputum acid-fast bacillus staining, often leading to misdiagnosis. This study found that the clinical symptoms of NTM-PD patients often include cough, sputum production, hemoptysis, chest tightness, shortness of breath, and fever. Imaging changes include pulmonary nodules, cavities, and bronchiectasis. It is evident that NTM-PD and pulmonary tuberculosis (PTB) share highly similar clinical and imaging characteristics, and thus possible misdiagnosis of both, which is consistent with conclusions from previous studies [19, 20]. Traditionally, NTM diagnosis requires positive mycobacterial culture for further identification, but the lengthy culture time often delays diagnosis. Therefore, there is an urgent clinical need for faster and more accurate diagnostic methods, especially for patients who cannot provide adequate sputum samples.

In recent years, third-generation sequencing technology based on single-molecule reading principles has emerged. Nanopore sequencing has garnered attention due to its advantages in read length, timeliness, and portability [13]. Initially marketed for genome sequencing, nanopore sequencing kits have rapidly evolved with advances in sequencing chemistry and computational capabilities, and are now widely

used in various clinical fields, including the detection of mycobacterial nucleic acids and gene resistance [13, 21, 22]. This method does not require enzymes and fluorescent labels, avoiding PCR amplification errors, and is simple, stable, and cost-effective, enabling real-time rapid sequencing [10].

Previous studies using nanopore sequencing for TB diagnosis have confirmed its good diagnostic performance. For example, Yu et al. [23] found that nanopore sequencing had a sensitivity, specificity, PPV, and NPV of 94.8%, 97.9%, 99.1%, and 88.7%, respectively, in a study of 164 suspected TB cases. Liu et al. [24] applied nanopore sequencing to BALF samples from 55 suspected TB patients and reported corresponding values of 75.9%, 80.8%, 81.5%, and 75.0%. Our earlier studies indicated that the corresponding indicators for nanopore sequencing in TB detection were 85.3%, 95.4%, 98.2%, and 68.3%, respectively [14]. However, studies on the clinical value of nanopore sequencing for NTM diagnosis are rare and mostly case reports. Huang et al. [25] successfully identified Mycobacterium marinum in pus samples from a 67-year-old patient with left hand swelling and suppuration using nanopore sequencing.

In this study, 83 suspected NTM-PD patients were included, with final clinical diagnoses of 27 NTM-PD cases, 43 PTB cases, and 13 OLD cases. There were no statistically significant differences in the general data among the three groups. Using the final clinical diagnosis as the reference, the sensitivities of nanopore sequencing, smear, and liquid culture for diagnosing NTM-PD were 88.9%, 11.1%, and 33.3%, respectively; the specificities were 87.5%, 98.2%, and 98.2%, respectively. Chi-square test and Bonferroni pairwise comparisons showed that the sensitivity of nanopore sequencing was significantly higher than that of smear and culture methods. The AUC value for nanopore sequencing was 0.882, significantly higher than those for smear (0.547) and liquid culture (0.658). Kong Jiao et al. [26] found that using second-generation metagenomic sequencing technology in 123 suspected NTM-PD patients, the sensitivity and specificity for NTM-PD diagnosis were 83.8% and 65.3%, respectively. The authors suggested that while second-generation sequencing has high sensitivity in diagnosing NTM-PD, its specificity is low and needs improvement. This study using third-generation sequencing (nanopore sequencing) found that the specificity for NTM-PD detection was 87.5%, lower than that of smear and culture methods in this study, consistent with Kong Jiao et al. [26]. Multiple comparisons of specificity among nanopore sequencing, smear, and culture methods were statistically significant ($P < 0.05$), but pairwise comparisons showed no statistical differences, likely due to the conservative results after Bonferroni correction and small sample size. The relatively low specificity should alert clinicians as some NTM can colonize the respiratory tract, and sample contamination is possible. Therefore, future efforts should focus on improving specificity to better serve clinical work.

NTM are widespread in the environment, but the pathogenicity of different NTM species varies. Typically, species such as MAC, Mycobacterium abscessus, Mycobacterium kansasii, Mycobacterium malmoense, Mycobacterium simiae, Mycobacterium scrofulaceum, Mycobacterium terrae, Mycobacterium fortuitum, and Mycobacterium marinum are potentially pathogenic when isolated from clinical samples, while species like Mycobacterium gordonae, Mycobacterium mucogenicum, Mycobacterium nonchromogenicum, and Mycobacterium terrae are generally non-pathogenic or weakly pathogenic, often considered as contaminants or transient colonizers [27]. Accurate and rapid identification of the species is crucial for prognosis and tailored treatment plans. Our previous study found that in Anhui, the most commonly isolated NTM species were Mycobacterium intracellulare and Mycobacterium abscessus [28]. In this study, both nanopore sequencing and culture methods primarily identified these two species. Among the 27 NTM-PD patients, nanopore sequencing detected 24 cases, while culture methods detected only 9 cases, with 6 cases positive by both methods. Using liquid culture as the reference standard for species identification, the accuracy of nanopore sequencing was 83.3%, higher than the 63.8% accuracy reported by Kong Jiao et al. using second-generation metagenomic sequencing for NTM-PD. This may be due to the ability of nanopore sequencing to read long DNA sequences in a single pass, better detecting large structural variations like deletions and duplications, providing clearer and more com-

plete genomic information. Additionally, this technology can achieve a streamlined process from sample input to sequencing output to data analysis, identifying diseases, mutations, or infection types in real-time, and rapidly obtaining results.

However, this study has some limitations. Firstly, the sample size was relatively small. Secondly, NTM PCR detection was not performed on the cases, a relatively mature method that could complement culture to improve positive detection rates. Therefore, future prospective cohort studies with larger sample sizes are needed to enhance the objectivity of the results and data.

In conclusion, nanopore sequencing technology has high diagnostic efficiency for NTM-PD and can directly identify species, providing direction for early pathogen identification, diagnosis, and treatment. However, specificity issues should be considered in clinical applications to avoid overdiagnosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Sheng-Sheng Liu, Department of Tuberculosis, Anhui Chest Hospital, No. 397 Ji Xi Road, Hefei 230000, Anhui, P. R. China. E-mail: liushengsheng1014@163.com

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