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ORIGINAL ARTICLE

Diagnostic Efficacy of Nanopore Sequencing Using Clinical Specimens for Nontuberculous Mycobacterial Pulmonary Disease

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SUMMARY

Background: **This study aimed to evaluate the diagnostic efficacy of nanopore sequencing using clinical specimens for the rapid diagnosis of nontuberculous mycobacterial pulmonary disease (NTM-PD) and to provide a new detection tool for the early diagnosis of NTM-PD.**
Methods: **Information regarding patients with suspected *Mycobacterium* infection with lung disease was retrospectively analyzed to determine the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) for nanopore sequencing compared with those for NTM culture.**
Results: **Overall, 871 patients were enrolled in the study. By using NTM culture as the reference standard for diagnosing NTM-PD, the sensitivity, specificity, PPV, NPV, and AUC were found to be 93.3% (95% CI: 86.1% - 97.5%), 97.2% (95% CI: 95.8% - 98.2%), 78.5% (95% CI: 69.5% - 85.9%), 99.2% (95% CI: 98.4% - 99.7%), and 0.95 (95% CI: 0.94 - 0.97) for nanopore sequencing, respectively. The overall accuracy of diagnosing NTM-PD was excellent for nanopore sequencing.**
Conclusions: **Nanopore sequencing analysis of clinical specimens offers high diagnostic accuracy for detecting NTM-PD. Furthermore, this approach can be used as the priority diagnostic tool for detecting NTM-PD.**
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KEYWORDS

NTM, pulmonary disease, third-generation sequencing, diagnostic accuracy, sensitivity

INTRODUCTION

Nontuberculous mycobacteria (NTM) refer to mycobacteria other than *Mycobacterium tuberculosis* (MTB) and *Mycobacterium leprae* [1]. To date, > 190 species of NTM have been identified [2]. Although most of these are nonpathogenic to humans, a few species of NTM can infect humans and cause lesions in tissues and organs, leading to NTM disease [3]. The incidence of this disease is increasing with the increase in the population of older adults and patients with immunodeficiencies (such as HIV infection) as well as with improvement in the level of laboratory diagnostic techniques [4,5]. Moreover, even in developed countries, the incidence of NTM disease is higher than that of MTB disease [6].

Furthermore, similar to tuberculosis (TB), NTM disease poses a threat to human public health [7].

Notably, NTM pulmonary disease (NTM-PD) is the most common type of NTM disease, accounting for approximately 70% - 80% cases of all NTM diseases [8]. In most cases, NTM-PD is associated with underlying lung diseases such as chronic obstructive pulmonary disease, bronchiectasis, pneumoconiosis, and TB [9]. Interactions between these diseases may exacerbate symptoms and reduce prognostic expectations [10]. The clinical symptoms and pulmonary imaging manifestations of NTM-PD and pulmonary TB are similar; therefore, it is difficult to effectively distinguish between them [11,12]. Thus, NTM-PD is often misdiagnosed as pulmonary TB and treated inappropriately, leading to delayed diagnosis and treatment and affecting the final prognosis [13,14]. Acid-fast bacilli (AFB) smear is the most commonly used test to detect mycobacterial infection. Although this method is convenient and inexpensive, AFB smears cannot distinguish between NTM and MTB infections; this is an important reason for the misdiagnosis of NTM-PD [15]. The diagnosis of NTM-PD depends on mycobacterial culture, but mycobacterial culture is usually time-consuming and takes several weeks to obtain results [16]. Thus, it is not conducive to the early and rapid diagnosis of NTM-PD.

Molecular tests play an increasingly important role in the rapid diagnosis of *Mycobacterium* infection and diagnosis of NTM disease [17,18]. Nanopore sequencing - also known as third-generation sequencing - is a new genetic sequencing technology that enables direct sequencing of single DNA/RNA molecules, with fast real-time reading and data analysis [19]. Moreover, it is increasingly used in clinical applications [20]. Compared with next-generation sequencing, nanopore sequencing is more convenient and flexible and demonstrates excellent diagnostic efficacy for the rapid diagnosis of clinical infectious diseases, including pulmonary TB [21-23]. However, the application of nanopore sequencing in NTM-PD diagnosis is limited, and its effectiveness relative to NTM-PD is unclear. Therefore, this study aimed to evaluate the diagnostic efficacy of nanopore sequencing using clinical specimens for the rapid diagnosis of NTM-PD and to develop a new detection tool for the early diagnosis of NTM-PD.

MATERIALS AND METHODS

This retrospective study evaluated the diagnostic accuracy of nanopore sequencing for NTM-PD at a provincial regional TB diagnosis and treatment center in Zhejiang Province. Data of patients with suspected *Mycobacterium* infection with lung disease hospitalized at the center between July 2021 and October 2022 were reviewed. The inclusion criteria for patients with suspected *Mycobacterium* infection with lung disease were as follows: those with respiratory symptoms (e.g. cough and hemoptysis) and/or systemic symptoms (e.g. fever), those with abnormal chest computed tomography findings (e.g. cavitary pulmonary shadow, multifocal bronchiectasis, and multiple small nodular lesions), and those who have not been treated with anti-NTM drugs. Patients with suspected *Mycobacterium* infection with lung disease for whom relevant respiratory clinical specimens were obtained; those who underwent concurrent AFB smear, *Mycobacterium* culture, and nanopore sequencing test; and those who had a complete clinical profile were included in the study. Patients who did not undergo the relevant tests, those with inconclusive test results, and those with incomplete clinical data were excluded from the study.

Clinical specimens examined in the study were from sputum and bronchoalveolar lavage fluid (BALF). For patients lacking sputum, BALF was obtained by fiberoptic bronchoscopic lavage of the lung lesion site. Written informed consent was obtained from the patients or their guardians before the procedure. Fresh sputum and BALF specimens were equally divided for AFB smear, NTM culture, and nanopore sequencing. Ethical approval for this study was obtained from the Human Research Ethics Committee of Hangzhou Red Cross Hospital (2024-YANSHEN-059, May 31, 2024), and this study is in accordance with the guidelines of Declaration of Helsinki. As a retrospective study analyzed using data that already existed, there would be no impact on patients, and therefore informed consent from patients was waived by the Ethics Committee.

Based on the diagnostic guidelines for NTM-PD [8,24], in this study, the diagnostic accuracy of the relevant tests was evaluated using NTM culture results as the reference standard. NTM-PD was defined as positive NTM culture of the same species from two separate sputum specimens or positive NTM culture in BALF. Non-NTM-PD was defined as negative NTM in a clinical specimen from the lung with a diagnosis of other pathogenic bacterial infection (e.g. TB) and an improved lung lesion without anti-NTM treatment.

AFB smear and NTM culture

Fresh clinical specimens were digested and decontaminated using N-acetyl-l-cysteine-NaOH. The decontaminated specimens were stained with Ziehl-Neelsen and then subjected to direct AFB smear microscopy. Further, a second decontaminated specimen was inoculated in Lowenstein-Jensen solid medium (Encode Medical Engineering Co., Ltd., China) and/or BACTEC MGIT 960 liquid medium (BD Diagnostic Systems, Sparks, MD, US) for NTM culture. Finally, the sample was incubated at a constant temperature of 35°C - 37°C [25]. Rapid identification of *Mycobacterium* species used a gene chip method for NTM culture-positive specimens [26]. The process of gene chip testing has been fully described previously [27]. Asymmetric polymerase chain reaction (PCR) was conducted using an ABI 9700 instrument following the specific instructions provided by the manufacturer. The PCR products obtained from this reaction were further analyzed by hybridization with a

gene chip manufactured by CaptialBio (Beijing, China). This hybridization step was carried out in a BioMixer II three-dimensional tilting agitator and hybridization oven, which ensures optimal mixing and uniform temperature distribution for efficient hybridization. To eliminate any non-specific background signals on the hybridized slides, a washing process was performed using an automated SlideWasher-8, an instrument developed by CapitalBio. Finally, the fluorescent signals generated from the microarrays on the gene chip were quantitatively analyzed using a LuxScan-10K confocal laser scanner (CapitalBio Corporation, Beijing, China).

Nanopore sequencing

The sputum sample (400 µL) was liquefied in NaOH solution and centrifuged, and the supernatant was discarded. The pellet was washed with phosphate-buffered saline and resuspended in 200 µL of lysis solution. Similarly, 5 mL of BALF fluid were centrifuged, supernatant was discarded, and pellet was resuspended in 200 µL of lysis solution. Samples were physically ground with grinding beads, and lysozyme solution was added. DNA was extracted from the lysates using a QIAamp DNA Microbiome Kit (cat. no. 51707, Qiagen, Hilden, Germany). The extracted DNA was used for the polymerase chain reaction (PCR) amplification of the rpoB gene region for *Mycobacterium* spp. The PCR primers used in the amplification process exhibited specificity for the nucleotide sequences Rpo5' (5'-TCA AGGAGAAGCGCTACGA-3') and Rpo3' (5'-GGATG TTGATCAGGGTCTGC-3') [18,22,28].

Touchdown PCR was performed using the following parameters: denaturation at 98°C for 3 minutes, six cycles of amplification at 95°C for 15 seconds, annealing at 66°C for 60 seconds (with a decrease of 1°C per cycle), and an elongation step at 72°C for 30 seconds. After the first six cycles, the annealing temperature was lowered to 61°C for the remaining 29 cycles. Finally, an extension step of 72°C for 5 minutes was performed. The amplified product was purified with magnetic beads and barcode-labeled using native barcoding expansion. After quality control, equal-quality products with different barcode labels were pooled, and the Ligation Sequencing Kit 1D R9 Version was used for subsequent procedures. The final library was up-sequenced using the GridION platform, and the sequencing data were collected using MinKnow v3.6.5 software. The paired raw sequencing reads were quality-filtered, and sequences of < 200 bp were removed. Repetitive regions were masked, and sequences were aligned with MiniMap 2 software. To differentiate between MTB and NTM, the filtered reads were compared with reference sequences for MTB and *Mycobacterium* using MiniMap 2 software, and the analysis results were obtained within 48 hours. Further, to eliminate the possibility of contamination, host DNA reads were removed by aligning with the human reference genome (GRCh 38) [21].

Statistical analysis

Mean values, quartiles, standard deviations, and true positive (TP), false positive (FP), false negative (FN), and true negative (TN) values were calculated using SPSS 24.0 (IBM Corp., Armonk, NY). Diagnostic accuracy indices, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the receiver operating characteristic curve (AUC) with 95% confidence intervals (CIs), were calculated using MedCalc Statistical v15.2.2 (MedCalc Software Bvba, Ostend, Belgium; http://www.medcalc.org), with four values for TP, FP, FN, and TN [29]. Levene test was used to evaluate the equality of variances, and Shapiro-Wilk normality test was used to evaluate the normality of the data distribution. Measurement data were compared using *t*-tests (normally distributed with equal variances) or Wilcoxon rank sum tests (non-normally distributed). McNemar's test was used to compare differences in paired data, chi-squared and Fisher's exact tests were used to compare differences in two ratios, and Z tests were used to compare differences in two AUCs. A p-value of < 0.05 was considered statistically significant.

RESULTS

Overall, 927 patients with suspected *Mycobacterium* infection with lung disease who underwent nanopore sequencing were examined. In 56 patients, relevant tests were not performed or data were incomplete. After excluding these patients, 871 patients were included in the study (Figure 1). The patient population included 517 males (59.4%), and the mean age was 50.6 ± 18.8 (range, 1 - 95) years. The types of respiratory clinical specimens included 101 (11.6%) sputum and 770 (88.4%) BALF, respectively. No other types of specimens were included, and no patient underwent nanopore sequencing of both specimen types. No patients with acquired immunodeficiency syndrome were included in the study. The clinical characteristics and demographics of included patients are shown in Table 1.

Based on the reference standard, 90 patients were diagnosed with NTM-PD, and the remaining 781 patients were diagnosed with non-NTM-PD. Positive AFB smear was obtained in 221 (25.4%) patients; conversely, 90 patients had positive NTM culture and 107 patients had positive nanopore sequencing. The number of positive and negative results for the three tests in terms of the proportion of all included patients is shown in Figure 2. The distribution and overlap of positive results for the three tests are shown in Figure 3. Positive MTB culture was obtained in 332 patients; further, after including NTM-positive patients, 422 patients had positive culture for *Mycobacterium*. Thus, 21.3% of positive *Mycobacterium* culture were for NTM (90/422). Among 221 AFB smear-positive patients, 38 (17.2%) were diagnosed with NTM-PD and 183 (82.8%) were diagnosed with pulmonary TB (PTB). The median number

Table 1. The clinical characteristics and demographics of included patients.

Characteristics	All (n = 871)
Age (year, mean ± SD)	50.6 ± 18.8
Male (n, %)	517 (59.4%)
HIV status (n, %)	
Negative	871 (100.0%)
Symptoms (n, %)	
Fever	329 (37.8%)
Cough	567 (65.1%)
Night sweat	175 (20.1%)
Hemoptysis	288 (33.1%)
Concomitant diseases	
Hypertension, n (%)	124 (14.2)
T2DM, n (%)	108 (12.4)
COPD, n (%)	60 (6.9)
Tuberculosis, n (%)	665 (76.3)
Bronchiectasis, n (%)	76 (8.7)

SD standard deviation, T2DM type 2 diabetes, COPD chronic obstructive pulmonary disease.

Table 2. Diagnostic efficacy of nanopore sequencing for the diagnosis of nontuberculous mycobacterial pulmonary disease.

Test	Sample		NTM culture +	NTM culture -	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)	AUC (95% CI)
Nanopore sequencing	All	+	84	23	93.3 (86.1 - 97.5)	97.2 (95.8 - 98.2)	78.5 (69.5 - 85.9)	99.2 (98.4 - 99.7)	0.95 (0.94 - 0.97)
		-	6	758					
	Sputum	+	9	1	100.0 (66.4 - 100.0)	98.9 (94.1 - 100.0)	90.0 (55.5 - 99.8)	100.0 (96.0 - 100.0)	0.99 (0.95 - 1.00)
		-	0	91					
	BALF	+	75	22	92.6 (84.6 - 97.2)	96.8 (95.2 - 98.0)	77.3 (67.7 - 85.2)	99.1 (98.1 - 99.7)	0.95 (0.93 - 0.96) *
		-	6	667					

Comparison between the sputum and BALF, * p = 0.009.
NTM nontuberculous mycobacterial, AFB acid-fast bacilli, PPV positive predictive value, NPV negative predictive value, AUC area under the curve, BALF bronchoalveolar lavage fluid.

of reads for 107 nanopore sequencing-positive cases was 657 (range, 1 - 56,179). The distribution of reads is presented in Figure 4.

Among 90 patients with NTM-PD, AFB smear was positive in 38 patients and nanopore sequencing was positive in 84; further, out of 781 non-NTM-PD cases, 183 and 23 had positive AFB smear and nanopore sequencing test, respectively. The median number of reads for positive nanopore sequencing in patients diagnosed with NTM-PD (known as TP) was significantly higher than that in patients diagnosed with non-NTM-PD (known as FP) (3,258 vs. 17; $p < 0.001$) (Figure 4). Among patients with NTM-PD (positive NTM culture), nanopore sequencing results were negative for NTM in six cases, including three cases in which pathogenic bacteria were not detected, one case that tested positive for *Pseudomonas aeruginosa*, and two cases that tested positive for TB (reads of 2 and 3, respectively).

Diagnostic accuracy of nanopore sequencing for NTM-PD

The overall sensitivity, specificity, PPV, NPV, and AUC for the diagnosis of NTM-PD (using NTM culture as the reference standard) were 93.3% (95% CI: 86.1%

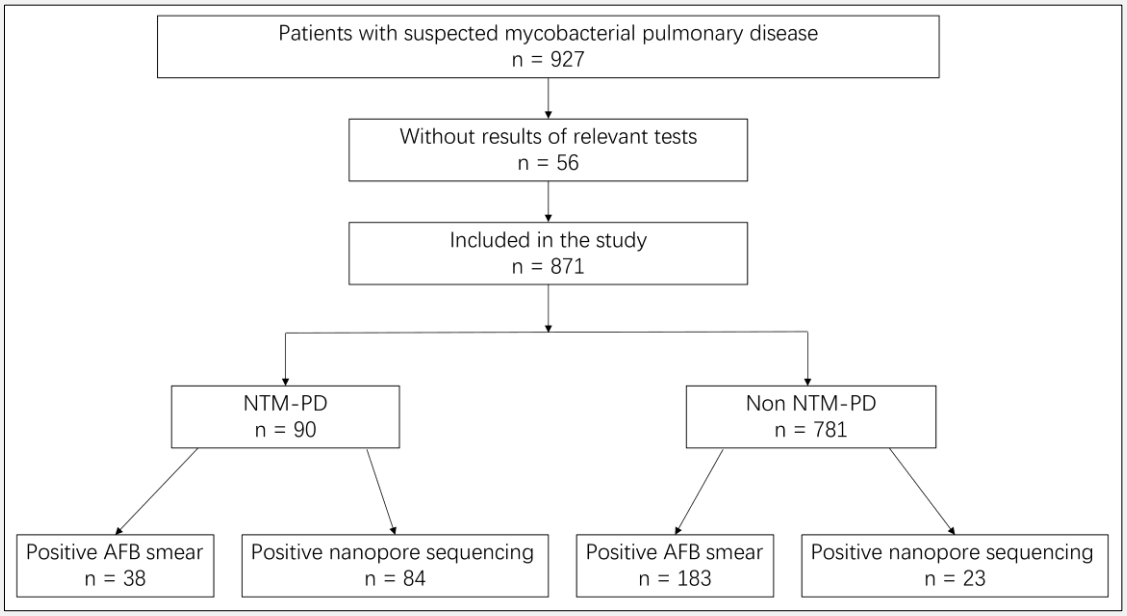

Figure 1. Screening process for inclusion of patients.

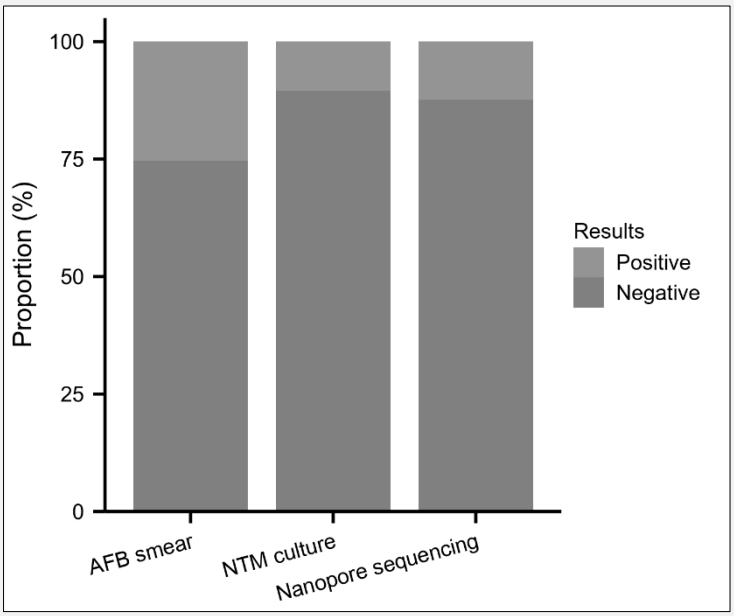

Figure 2. The number of positive and negative results for the three tests in terms of the proportion of all included patients.

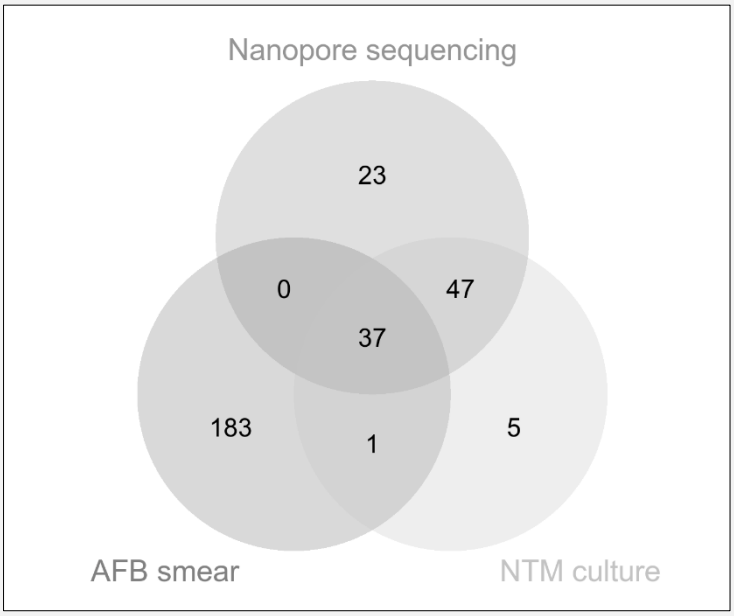

Figure 3. The distribution and overlap of positive results for the three tests.

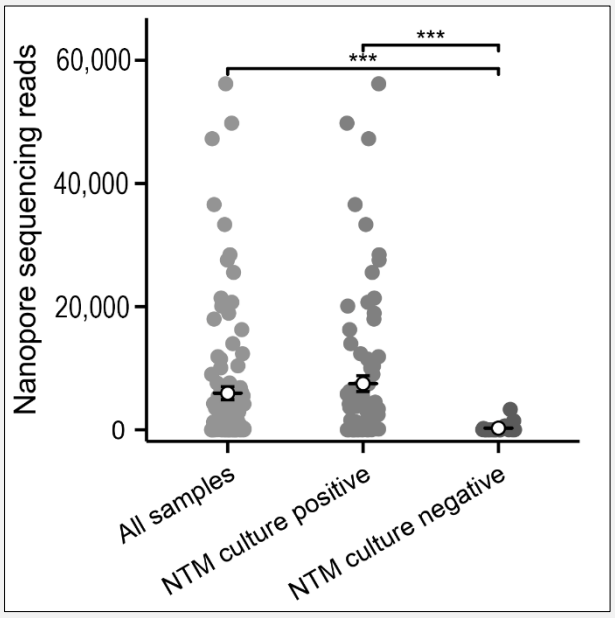

Figure 4. The distribution of reads for different classification of positive nanopore sequencing patients.

*** **p < 0.001.**

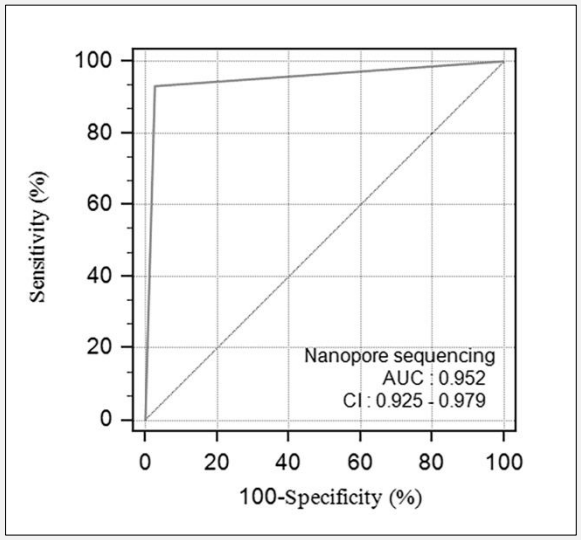

Figure 5. The receiver operating characteristic curves for the diagnosis of nontuberculous mycobacterial pulmonary disease using nanopore sequencing.

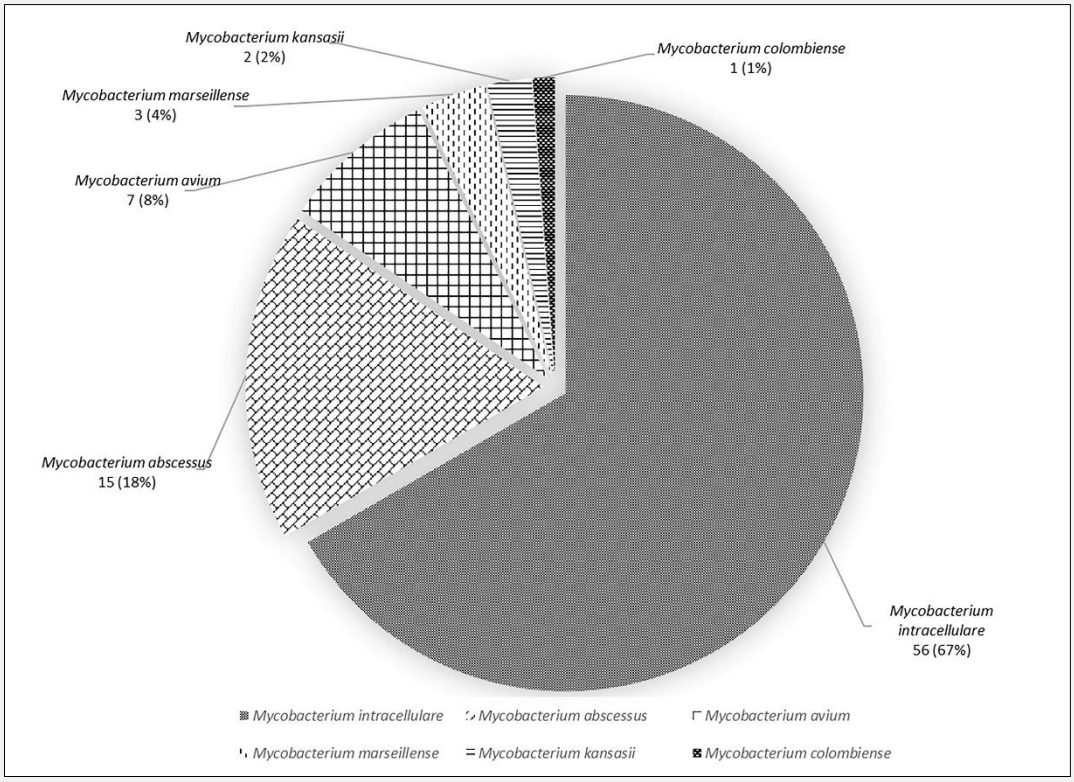

Figure 6. The distribution of nontuberculous mycobacterial species.

- 97.5%), 97.2% (95% CI: 95.8% - 98.2%), 78.5% (95% CI: 69.5% - 85.9%), 99.2% (95% CI: 98.4% - 99.7%), and 0.95 (95% CI: 0.94 - 0.97) for nanopore sequencing, respectively. These results are presented in Table 2. The receiver operating characteristic curves for the diagnosis of NTM-PD using nanopore sequencing are shown in Figure 5. The overall accuracy of nanopore sequencing for diagnosing NTM-PD was excellent. The diagnostic accuracy of AFB smear for NTM-PD is demonstrated in Supplementary Table 1, and the results are unsatisfactory. The sensitivity, specificity, PPV, NPV, and AUC of nanopore sequencing in sputum and BALF are likewise demonstrated in Table 2. Diagnostic accuracy of sputum for nanopore sequencing was significantly better than that of BALF for nanopore sequencing ($p < 0.05$, Table 2).

NTM species identification

Nanopore sequencing identified NTM species in 84 out of 90 patients with NTM-PD. Notably, six NTM species were identified, and the most common species were *Mycobacterium intracellulare* and *Mycobacterium abscessus*. The distribution of these species is shown in Figure 6.

DISCUSSION

It is important to make an accurate and timely diagnosis of NTM-PD, because both misdiagnosis and missed diagnosis are possible [30]. The nonspecific symptoms and overlapping characteristics with other respiratory diseases further complicate the correct identification of this disease. In addition, NTM-PD is often diagnosed at a later stage when significant damage to the lungs has already occurred, making treatment more complicated and less effective. Therefore, there is an urgent need for improved diagnostic tools and criteria to ensure the early and accurate detection of NTM-PD.

The AFB smear test is commonly used to detect MTB and NTM infections in the lungs [13]. However, this test has a low positive rate and cannot differentiate between MTB and NTM. Based on the results of the present study, only 25.4% of the specimens tested positive for NTM using AFB smear, and only 17.2% were finally diagnosed with NTM-PD. This finding highlights the low diagnostic accuracy of AFB smear for NTM-PD and its inability to effectively identify patients with NTM-PD early. Although the AFB smear test is quick, it does not meet the demands of an accurate diagnostic test. The result of a single AFB smear test is not sufficient to diagnose NTM-PD; thus, further culture or molecular tests are needed to clarify the diagnosis. Notably, this test is only useful for distinguishing between mycobacterial and nonmycobacterial infections. However, it is not reliable enough to confirm the diagnosis of NTM-PD; NTM infections are caused by a large and diverse group of bacteria that are not easily distinguishable by smear microscopy alone. Therefore, it is important to conduct additional tests to provide a more definitive diagnosis and ensure appropriate treatment.

NTM culture is crucial for the diagnosis and treatment of NTM-PD [8,24]. In this study, a total of 23 patients tested negative for culture but positive for nanopore sequencing. These patients did not show any positive results for NTM when tested using other PCR techniques. These patients had improved lung lesions without anti-NTM therapy, and no recurrence was observed during follow-up, and we considered this group of patients to be non-NTM-PD. Therefore, we used NTM culture as the reference standard for this study. However, it can take a long time to obtain NTM culture results, which makes it not conducive for early and accurate diagnosis [8]. This delay can lead to misdiagnosis, inadequate treatment, prolonged illness, and even death. Based on the results of the present study, 21.3% of positive *Mycobacterium* specimens were NTM; this finding is similar to the findings of previous studies in similar regions [31]. This result suggests that the infection rate of NTM-PD is relatively high in eastern China, and there is a greater demand for early and accurate detection of NTM-PD in this area. The increased infection rates may be attributed to various factors, such as environmental conditions, population density, and healthcare access [32]. Therefore, it is crucial to develop effective diagnostic tools that can accurately detect and identify NTM-PD in such high-risk regions to facilitate early diagnosis.

Currently, sequencing technology is an advanced means of detecting infectious diseases, and both next-generation sequencing and third-generation sequencing (nanopore sequencing) play increasingly important roles in the early and accurate diagnosis of infectious diseases [23,33,34]. The application of next-generation sequencing for the diagnosis of NTM-PD is gradually increasing and has yielded satisfactory results [35]. In particular, next-generation sequencing is a valuable tool for identifying various NTM species in clinical specimens [35]. Compared with next-generation sequencing, third-generation sequencing has several advantages [20,21]. Third-generation sequencing has excellent diagnostic accuracy for the rapid detection of PTB [21]. However, research into the use of third-generation sequencing for diagnosing NTM-PD is currently lacking, and our study aimed to fill this gap. Notably, this study focused on the application of third-generation sequencing for the diagnosis of NTM-PD in order to provide a more accurate and efficient means of diagnosing this disease.

According to the results of this study, nanopore sequencing demonstrated excellent diagnostic accuracy, sensitivity, and specificity for the detection of NTM-PD, allowing for early and accurate screening of the disease. The results of this study were similar to the diagnostic accuracy of next-generation sequencing for detecting NTM-PD in a previous study [36], but superior to the diagnostic accuracy of real-time PCR for detecting NTM-PD [37]. For the different types of specimens, the results of our study suggest that the accuracy of

nanopore sequencing in sputum is higher than in BALF, which is different from other studies [38]. This may be related to the fact that the present study was a retrospective study and there may have been a bias in the inclusion of patients, where sputum testing was performed on patients with significant symptoms such as coughing and sputum, whereas BALF testing was performed on patients with insignificant symptoms. However, there are some limitations to this method. In this study, the PPV of nanopore sequencing was relatively low, possibly due to PCR amplification of the extracted nucleic acids before nanopore sequencing. This may have caused the FP results to decrease PPV. Further analysis revealed that the median reads of nanopore sequencing for FP were significantly lower than those for TP. Therefore, when interpreting nanopore sequencing reports, higher sequence reads could indicate higher reliability. Accordingly, this finding should be considered when analyzing and interpreting nanopore sequencing data. In six culture-positive patients for NTM, nanopore sequencing for NTM was negative. Among them, three patients had no pathogens detected by nanopore sequencing, whereas one tested positive for *Pseudomonas aeruginosa* and two patients had low sequence numbers for MTB; BALF specimens were used for all six patients. The low content of NTM in these specimens may be attributed to improper lung lavage during bronchoscopy or errors during PCR amplification. Similar to next-generation sequencing, nanopore sequencing can detect NTM sequences as well as directly identify NTM species, which is essential for NTM diagnosis. In the present study, the species type was identified by nanopore sequencing in 84 cases of NTM. Notably, the distribution of species in our study was similar to that in previous studies in similar regions.

Although the present study provides important insight into the use of nanopore sequencing for the initial screening of NTM, it is important to acknowledge the study's limitations. First, the major limitation of the study is patient selection bias, which is often difficult to avoid in retrospective studies. Therefore, the results may not be generalizable to other patient groups or settings. Second, no outcomes related to NTM resistance were evaluated in this study. Third, the study was conducted in a region with a high TB burden; therefore, the results may not be applicable to environments with lower TB burdens. Fourth, the current cost of nanopore sequencing is still high, which limits its clinical application, especially for low-income populations. This could also be a source of bias. Finally, only NTM data were evaluated in the study, and no data related to MTB, the causative agent of TB, were analyzed. Therefore, the results cannot be extrapolated to MTB-related results. Despite these limitations, the results of this study highlight the potential application of nanopore sequencing to diagnose NTM infection. Further studies are needed to confirm and expand these results in other populations and settings. Additional studies that can better guide clinical practice and improve the management of NTM infections are warranted by overcoming these limitations.

CONCLUSION

Our results revealed that clinical specimens analyzed using nanopore sequencing offer high diagnostic accuracy for detecting NTM-PD. Given the reliability of nanopore sequencing, this approach can be recommended as the priority diagnostic tool for detecting NTM-PD. In particular, it is useful in cases where traditional methods are insufficient or yield unreliable results. By prioritizing nanopore sequencing as a diagnostic tool, clinicians can improve the accuracy and efficiency of NTM-PD diagnosis.

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Ethical Approval Statement:
Ethical approval for this study was obtained from the Human Research Ethics Committee of the Hangzhou Red Cross Hospital (2024-YANSHEN-059, May 31, 2024), and this study is in accordance with the guidelines of Declaration of Helsinki. As a retrospective study analyzed using data that already existed, there would be no impact on patients, and therefore informed consent from patients was waived by the Ethics Committee.

Availability of Data and Materials:
Data are available from the corresponding author on reasonable request.

Source of Funds:
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Declaration of Interest:
The authors declare that they have no conflicts of interest.

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