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ORIGINAL RESEARCH

Diagnostic Performance of Nucleotide MALDI-TOF-MS for the Rapid Diagnosis of Pulmonary Tuberculosis and the Detection of Drug Resistance

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Objective: This study aims to evaluate the diagnostic performance of nucleotide MALDI-TOF-MS in the rapid diagnosis of pulmonary tuberculosis and the detection of drug resistance.

Methods: A retrospective study was conducted on suspected pulmonary tuberculosis patients (total of 110) admitted to Affiliated Hospital of Hangzhou Normal University between November 2020 to April 2024. The age of all patients range of the patients was 19 to 90 years, and the mean age was 55.1±20.9 years. Of the enrolled participants, 59 (53.6%) were males and 51 (46.4%) were females. The study involved calculating and comparing the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of nucleotide MALDI-TOF-MS, MGIT960 culture, and Xpert MTB/RIF.

Results: According to the clinical diagnosis reference standard, the sensitivity, specificity, PPV, and NPV of nucleotide MALDI-TOF-MS for detecting Mycobacterium tuberculosis (MTB) were 84.2% (95% CI: 74.4–94.0%), 92.5% (85.1–99.8%), 92.3% (84.8–99.8%), and 84.5% (74.9–94.4%). The MGIT960 culture method served as the reference standard, and the nucleotide MALDI-TOF-MS demonstrated a sensitivity of 100.0% (95% CI: 88.3–100%), specificity of 71.6% (61.6–81.6%), PPV of 55.8% (41.8–69.7%), and NPV of 100.0% (93.8–100.0%) in identifying MTB. Regarding drug resistance, the nucleic acid MALDI-TOF-MS technique shows a high level of agreement with conventional culture methods in identifying resistance to isoniazid, rifampicin, streptomycin and ethambutol.

Conclusion: Nucleotide MALDI-TOF-MS demonstrates good diagnostic efficacy in the rapid diagnosis of pulmonary tuberculosis and the assessment of drug resistance, positioning it as a promising technique for the diagnosis of Mycobacterium tuberculosis.

Keywords: tuberculosis, nucleotide MALDI-TOF-MS, drug resistance

Introduction

The World Health Organization (WHO) identifies tuberculosis (TB) as a persistent and significant global health issue, with over 10 million new cases and more than 1.25 million deaths annually.[1] Despite advancements in diagnostic methodologies, delays in diagnosis remain prevalent. Approximately 25% of tuberculosis cases worldwide in 2023 were not detected or documented, and only 44% of people with multidrug-resistant or rifampicin-resistant tuberculosis (MDR/RR-TB) received suitable treatment.[2] Boosting the detection rates of tuberculosis pathogens and drug-resistant strains is essential for managing and controlling the disease effectively.[3] Therefore, there is an urgent need for the research and development of advanced diagnostic technologies for mycobacterium tuberculosis (MTB).[4,5]

Currently, the differential diagnosis of mycobacteria in clinical laboratories primarily relies on acid-fast staining and mycobacterial Culture techniques. Acid-fast staining offers advantages such as rapid processing, low cost, and ease of operation; however, it suffers from low sensitivity and limited specificity. In contrast, mycobacterium culture is considered the gold standard for tuberculosis diagnosis due to its higher sensitivity compared to acid-fast staining and

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its 100% specificity. Additionally, it facilitates drug susceptibility testing. Nevertheless, Mycobacterium culture is associated with drawbacks, including prolonged processing time, elevated costs, and significant technical demands.[6,7] These drawbacks constitute major impediments to timely disease diagnosis and effective clinical management. A variety of molecular techniques are presently accessible for the expeditious identification of Mycobacterium species. These include matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), real-time polymerase chain reaction (PCR), line probe assays, nucleic acid hybridization assays and DNA sequencing.[8–10] Each of these methods possesses distinct advantages and limitations.[11] In the context of tuberculosis diagnosis, acid-fast staining, Mycobacterium culture, and molecular testing together constitute a comprehensive diagnostic framework. These methods are mutually complementary and sequentially interconnected, each fulfilling a distinct and vital role.

MALDI-TOF-MS represents an innovative molecular diagnostic methodology. Through the execution of multiple PCR-specific amplifications targeting gene segments, followed by single nucleotide extension reactions, the resultant reaction product is integrated with a matrix. Subsequently, a mass spectrum is acquired, yielding critical information regarding species identification and drug resistance.[12] In comparison to conventional diagnostic techniques, MALDI-TOF-MS streamlines the identification process, reduces the time required for identification, and offers advantages such as high throughput, rapidity, sensitivity, high resolution, and cost-effectiveness.[13] Several studies have shown promising applications of this technique in the diagnosis of mycobacterium tuberculosis and non-mycobacterium tuberculosis and their drug resistance.[14–18] Nevertheless, the assessment of this assay using respiratory specimens in practical, real-world settings across various regions in China remains significantly underexplored. In this study, we evaluated the diagnostic performance of the nucleotide MALDI-TOF-MS assay on respiratory specimens collected from patients with a high suspicion of pulmonary tuberculosis (PTB) in the southeastern region of China.

Materials and Methods

Study Design and Participants

Between November 2020 and April 2024, 110 patients suspected of PTB had their bronchoalveolar lavage fluid (BALF) or sputum, collected at the Affiliated Hospital of Hangzhou Normal University. Relevant medical test results and clinical diagnoses were collected from the electronic medical system. This research was carried out following the World Medical Association's Declaration of Helsinki and the Good Clinical Practice Guidelines. Research approval was obtained from the Ethics Committee of the Hangzhou Normal University Affiliated Hospital. The Research Ethics Committee of the study hospital approved the waiver of written informed consent due to the retrospective nature of the study. All patient data have been de-identified to ensure privacy and confidentiality. No personally identifiable information is present in the manuscript, figures, or tables.

The criteria for diagnosing PTB are based on the "Diagnostic Criteria for Pulmonary Tuberculosis WS288-2017". The diagnostic criteria for NTM lung disease refer to "Chinese Medical Association Tuberculosis Branch Guideline for Diagnosis and Treatment of Nontuberculous mycobacteria Disease (2020 Edition)"; Among the 110 patients with a finally clinical diagnosis, 57 were diagnosed with tuberculosis, 51 with non-tuberculous mycobacterial lung disease, and 2 with pneumonia.

MTB Detection via Acid-Fast Staining Technique

Approximately 0.05mL of BALF or sputum was uniformly spread across the surface of a glass microscope slide. The slide was then allowed to air dry before being heat-fixed. Subsequently, the Ziehl-Neelsen staining technique was employed using a Mycobacterium tuberculosis acid-fast staining kit, following the manufacturer's instructions meticulously. Smears that demonstrated a grade of 1+ or higher were considered positive.

Xpert MTB/RIF Assay

The samples were processed on the day of collection, with 1mL tested using an Xpert cartridge in accordance with the manufacturer's guidelines. For MTB/RIF testing, 1.4mL of the MTB/RIF sample reagent was combined with 0.7mL of BALF

or sputum, and subsequently processed following the manufacturer's instructions. Rapid molecular assays were performed utilizing the GeneXpert System (Cepheid, Sunnyvale, CA, USA), adhering to all protocols supplied by the Cepheid company.

MTB Cultured Through the BACTEC MGIT 960 Technique

The specific operation steps of the BACTEC MGIT 960 system are as follows: Step 1: Sample Pre-treatment Digestion: Prepare the NALC-NaOH digestion solution by combining equal volumes of a 4% NaOH solution and a 2.9% sodium citrate solution, followed by the addition of NALC powder to achieve a final concentration of 0.5%. Introduce the sample (eg, sputum) to an equal volume of the digestion solution and mix thoroughly in 50 mL sterile centrifuge tubes using vortexing for 15–30 seconds. Allow the mixture to stand at room temperature for 15 minutes. Centrifugation and Neutralization: Add pre-cooled phosphate-buffered saline (PBS) to neutralize the solution, then centrifuge at 3000×g for 15 minutes. Resuspension: Discard the supernatant, resuspend the precipitate in a small volume of PBS, and prepare a smear for acid-fast staining. Step 2: Inoculation and Machine Loading Add Supplements: Introduce 0.8 mL of MGIT growth supplement (OADC) and 0.1 mL of pathogen inhibition supplement (PANTA) into the MGIT culture tube. Inoculation: Inject 0.5 mL of the treated sample solution into the culture tube. Machine Loading: Thoroughly clean the culture tube and place it in the detection slot of the instrument. Step 3: Result Interpretation A positive result is confirmed if both the instrument and acid-fast staining indicate the presence of acid-fast bacteria. Otherwise, the result is negative. Positive strains underwent further analysis through phenotypic drug susceptibility testing (pDST) for isoniazid (INH), rifampin (RIF), ethambutol (EMB), and streptomycin (S) utilizing the absolute concentration method. The critical concentrations established for rifampin (RIF), isoniazid (INH), ethambutol (EMB), and streptomycin (SM) were 1.0 mg/L, 0.2 mg/L, 5.0 mg/L, and 2.0 mg/L, respectively.

Nucleotide MALDI-TOF-MS Assay

In accordance with the operating instructions for the MassARRAY Analyzer four, both the flight mass spectrometry detection and the subsequent results readout were conducted. The detailed operation procedures can be found in the relevant literature[17] or in the Supplementary Material.

Statistical Analysis

Statistical analyses were conducted utilizing SPSS software, version 25.0, with statistical significance established at a p-value threshold of <0.05. Descriptive statistics were computed and are presented as frequencies (percentages) for categorical variables and as medians with interquartile ranges (IQR) for continuous variables. Sensitivity, specificity, positive predictive value, and negative predictive value for the various tests were assessed in comparison to the reference standard.

Results

Characteristics of Study Population

As shown in the flow chart (Figure 1), we retrospectively screened suspected PTB or NTM lung disease cases who visited Affiliated Hospital of Hangzhou Normal University from November 2020 to April 2024. BACTEC MGIT 960 culture, Xpert MTB/RIF and Nucleotide MALDI-TOF-MS were performed in all patients.

Demographic and clinical features of the study population were presented in Table 1. The age of all patients range of the patients was 19 to 90 years, and the mean age was 55.1±20.9 years. Of the enrolled participants, 59 (53.6%) were males and 51 (46.4%) were females. In the study cohort of 110 patients, MGIT 960 culture results indicated that 29 individuals (26.4%) tested positive for Mycobacterium tuberculosis, whereas 81 individuals (73.6%) tested negative. The Xpert MTB/RIF test identified 39 individuals (35.5%) as MTB positive and 71 individuals (64.5%) as MTB negative. Additionally, the MALDI-TOF-MS test results showed that 48 individuals (43.6%) were MTB positive, while 62 individuals (56.4%) were MTB negative. Among the 110 patients with a finally clinical diagnosis, 57 were diagnosed with tuberculosis, 51 with non-tuberculous mycobacterial lung disease, and 2 with pneumonia.

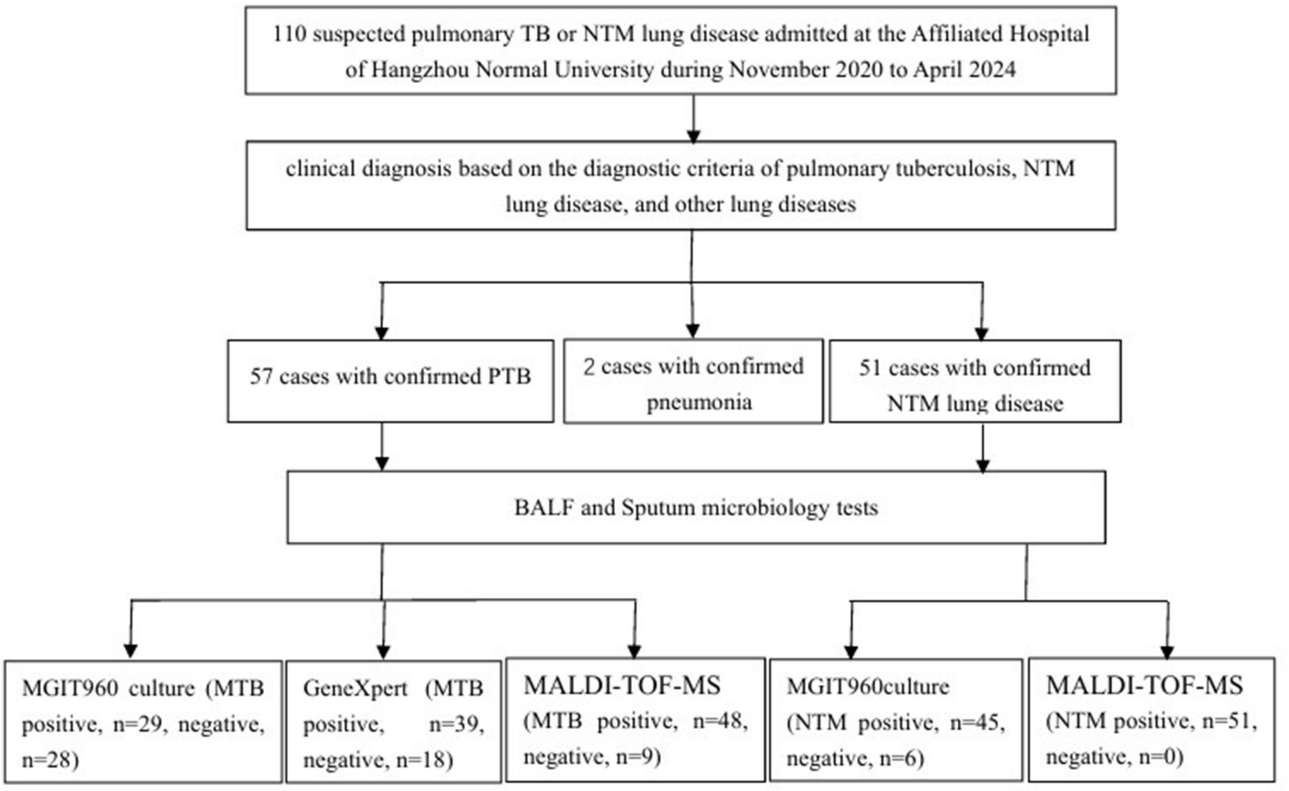

Figure 1 The methodologies employed include the MGIT960 culture medium, the Xpert MTB/RIF detection system, and the detection flowchart of MALDI-TOF-MS technology.

Nucleotide MALDI-TOF-MS's Efficiency in Detecting Pulmonary Tuberculosis Pathogens

Using the MGIT960 culture technique as the benchmark standard, 29 samples (26.4%, 29/110) tested positive for Mycobacterium tuberculosis, while the remaining samples tested negative. The sensitivity and negative predictive value (NPV) of both the nucleotide MALDI-TOF-MS and Xpert MTB/RIF assays were 100%. However, the specificity of the

Table 1 Basic Information of the Study Population

Characteristics	Patients (n = 110)
Age (years)	55.1±20.9
18–39	33 (30.0%)
40–59	19 (17.3%)
60–79	47 (42.7%)
≥80	11 (10.0%)
Sex (n, %)	
Male	59 (53.6%)
Female	51 (46.4%)
Respiratory specimen type (n, %)	
BALF	62 (60.7%)
Sputum	48 (35.7%)
MGIT 960 culture (n, %)	
MTB positive	29 (26.4%)
MTB negative	81 (73.6%)

(*Continued*)

Table 1 (Continued).

Characteristics	Patients (n = 110)
Xpert MTB/RIF (n, %)	
MTB positive	39 (35.5%)
MTB negative	71 (65.5%)
MALDI-TOF-MS	
MTB positive	48 (43.6%)
MTB negative	62 (56.4%)
Clinical diagnosis	
Pulmonary TB	57 (51.8%)
NTM lung disease	51 (46.4%)
Pneumonia	2 (1.8%)

nucleotide MALDI-TOF-MS assay was 71.6% (95% CI: 61.6–81.6%), which is slightly lower than that of the Xpert MTB/RIF assay, which was 85.2% (95% CI: 77.3–93.1%). The positive predictive value (PPV) of the nucleotide MALDI-TOF-MS assay was 55.8% (95% CI: 41.8–69.7%), also slightly lower than that of the Xpert MTB/RIF assay, which was 70.7% (95% CI: 56.2–85.3%) (refer to Table 2). Using the clinical diagnosis as the reference standard, the nucleotide MALDI-TOF-MS assay demonstrated an overall sensitivity of 84.2% (95% CI: 74.4–94.0%), which was significantly higher than that of the MGIT960 culture (50.9%, 95% CI: 37.5–64.3%) and the Xpert MTB/RIF assay (68.4%, 95% CI: 56.0–80.9%). The diagnostic specificities of the MGIT960 culture, Xpert MTB/RIF, and MALDI-TOF-MS were all above 90.0%. The PPV for these methods were 100.0%, 100.0%, and 92.3%, respectively, while their NPV were 74.6%, 74.6%, and 84.5%, respectively (refer to Table 3).

Using Nucleotide MALDI-TOF-MS to Predict Drug Resistance in Pulmonary Tuberculosis

A total of 29 patients in this study were positive for MGIT 960 culture. Utilizing the MGIT 960 culture results, we conducted an analysis to evaluate the efficacy of nucleotide MALDI-TOF-MS in predicting MTB resistance to anti-tuberculosis drugs. Drug susceptibility testing was performed using the absolute concentration method to assess resistance to isoniazid, rifampicin, ethambutol, and streptomycin. The results identified 16 (55.2%, 16/29) strains resistant to isoniazid, 15 (51.7%, 15/29) strains resistant to rifampicin, 8 (27.6%, 8/29) strains resistant to ethambutol, and 12 (41.4%, 12/29) strains resistant to streptomycin. For predicting isoniazid resistance, nucleotide MALDI-TOF-MS showed 81.3% sensitivity, 92.3% specificity, 92.9% PPV, 80.0% NPV, and an overall accuracy of 86.2%. For rifampicin resistance, these values were 93.3%, 92.9%, 93.3%, 92.9%, and 93.1%, respectively. For ethambutol resistance, the values were 50.0%, 100.0%, 100.0%, 84.0%, and 86.2%, respectively. Finally, for streptomycin resistance, the values were 75.0%, 100.0%, 100.0%, 85.0%, and 89.7%, respectively. Using the Kappa test, the consistency between nucleotide MALDI-TOF-MS and the culture method was evaluated, with rifampicin showing the highest agreement at 0.86, followed by streptomycin at 0.78, isoniazid at 0.73, and ethambutol at 0.59 (Table 4).

Table 2 The Diagnostic Effectiveness of the Nucleotide MALDI-TOF-MS Test Using MGIT 960 Culture Outcomes as a Benchmark

Test Methods	Results	MGIT960 Culture		Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)	Accuracy (%, 95% CI)	Kappa (95% CI)
		MTB + (n=29)	MTB − (n=81)						
Xpert MTB/RIF	+	29	12	100.0 (88.3–100.0)	85.2 (77.3–93.1)	70.7 (56.2–85.3)	100.0 (94.7–100.0)	89.1 (83.2–95.0)	0.75 (0.63–0.88)
	−	0	69						
MALDI-TOF-MS	+	29	23	100.0 (88.3–100.0)	71.6 (61.6–81.6)	55.8 (41.8–69.7)	100.0 (93.8–100.0)	79.1 (71.4–86.8)	0.57 (0.43–0.71)
	−	0	58						

Notes: "+": The detection of MTB is positive; "−": The detection of MTB is negative.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

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Table 3 The Diagnostic Effectiveness of Culture, Xpert MTB/RIF, and Nucleotide MALDI-TOF-MS Was Evaluated Using Clinical Diagnosis as the Benchmark

Test Methods	Results	Clinical Diagnosis		Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)	Accuracy (%, 95% CI)	Kappa (95% CI)
		PTB (n=57)	Non-PTB (n=53)						
MGIT960	+	29	0	50.9	100	100.0	74.6	74.5	0.50
culture	−	28	53	(37.5–64.3)	(93.2–100.0)	(88.3–100.0)	(64.3–85.0)	(66.3–82.8)	(0.36–0.64)
Xpert	+	39	0	68.4	100	100.0	74.6	83.6	0.68
MTB/RIF	−	18	53	(56.0–80.9)	(93.2–100.0)	(91.0–100.0)	(64.3–85.0)	(76.6–90.7)	(0.55–0.81)
MALDI-	+	48	4	84.2	92.5	92.3	84.5	88.2	0.76
TOF-MS	−	9	49	(74.4–94.0)	(85.1–99.8)	(84.8–99.8)	(74.9–94.4)	(82.1–94.3)	(0.64–0.88)

Notes: "+": The detection of MTB is positive; "−": The detection of MTB is negative.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Table 4 Evaluation of the Detection Value of MALDI-TOF-MS in the Assessment of Anti-Tuberculosis Drug Resistance

MALDI-TOF-MS	MGIT960 Culture		Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)	Accuracy (%, 95% CI)	Kappa (95% CI)
	Resistant	Sensitive						
INH	16	13						
+	13	1	81.3 (57.1–93.4)	92.3 (64.2–98.5)	92.9 (66.3–98.7)	80.0 (57.1–93.4)	86.2 (69.4–94.4)	0.73 (0.48–0.97)
−	3	12						
RIF	15	14						
+	14	1	93.3 (71.5–99.4)	92.9 (77.9–99.1)	93.3 (71.5–99.4)	92.9 (77.9–99.1)	93.1 (80.6–98.4)	0.86 (0.79–0.94)
−	1	13						
EMB	8	21						
+	4	0	50.0 (25.6–74.4)	100.0 (83.8–100.0)	100.0 (65.1–100.0)	84.0 (67.0–93.4)	86.2 (69.4–94.4)	0.59 (0.25–0.93)
−	4	21						
S	12	17						
+	9	0	75.0 (51.9–89.5)	100.0 (80.6–100.0)	100.0 (65.1–100.0)	85.0 (65.7–94.8)	89.7 (74.5–96.2)	0.78 (0.55–0.97)
−	3	17						

Notes: "+": drug resistant; "−": drug sensitive.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; S, streptomycin.

Discussion

The nucleotide MALDI-TOF-MS assay represents a straightforward, rapid, and multiplexed technique characterized by high specificity, sensitivity, and adaptability across various research domains.[19] This study assessed the diagnostic efficacy of the nucleotide MALDI-TOF-MS assay on respiratory specimens obtained from patients with a strong clinical suspicion of PTB in the southeastern region of China. The findings demonstrated that the nucleotide MALDI-TOF-MS assay exhibits high sensitivity and specificity in the detection of mycobacterium tuberculosis, suggesting its potential as a viable method for mycobacterial identification.

Studies have shown that nucleotide MALDI-TOF-MS is effective in detecting MTB. Li et al found that in 38 patients suspected of pulmonary tuberculosis, 33 were confirmed cases. The positive detection rates for MALDI-TOF-MS, GeneXpert MTB/RIF, BACTEC MGIT960, and acid-fast staining were 72.7%, 63.6%, 54.5%, and 27.3%, respectively.[20] Another study of 87 patients with suspected pulmonary tuberculosis, who were smear-negative or sputum-free, showed that MALDI-TOF-MS had a sensitivity of 68.5%, compared to SAT at 13.0%, Xpert MTB/RIF at 40.7%, and BACTEC MGIT960 at 27.8%.[21] In this study, taking clinical diagnosis as the reference standard, the total sensitivity of MALDI-TOF-MS detection was 84.2%, which was significantly higher than that of MGIT960 culture (50.9%) and Xpert MTB/RIF (68.4%). The results indicate that MALDI-TOF-MS outperforms MGIT960 culture and Xpert MTB/RIF in detecting Mycobacterium tuberculosis, reducing missed detections.

When research relies on clinicians' judgment as the reference standard, it may compromise reliability and validity. The BACTEC MGIT960 culture is the gold standard for diagnosing Mycobacterium tuberculosis, providing more credible results. Wang et al used MALDI-TOF-MS to detect nucleic acids in respiratory samples from 214 suspected tuberculosis patients. With BACTEC MGIT960 as the diagnostic benchmark, tuberculosis was confirmed in 143 cases. The sensitivity of MALDI-TOF-MS for the identification of Mycobacterium tuberculosis was found to be 92.2%, with a specificity of 74.1% and an accuracy of 82.7%.[16] The results of this study are consistent with those of the aforementioned studies. When employing the MGIT960 culture method as the reference standard, the sensitivity, specificity, and accuracy of MALDI-TOF-MS in detecting Mycobacterium tuberculosis were determined to be 100.0%, 71.6%, and 79.1%, respectively.

In 2013, WHO updated the diagnostic criteria for TB, recommending that positive detection results from Xpert MTB/RIF and LAMP-TB technologies be considered as positive bacteriological tests.[20] These results can serve as the basis for diagnosing pathogen-positive TB. Previous studies have demonstrated that Xpert MTB/RIF exhibits high sensitivity and specificity.[22–24]

Our research indicates that using clinical diagnosis as a reference, nucleic acid MALDI-TOF-MS has a sensitivity, specificity, and accuracy of 84.2%, 92.5%, and 88.2%, respectively, while Xpert MTB/RIF scores 68.4%, 100%, and 83.6%. With MGIT960 culture as the reference, both methods show 100% sensitivity, but MALDI-TOF-MS has 71.6% specificity and 79.1% accuracy, compared to Xpert MTB/RIF's 85.2% specificity and 89.1% accuracy. Xpert MTB/RIF performs slightly better for MGIT960-positive patients, but MALDI-TOF-MS excels in clinical diagnosis scenarios, potentially reducing missed diagnoses. More extensive studies are needed to confirm MALDI-TOF-MS's potential in identifying Mycobacterium tuberculosis.

In the context of drug resistance, the nucleotide MALDI-TOF-MS assay demonstrates the ability to directly identify various Mycobacterium species from clinical specimens, while concurrently detecting mutations in genes associated with drug resistance.[25] The findings from this study reveal that the nucleic acid MALDI-TOF-MS method exhibits a high degree of concordance with traditional culture methods in detecting resistance to rifampicin, isoniazid, ethambutol, and streptomycin. With the exception of ethambutol, the Kappa coefficients for these drugs exceed 0.7, and the accuracy rates surpass 86%. These results align with those reported in previous studies, suggesting that the nucleic MALDI-TOF-MS method can reliably predict Mycobacterium tuberculosis resistance to these four drugs, thereby holding significant clinical application potential.[13–15,26,27] A major concern is the discrepancy between phenotypic and genotypic drug resistance, as heteroresistance and strains with low-level resistance could contribute to these differences.[28–30] Furthermore, certain strains exhibit resistance without detectable mutation sites, necessitating further investigation to elucidate the underlying mechanisms of resistance.

Our study is subject to several limitations. Firstly, as a retrospective investigation conducted at a single center, the data and results may lack generalizability and could be subject to bias. Secondly, the study did not account for certain potential confounding factors that could influence TB diagnosis, such as lifestyle factors, or the use of other antibiotics. Third, in instances where discrepancies were observed between the nucleotide MALDI-TOF-MS assay and culture-based detection in identifying drug resistance, sequencing was not performed to elucidate the specific causes of these discrepancies. Fourth, the high cost of this detection method is a significant drawback. This elevated cost restricts the widespread application of the Nucleotide MALDI-TOF-MS assay. To achieve a more precise evaluation of the nucleotide MALDI-TOF-MS assay's performance, future research should involve prospective, multicenter studies.

Disclosure

The authors have no conflicts of interest to declare.

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