Am J Transl Res 2026;18(2):1012-1025
www.ajtr.org /ISSN:1943-8141/AJTR0169329

Original Article

The diagnostic value of targeted next-generation sequencing for smear-negative and sputum-scarce pulmonary tuberculosis

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Received October 14, 2025; Accepted December 30, 2025; Epub February 15, 2026; Published February 28, 2026

Abstract: Objective: To evaluate the diagnostic performance of targeted next-generation sequencing (tNGS) in pulmonary tuberculosis (PTB). Methods: This retrospective study included 164 suspected PTB patients admitted to Anhui Chest Hospital between October 2022 and October 2024. All patients were smear-negative or sputum-scarce. Clinical specimens were tested simultaneously with tNGS, SAT-TB, GeneXpert MTB/RIF, and Mycobacterium tuberculosis (MTB) culture. Results: Among 108 diagnosed PTB patients, the sensitivity of tNGS was significantly superior to that of other methods. The negative predictive value (NPV) and accuracy of tNGS were 66.67% and 82.93%, which were superior to other methods. In bronchoalveolar lavage fluid (BALF) specimens, the positive detection rate of tNGS reached 48.12%, significantly higher than that of other methods (P<0.0001). In pleural effusion specimens, the positive detection rate of tNGS was 38.10%, which was significantly better than that of SAT-TB and GeneXpert MTB/RIF (P<0.01). ROC curve analysis showed that the AUC value of tNGS reached 0.87, significantly higher than that of the culture method, GeneXpert MTB/RIF, and SAT-TB. The combined detection strategy further increased the sensitivity to 79.63%, and the overall diagnostic accuracy reached 86.59%. Conclusions: tNGS demonstrates superior diagnostic performance for tuberculosis diagnosis, particularly in alternative specimens such as BALF and pleural effusion.

Keywords: Mycobacterium tuberculosis, target next-generation sequencing, diagnostics, pulmonary disease

Introduction

Pulmonary tuberculosis (PTB) is a major worldwide public health challenge [1]. According to the World Health Organization (WHO) 'Global Tuberculosis Report 2024', there were more than 10.8 million new cases of tuberculosis worldwide in 2023, and about 1.25 million people died from the disease. It is the leading cause of death from infectious diseases worldwide. In 2023, China reported approximately 741,000 new cases of tuberculosis, ranking third in the world in the number of cases [2]. Delays or misdiagnoses frequently occur due to insufficient sputum specimens or negative acid-fast bacillus (AFB) smears, thereby increasing transmission risk and the overall public health burden [3]. Therefore, it is of great significance to develop new molecular diagnostic technologies with higher sensitivity and broader detection range.

Traditional PTB diagnostics rely on sputum smear microscopy, *Mycobacterium tuberculosis* (MTB) culture, and molecular detection methods [4]. Sputum smear microscopy is widely used because it is simple and inexpensive, but its sensitivity is low, with a positivity rate of only 22-43% [5]. Although MTB culture remains the diagnostic "gold standard", the process requires several weeks, delaying both early diagnosis and timely initiation of therapy [6]. Molecular detection methods based on nucleic acid amplification (such as GeneXpert MTB/RIF) can be completed within a few hours and indicate rifampicin resistance, but the sensitivity in low-volume specimens is still insufficient [7]. For alternative samples such as pleural effusion and bronchoalveolar lavage fluid

https://doi.org/10.62347/JPAB9669

(BALF), the detection performance of existing diagnostic reagents is generally poor, which highlights the urgent need for new molecular detection tools [8].

Next-generation sequencing (NGS) has shown great potential in the detection of infectious disease pathogens [9]. Metagenome-based NGS (mNGS) can perform unbiased detection of all nucleic acids in samples, significantly improving the diagnostic ability of complex pathogen infections [10, 11]. However, the high cost and limited sensitivity limit the conventional application of mNGS technology [12]. In contrast, targeted NGS (tNGS) utilizes specific primers or probes to enrich MTB and related drug resistance gene regions, improving sensitivity and specificity. This approach also boasts a faster turnaround time and lower costs [13]. In addition, tNGS has demonstrated significant advantages in diagnosing patients with smear-negative or oligosputum pulmonary tuberculosis. Especially in alternative specimens such as BALF, its positive detection rate is significantly higher than that of the GeneXpert method. Nanopore targeted sequencing (NTS) can be used for direct detection of Mycobacterium tuberculosis in BALF and it can accurately identify the drug resistance mutation sites for commonly used drugs [14]. In addition, Yang et al. reported that tNGS can effectively detect Mycobacterium tuberculosis DNA in smear and culture-negative pulmonary tuberculosis patients, showing high diagnostic sensitivity and strong clinical application potential [15]. These findings provide evidence-based support for the clinical application of real-time sequencing technology.

There are some problems to be solved. First, most studies have a small sample size and lack of comparison between different specimens. Second, the existing research mainly focuses on mNGS, while the evidence for the application of tNGS is still insufficient. Third, a systematic comparison between targeted sequencing and existing mainstream detection methods is not yet sufficient. To fill the above gap, this study included 164 patients with suspected PTB who were smear-negative or had no sputum. The diagnostic value of tNGS in different types of specimens was evaluated. This study may provide evidence for the clinical application of tNGS in patients with PTB, helping to formulate precise and practical treatment plans.

Materials and methods

Study population

This was a retrospective study. A total of 164 suspected cases of active PTB admitted to Anhui Chest Hospital from October 2022 to October 2024 were included. All cases were patients with negative sputum smears or little sputum. The collected clinical specimens included 10 sputum specimens, 133 bronchoalveolar lavage fluid specimens and 21 pleural effusion specimens. All patients underwent standard diagnostic procedures and were followed up until a final diagnosis was obtained. This study was approved by the Ethics Committee of Anhui Chest Hospital (KJ2024-137).

Inclusion criteria:(1) Age ≥18 years; (2) Patients clinically highly suspected of having PTB, presenting with symptoms related to tuberculosis infection such as subacute cough, fever, night sweats, and weight loss; (3) Chest computed tomography (CT) or X-ray imaging showing suspected tuberculosis lesions such as miliary nodules, patchy shadows, or lung cavities; (4) At least three negative sputum AFB smears, or collection of alternative specimens (BALF or pleural effusion) in patients unable to produce sputum; and (5) Concurrent testing of specimens with tNGS, SAT-TB, GeneXpert MTB/RIF, and MTB culture.

Exclusion criteria: (1) A prior history of PTB diagnosis; (2) Incomplete clinical or laboratory data; (3) Indeterminate clinical diagnosis; (4) Patients who had received anti-tuberculosis treatment before sample collection.

In order to verify the adequacy of the sample size, the post-effect analysis (G*Power 3.1 software) was used for verification: the sensitivity difference between tNGS and GeneXpert (74.07% vs. 16.67%) was used as the main effect index, α=0.05 and β=0.2, and the minimum sample size was calculated to be 126 cases. 164 cases were actually enrolled, and the test efficacy was >80%, indicating that the sample size fully supported the main conclusions.

Clinical diagnostic criteria

PTB diagnosis was established in accordance with the Chinese National Health Industry Standard WS 288-2017 [16]. A diagnosis of PTB was confirmed if any of the following criteria were met: (1) Positive MTB culture or a positive result from a molecular biological assay. (2) Histopathologic findings from a lung tissue biopsy consistent with TB lesions. (3) For clinically suspected cases with negative mycobacterial detection, a confirmed diagnosis was made if pulmonary lesions on imaging showed significant reduction or complete resolution after three months of standardized anti-tuberculosis treatment. Significant absorption is defined as a ≥50% reduction in lesion size on imaging. Treatment adherence is verified through outpatient follow-up and patient medication diaries. All CT/X-ray images were independently assessed by two physicians with experience in respiratory imaging diagnosis using a blinded approach. In cases of disagreement, a third senior-level physician reviewed and determined the final assessment. Criteria for identifying suspected tuberculosis lesions referenced WS 288-2017 Diagnosis of Pulmonary Tuberculosis, encompassing typical manifestations such as miliary nodules, patchy opacities, and cavities. Inter-rater agreement yielded a Kappa coefficient of 0.82 (*P*<0.001), indicating excellent consistency.

Specimen collection

Sputum: Morning, overnight, and spot sputum samples were collected from patients capable of expectorating, with pre-collection mouth rinsing to reduce oral contamination.

BALF: A bronchoscope was passed from the patient's nasal cavity through the pharynx to the trachea and bronchus. A suitable amount of sterile 0.9% sodium chloride solution was injected and the lavage fluid was retrieved. To reduce contamination, the second tube of BALF was sent for testing. In this study, BALF specimen collection strictly adhered to standardised procedures: ① Volume of sterile 0.9% sodium chloride solution instilled: 20-30 mL per lobe, totalling 80-120 mL; ② Volume of fluid recovered: 40-60 mL recovered, with a recovery rate >50%; ③ Dilution factor: Calculated based on the injected and recovered volumes, the dilution factor ranged from 1.5 to 3 times. All specimens fell within this range, with no instances of extreme dilution.

Pleural effusion: Collected under ultrasound-guided thoracentesis with aseptic precautions.

All samples were temporarily stored at 4°C for no more than 8 hours after collection, and then transferred to an ultra-low temperature freezer at -80°C until detection. The sample storage time ranged from 7 to 90 days. In order to evaluate the potential impact of storage time on the detection performance of tNGS, the samples were divided into three groups according to the storage time: short-term (≤30 days), medium-term (31-60 days) and long-term (>60 days). The positive rate of tNGS in each group was compared. The results showed that there was no significant difference in the positive rate of tNGS in each group, indicating that the storage time did not significantly affect the sensitivity of tNGS detection within the time range of this study. All tests used four identical specimens collected from the same patient by the same trained clinician for tNGS, SAT-TB, GeneXpert MTB/RIF detection and MTB culture to ensure sample consistency and comparability of test results.

Detection assays

(1) tNGS: Approximately 1 mL of each sample was heat-inactivated at 95°C for 10 min, followed by centrifugation at 16,000×g for 5 min. The pellet was subjected to DNA extraction and purification using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, USA). DNA concentration and quality were measured with a Qubit Fluorometer 4.0 (Thermo Fisher Scientific, USA). A panel of ~20 bp oligonucleotide probes targeting non-repetitive regions of MTB and nontuberculous mycobacteria (NTM) was designed using an in-house platform and validated by BLASTn searches against the NCBI human and microbial genome databases. The tNGS panel targets specific genomic regions of the MTB complex and common drug resistance-associated genes, which are distinct from the 16S rRNA target of the SAT-TB assay. Reference sequences from 10 MTB and 39 pathogenic NTM strains were retrieved from the NCBI nucleotide database. Multiplex amplification of target sequences was performed on a TGreat Expert gradient thermocycler (Tiangen, China), and qualified PCR librar-

ies were sequenced on the Illumina NextSeq 550 platform (USA). Positive and negative controls (including no-template controls, NTCs) were processed simultaneously through the same tNGS workflow and bioinformatic analysis. Raw data were filtered to remove adapters, low-quality reads, low-complexity reads, and reads shorter than 35 bp. Human-derived reads were removed by mapping to the human reference genome (GRCh38) [17]. The remaining reads were aligned to a reference database of mycobacterial genomes and an antibiotic resistance database using the Burrows-Wheeler Aligner [18]. The number of MTB/NTM reads and reads per million (RPM) were calculated. Positive criteria were defined as follows: MTB/NTM identification: if the RPM of the NTC was non-zero, a sample was considered positive when the sample-to-NTC RPM ratio was ≥5. In this study, the RPM values for NTC ranged from 0 to 1.2, with no instances approaching the threshold.

(2) SAT-TB: Detection of Mycobacterium tuberculosis was performed using a commercial SAT-TB kit (Shanghai Rendu Biotechnology Co., China), which is based on simultaneous isothermal amplification of 16S rRNA and fluorescence detection. Specimens (1.5 mL) were pretreated with NaOH (final concentration 4%) for liquefaction and decontamination. After centrifugation, washing and resuspension, the prepared samples were processed according to the manufacturer's instructions. The nucleic acid was released by ultrasonic treatment, and the amplification reaction was performed in a microcentrifugal tube containing SAT-TB reagent, followed by fluorescence detection using an isothermal fluorescence detector.

(3) GeneXpert MTB/RIF: GeneXpert MTB/RIF testing (Cepheid Company, United States) was carried out in accordance with the procedures. In brief, the specimen was mixed with the sample processing solution at a ratio of 1:2 for liquefaction and inactivation treatment, then it was transferred to a disposable test box. The system automatically completes the remaining process from sample processing to fluorescence detection.

(4) MTB culture: Specimens were processed using the standard N-acetyl-L-cysteine (NALC) - NaOH method for decontamination and concentration. After processing, 0.5 mL of each specimen suspension was inoculated into BBL MGIT tubes and cultured in the BACTEC MGIT 960 system (Becton Dickinson, USA). Tubes were automatically monitored for growth for up to 42 days according to the manufacturer's instructions. Positive cultures were further identified as MTB complex by standard biochemical or molecular methods.

Statistical analysis

Data analysis was performed using SPSS 26.0 and GraphPad Prism 9.0 software. Categorical variables were expressed as frequency and percentage [n (%)], and chi-square test was used for comparison between groups. McNemar test was used to compare the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of tNGS, SAT-TB, GeneXpert MTB/RIF and MTB culture. The diagnostic efficacy of each method was evaluated by drawing a receiver operating characteristic (ROC) curve, and the area under the curve (AUC) and its 95% confidence interval (CI) were calculated. $P < 0.05$ was considered significant.

Results

Patient classification

This study included 164 patients with suspected active PTB. The clinical specimens included 10 sputum samples, 133 BALF samples and 21 pleural effusion samples. Based on the final comprehensive clinical diagnosis, 108 patients were confirmed as PTB, while 56 patients were classified as non-PTB, including 53 with other pulmonary infections and 3 with NTM infections (**Figure 1**).

Baseline characteristics of patients

The baseline clinical characteristics of the 164 patients with suspected PTB are summarized in **Table 1**. The male-to-female ratio differed significantly between the PTB and non-PTB groups ($\chi^2=5.109$, $P=0.024$). Relationship between gender and specimen quality: ① Sputum specimens: The rate of acceptable specimens was 100% for male patients and 100% for female patients, showing no difference. ② BALF specimens: The mean MTB load was 120±50 for male patients and 110±45 for female patients, with no significant

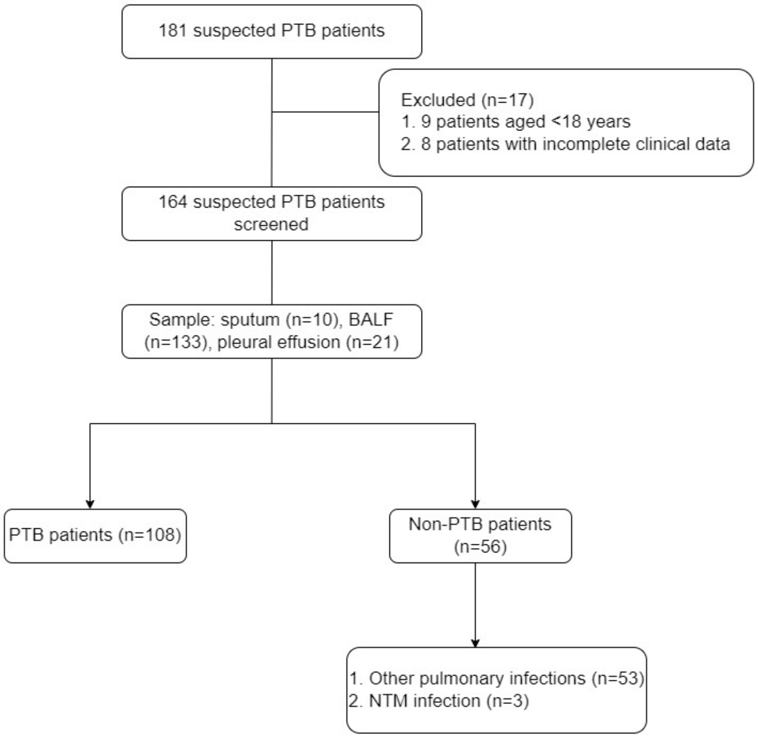

Figure 1. Flow chart of patient enrollment and classification. PTB: pulmonary tuberculosis; BALF: bronchoalveolar lavage fluid; NTM: nontuberculous mycobacteria.

difference (P=0.320). Patients aged ≥65 years were more frequently observed in the non-PTB group, although the difference did not reach significance ($\chi^2=3.106$, P=0.078). Common clinical symptoms such as fever, cough/sputum production, hemoptysis/bloody sputum, and chest tightness/dyspnea showed no significant differences between the two groups (*P*>0.05). In terms of comorbidities and underlying conditions, there were no significant differences between the PTB and non-PTB groups with respect to smoking history, alcohol consumption, hypertension, diabetes, bronchiectasis, malignancy, hypoproteinemia, anemia, or leukopenia (*P*>0.05).

MTB detection results across different diagnostic methods

Of the 108 patients confirmed as PTB by comprehensive clinical diagnosis, 80 tested positive with tNGS, 15 with SAT-TB, 18 with GeneXpert MTB/RIF, and 37 with culture. The corresponding numbers of negative results were 28, 93, 90, and 71, respectively. In contrast, among the 56 patients clinically diagnosed as non-PTB, none of the four assays yielded MTB-positive results, with all results being negative (**Table 2**; **Figure 2**). The tNGS panel employed in this study encompassed core drug resistance genes: rpoB (rifampicin), katG/inhA (isoniazid), embB (ethambutol), and gyrA/gyrB (quinolones). Among 80 tNGS-positive PTB patients, drug resistance gene mutations were detected in 23 cases: 15 rpoB mutations, 6 katG mutations, and 2 embB mutations.

Positive detection rates of MTB across different clinical specimens

The MTB detection results of the four methods in different clinical specimens are shown in **Table 3** and **Figure 3**. In sputum specimens, tNGS demonstrated the highest positive rate (80.00%), followed by GeneXpert MTB/RIF (70.00%), SAT-TB (50.00%), and MTB culture (40.00%). Although tNGS showed a higher pathogen detection rate, the difference between it and the other three methods was not significant. In BALF samples, the positive detection rate of tNGS was 48.12%, which was significantly better than SAT-TB (6.02%), GeneXpert MTB/RIF (6.77%), and MTB culture (20.30%) (P<0.0001). These data strongly confirm the outstanding diagnostic value of tNGS in BALF detection. For pleural effusion samples, the positive detection rate of tNGS reached 38.10%, which was not only significantly higher than SAT-TB and GeneXpert MTB/RIF (P<0.01), but also higher than that of the culture method (28.57%). However, the difference in the latter did not reach a significance. It is noteworthy that despite this study exclusively including challenging cases with negative sputum smears, the culture positivity rate for sputum specimens remained higher than that for BALF and pleural effusion specimens. This may reflect that in patients capable of producing sputum, even when smear-negative, their sputum specimens still harbor a certain quantity of pathogens.

Table 1. Baseline characteristics of patients with suspected PTB

	Total (n=164)	PTB (n=108)	Non-PTB (n=56)	χ^2	*P*
Gender				5.109	0.024
Male	107	77	30		
Female	57	31	26		
Age				3.106	0.078
18-64	114	80	34		
≥65	50	28	22		
Fever				3.507	0.061
No	128	89	39		
Yes	36	19	17		
Cough/sputum production				0.254	0.615
No	63	40	23		
Yes	101	68	33		
Hemoptysis/bloody sputum				0.003	0.956
No	138	91	47		
Yes	26	17	9		
Chest tightness/dyspnea				0.132	0.717
No	120	80	40		
Yes	44	28	16		
Smoking history				2.622	0.103
No	132	83	49		
Yes	32	25	7		
Alcohol consumption				1.869	0.172
No	148	95	53		
Yes	16	13	3		
Hypertension				1.274	0.259
No	139	94	45		
Yes	25	14	11		
Diabetes				0.010	0.922
No	137	90	47		
Yes	27	18	9		
Bronchiectasis				2.512	0.113
No	139	95	44		
Yes	25	13	12		
Malignancy				0.576	0.448
No	159	106	53		
Yes	5	2	3		
Hypoproteinemia				0.738	0.390
No	113	72	41		
Yes	51	36	15		
Anemia				0.976	0.323
No	82	57	25		
Yes	82	51	31		
Leukopenia				0.069	0.792
No	145	96	49		
Yes	19	12	7		

PTB: pulmonary tuberculosis.

Table 2. Detection results of tNGS, SAT-TB, GeneXpert MTB/RIF, and MTB culture in PTB and non-PTB patients

Detection method	Diagnostic result	Number	Clinical comprehensive diagnosis results	
			PTB	Non-PTB
tNGS	Positive	80	80	0
	Negative	84	28	56
	Total	164	108	56
SAT-TB	Positive	15	15	0
	Negative	149	93	56
	Total	164	108	56
GeneXpert MTB/RIF	Positive	18	18	0
	Negative	146	90	56
	Total	164	108	56
MTB culture	Positive	37	37	0
	Negative	127	71	56
	Total	164	108	56

tNGS: targeted next-generation sequencing; MTB: Mycobacterium tuberculosis; PTB: pulmonary tuberculosis.

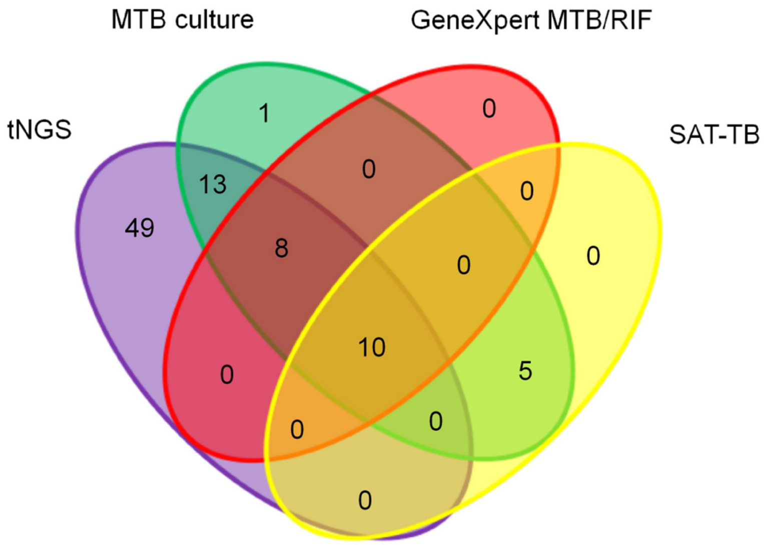

Figure 2. Venn diagram of PTB-positive results obtained for the four assays. tNGS: targeted next-generation sequencing; MTB: Mycobacterium tuberculosis.

Evaluation of diagnostic efficacy for MTB of each detection method

The diagnostic performances of the four detection methods are summarized in **Table 4**. The sensitivity of tNGS reached 74.07%, significantly higher than that of the other three methods (P<0.05). The specificity and PPV of all methods reached 100%. Due to the significant differences in sensitivity among various methods, their NPV and accuracy performances varied. tNGS performed the best in both indicators and was significantly superior to other control methods.

ROC curve analysis

Next, we evaluated the diagnostic effects of each detection method. We present it by drawing the ROC curve. The result of this part is shown in **Figure 4**. The AUC of tNGS is 0.87 (95% CI: 0.83-0.91). The MTB culture method came second, with an AUC of 0.67 (95% CI: 0.63-0.72). The AUCs of GeneXpert MTB/RIF and SAT-TB were 0.58 (95% CI: 0.55-0.62) and 0.57 (95% CI: 0.54-0.60), suggesting that the diagnostic value of both was limited. These results clearly indicate that the comprehensive performance of tNGS for the diagnosis of PTB was superior to that of other conventional detection methods.

Diagnostic efficacy of combined detection

This study evaluated two strategies: parallel detection and series detection. Parallel testing is defined as a positive result if any one of the testing methods is positive, while series testing is determined as positive only when all the testing methods show positive results. As shown in **Table 5**, the results indicate that the parallel detection strategy significantly improves the diagnostic efficiency. Its accuracy rate reached 86.59%, the sensitivity was 79.63%, and it maintained a specificity and PPV of 100%, with a NPV of 71.79%. In contrast, the overall diagnostic efficacy of the serial detection strategy was significantly reduced. Its accuracy, sensitivity, and NPV were only 40.24%, 9.26%, and 36.36%, significantly lower than parallel detection (**Table**

5). Results indicate that parallel testing substantially improved sensitivity by reducing the false-negative rate. Further ROC curve analysis validated these differences: parallel detection achieved an AUC of 0.90 (95% CI: 0.86-0.94), whereas serial detection yielded only 0.55 (95% CI: 0.52-0.5) (**Figure 5**).

Table 3. MTB detection results of tNGS, SAT-TB, GeneXpert MTB/RIF, and MTB culture in different clinical specimens

Sample	Detection method	Diagnostic result	Detection rate (%)
Sputum	tNGS	8/10	80.00
	SAT-TB	5/10	50.00
	GeneXpert MTB/RIF	7/10	70.00
	MTB culture	4/10	40.00
BALF	tNGS	64/133	48.12
	SAT-TB	8/133	6.02
	GeneXpert MTB/RIF	9/133	6.77
	MTB culture	27/133	20.30
Pleural effusion	tNGS	8/21	38.10
	SAT-TB	2/21	9.52
	GeneXpert MTB/RIF	2/21	9.52
	MTB culture	6/21	28.57

tNGS: targeted next-generation sequencing; MTB: Mycobacterium tuberculosis; BALF: bronchoalveolar lavage fluid.

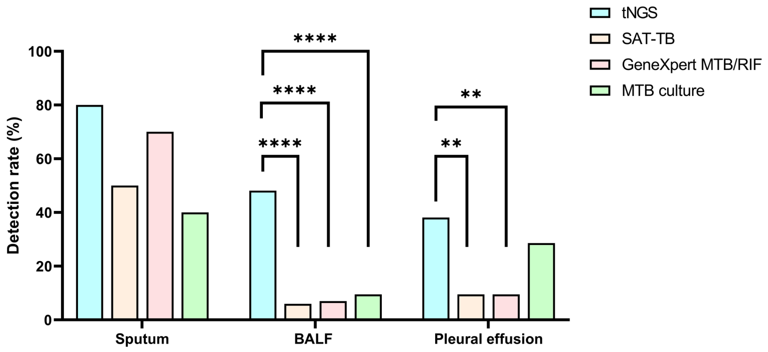

Figure 3. Positive detection rates of MTB across different specimen types using four diagnostic methods: tNGS, SAT-TB, GeneXpert MTB/RIF, and MTB culture. tNGS: targeted next-generation sequencing; MTB: Mycobacterium tuberculosis. ***P*<0.01; *****P*<0.0001 compared to tNGS.

Table 4. Comparative diagnostic performance of tNGS, SAT-TB, GeneXpert MTB/RIF, and culture for MTB

Detection method	Sensitivity (%)	Accuracy (%)	Specificity (%)	PPV (%)	NPV (%)
tNGS	74.07	82.93	100	100	66.67
SAT-TB	13.89*	43.29*	100	100	37.58*
GeneXpert MTB/RIF	16.67*	45.12*	100	100	38.36*
MTB culture	34.26*	56.71*	100	100	44.09*

Sensitivity = true positives ÷ (true positives + false negatives) × 100%; Accuracy = (true positives + true negatives) ÷ total cases × 100%; Specificity = true negatives ÷ (true negatives + false positives) × 100%; PPV = true positives ÷ (true positives + false positives) × 100%; NPV = true negatives ÷ (true negatives + false negatives) × 100%. PPV: positive predictive value; NPV: negative predictive value; tNGS: targeted next-generation sequencing; MTB: Mycobacterium tuberculosis. $^{*}P$<0.05 vs. tNGS.

Discussion

PTB is one of the most important infectious diseases in the world, and its accurate diagnosis is still a major challenge for public health [19]. Traditional Mycobacterium tuberculosis detection methods play an important role in clinical practice, but each method has obvious limitations: the sensitivity of acid-fast staining smears is insufficient, and the culture cycle of strains is long; although GeneXpert and SAT-TB can be quickly detected, the sensitivity is still not ideal in low-volume specimens or non-sputum specimens [20-22]. This issue is particularly pronounced in smear-negative or sputum-absent patients, often leading to missed diagnoses or treatment delays that exacerbate transmission risk and the public health burden. In recent years, NGS has emerged as a promising tool for pathogen detection [23]. This study aimed to evaluate the diagnostic performance of tNGS in different clinical specimens for PTB and compare it to three other methods, in order to provide more evidence for the clinical application of tNGS in PTB diagnosis.

As a rapid pathogen diagnostic tool, the clinical value of

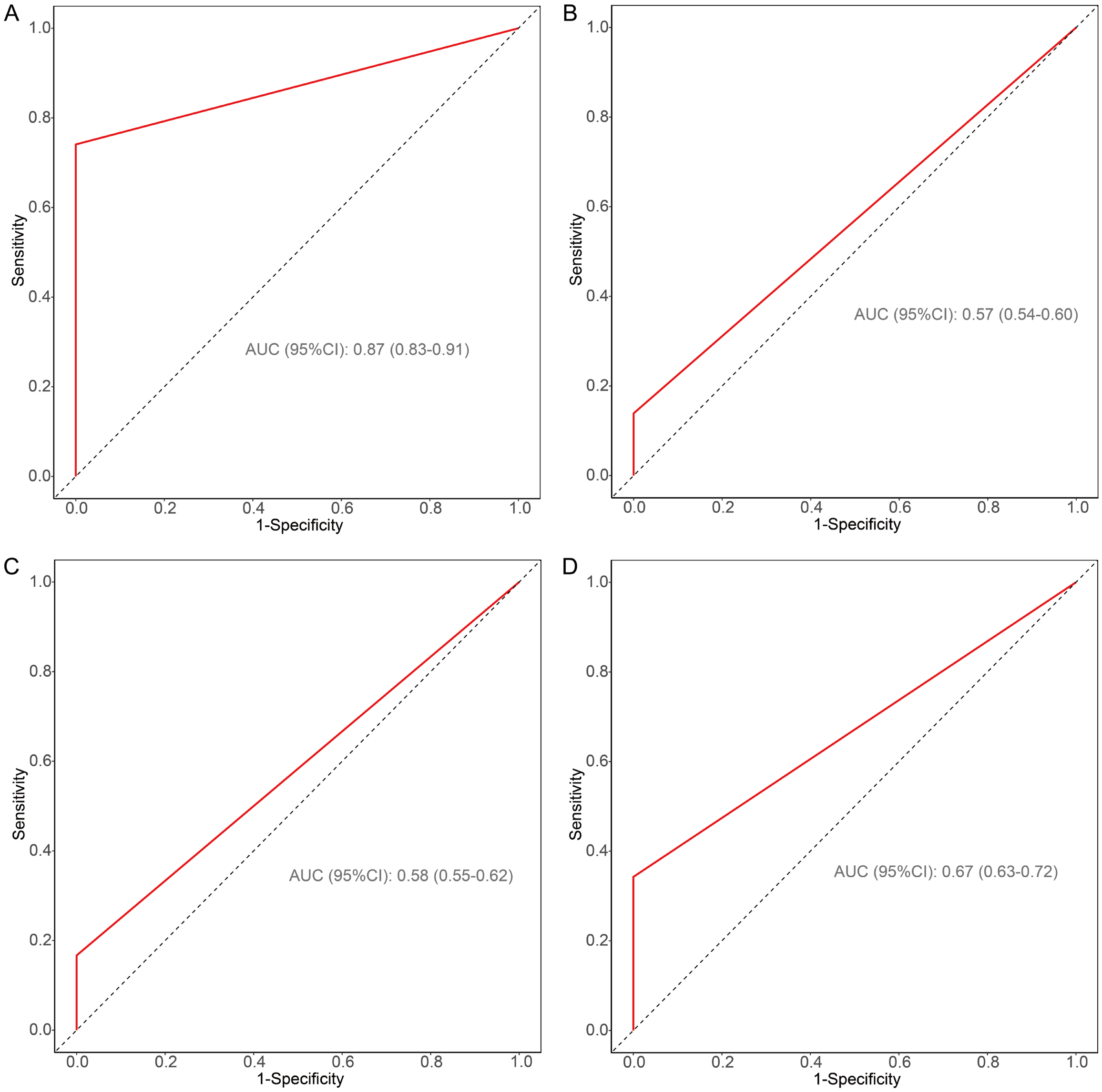

Figure 4. ROC curves of tNGS, SAT-TB, GeneXpert MTB/RIF, and MTB culture for the diagnosis of MTB. A. tNGS; B. SAT-TB; C. GeneXpert MTB/RIF; D. MTB culture. ROC: receiver operating characteristic; tNGS: targeted next-generation sequencing; MTB: Mycobacterium tuberculosis.

Table 5. Diagnostic efficacy of combined detection

Testing strategy	Sensitivity (%)	Accuracy (%)	Specificity (%)	PPV (%)	NPV (%)
Parallel	79.63	86.59	100	100	71.79
Series	9.26*	40.24*	100	100	36.36*

Sensitivity = true positives ÷ (true positives + false negatives) × 100%; Accuracy = (true positives + true negatives) ÷ total cases × 100%; Specificity = true negatives ÷ (true negatives + false positives) × 100%; PPV = true positives ÷ (true positives + false positives) × 100%; NPV = true negatives ÷ (true negatives + false negatives) × 100%. PPV: positive predictive value; NPV: negative predictive value. *$P<0.05$ vs. parallel.

tNGS is gaining increasing recognition. Previous studies have demonstrated that tNGS can identify the vast majority of microorganisms in respiratory specimens, with sensitivity and specificity typically surpassing traditional detection methods [24]. Across multiple clinical settings, tNGS demonstrates promising applications: for instance, Cai et al. found tNGS to be diagnostically compara-

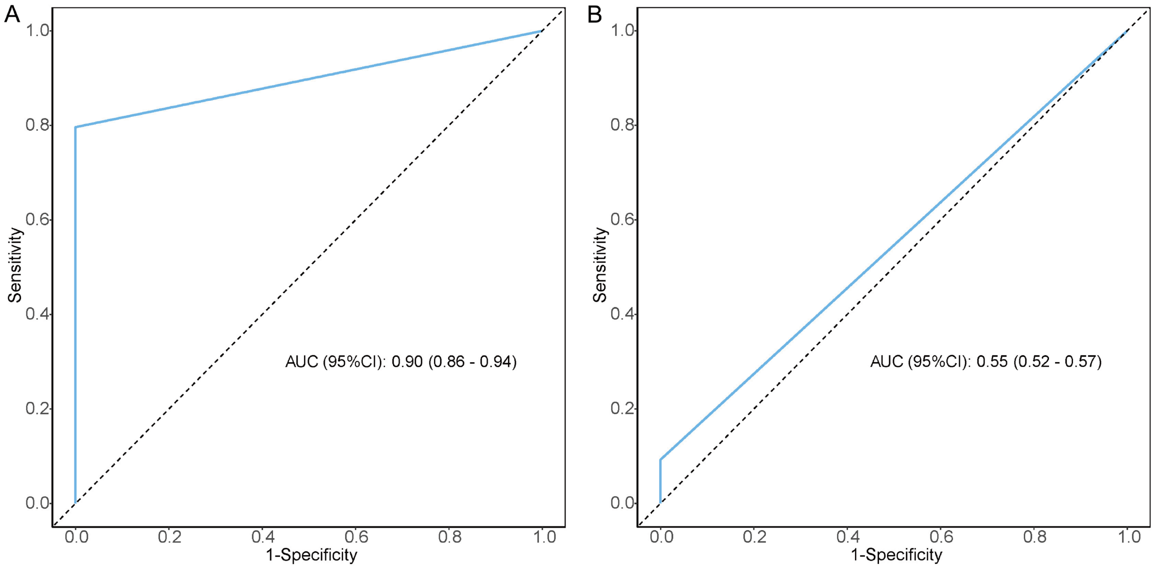

Figure 5. ROC curves of parallel and series detection strategies. A. Parallel; B. Series. AUC: area under the curve; ROC: receiver operating characteristic.

ble to mNGS in bloodstream infections while delivering results and treatment guidance more rapidly [25]. In this study, tNGS exhibited the highest sensitivity among all tested methods, significantly surpassing SAT-TB, GeneXpert, and MTB culture. These findings indicate that tNGS possesses distinct advantages across various specimen types, particularly for smear-negative or low-bacterial-load cases. Huang et al. reached similar conclusions, confirming tNGS's superior sensitivity over GeneXpert and culture methods, effectively reducing missed diagnoses in low-bacterial-load patients [26]. Yang et al. reported that nanopore targeted sequencing (NTS) can directly detect Mycobacterium tuberculosis in clinical specimens without culture, demonstrating high diagnostic accuracy for multidrug-resistant and extensively drug-resistant tuberculosis. Its sensitivity for detecting most anti-tuberculosis drug resistance mutations exceeded 70%, significantly outperforming conventional methods [27]. Wang et al.'s study further showed that tNGS achieved 94.94% sensitivity in confirmed pulmonary tuberculosis patients, with 92.86% concordance with Xpert MTB/RIF for rifampicin resistance detection. Its detection limit reached as low as 10 copies/mL, enabling stable performance even in low-bacterial-load clinical samples. It can simultaneously identify non-tuberculous mycobacteria (NTM) and other respiratory pathogens [28]. Notably, tNGS delivers comprehensive pathogen and resistance profiling within 24-48 hours, enabling early initiation of personalized treatment. In contrast, conventional culture methods require 2-6 weeks, frequently delaying therapeutic decisions [29]. In this study, 28 of 108 clinically confirmed pulmonary tuberculosis patients yielded negative tNGS results. Analysis of these cases revealed concurrent negative outcomes in other molecular assays and cultures, suggesting potentially extremely low Mycobacterium tuberculosis loads in pulmonary lesions - below the detection threshold of tNGS technology. Furthermore, despite DNA quality and sequencing data quality control measures applied to all samples, false-negative results due to uneven distribution of pathogen nucleic acids within samples or variations in extraction efficiency cannot be entirely ruled out. Future studies should employ more sensitive detection techniques or repeat sampling for further validation.

Another finding of this study was that no false positive results were found in all non-PTB patients. The specificity and positive predictive value of tNGS were both 100%. This indicates that when tNGS detects the positive nucleic acid of Mycobacterium tuberculosis, the results are highly consistent with a clinical diagnosis of pulmonary tuberculosis. This finding further confirms the reliability of nucleic acid detection as a diagnostic tool [30]. It is worth noting that tNGS was significantly superior to other detection methods in terms of sensitivity. Therefore, for patients with a high clinical suspicion of PTB, a negative tNGS result can more effectively help rule out the diagnosis of tuberculosis. This further avoids unnecessary empirical anti-tuberculosis treatment.

BALF and pleural effusion have more prominent value in the diagnosis of PTB. The collection of both specimens bypassed the oropharynx. This approach not only reduces the risk of upper respiratory tract microbiota contamination but also more accurately reflects the infection status [31]. The concentration of pathogens here is usually higher, while the content of human nucleic acids is relatively low. These characteristics result in less interference during detection and naturally more reliable results [32]. In contrast, the bacterial content of pleural effusion is low, but it is still of great significance in tuberculous pleurisy. At this time, tNGS technology can improve the detection rate and provide information on drug resistance, thus providing support for diagnosis and treatment [33].

Tao et al. showed that in patients with clinical manifestations suggestive of PTB but negative sputum tests, tNGS analysis of BALF could rapidly and accurately identify MTB and other atypical pathogens. The study highlighted that tNGS achieved a high sensitivity. Effectively overcoming the diagnostic limitations of traditional methods [34]. The study highlighted frequent co-infections and macrolide resistance, underscoring the value of tNGS in complex or low-burden infections [35]. In addition, MTB load in pleural effusion is low, and positivity rates of traditional methods are generally unsatisfactory [36]. Zheng et al. found that GeneXpert and culture showed low positivity in pleural effusion. tNGS can detect MTB and drug resistance sites and is valuable for the diagnosis of smear-negative tuberculous pleurisy [33]. However, when tNGS detects fungi or viruses, interpretation still requires careful integration of patient immune status and imaging findings; otherwise, overdiagnosis may occur [37]. Although tNGS has shown excellent efficacy, our combined test results support the clinical strategy of integrating different detection methods. We believe that this method is of great value for difficult cases with clinically suspected pulmonary tuberculosis but negative single test results.

Despite its promising performance, challenges remain for broader clinical adoption of tNGS. First, our study was retrospective, single-centered, and included a relatively small sample size, especially for sputum and pleural effusion specimens, limiting generalizability. Although the *post hoc* statistical power analysis showed that the overall sample size of this study was sufficient to support the main conclusions about the overall diagnostic performance of tNGS, in the subgroup analysis of different specimen types (especially sputum specimens), a smaller sample size may lead to insufficient statistical power. Larger, multicenter, prospective studies are required to validate these findings and further explore the role of tNGS in resistance profiling and differentiation of colonization from infection. Second is the cost issue. Although the cost of tNGS is lower than mNGS, it is still higher than GeneXpert and culture, which limits its application in grassroots and resource-limited areas. Therefore, we believe that cost-effectiveness analysis is also needed in the future to evaluate the feasibility of tNGS promotion in different regions and medical environments. Third, the volume of saline instilled and retrieved for BALF was not standardized, and the degree of contamination was not quantitatively assessed, which might have introduced variability. Finally, despite tNGS demonstrating excellent diagnostic performance, its cost remains a factor to consider for clinical implementation. Based on our center's experience, the reagent cost for a single tNGS test is approximately RMB 800-1000, higher than that of GeneXpert MTB/RIF and SAT-TB. When factoring in instrument depreciation and labor costs, the total expense increases further. However, a comprehensive health economic assessment should consider its indirect benefits as a whole, such as the cost avoided through early accurate diagnosis, including the cost of misdiagnosis and treatment, the saving brought by shortening the length of hospital stay, and the social benefits generated by blocking the spread of the disease. Future prospective studies need to systematically evaluate the cost-effectiveness of tNGS for tuberculosis diagnosis, especially its feasibility in different levels of medical institutions. We expect that with the maturity of technology and the reduction of costs, tNGS can be gradually incorporated into the routine diagnostic process of tuberculosis. If the above problems can be effectively solved, tNGS is expected to become an important component of the PTB diagnostic system. At that time, this technology will not only be popularized and applied in primary hospitals, but also help to solve the problem of difficult diagnosis of pulmonary tuberculosis, and it is expected to supplement the existing methods. On this basis, with the technical advantage of quickly providing drug resistance information, tNGS will further improve the level of individualized treatment of tuberculosis, and ultimately provide more accurate and efficient diagnostic support for the prevention and control of PTB.

Conclusion

tNGS has excellent diagnostic efficacy for pulmonary tuberculosis, especially in key specimens such as BALF and pleural effusion. Our research results have laid a solid foundation for the clinical application of tNGS. Future research should focus on multi-center verification, cost-effectiveness evaluation, and clinical application expansion to promote the wider application of tNGS for PTB.

Acknowledgements

This work was supported by Anhui Provincial Natural Science Foundation Project (2208085MH193).

Disclosure of conflict of interest

None.

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