Infection and Drug Resistance

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ORIGINAL RESEARCH

Application Research of Targeted Next-Generation Sequencing Technology Based on Bronchoalveolar Lavage Fluid in the Diagnosis of Pulmonary Tuberculosis and Drug-Resistance Detection

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Purpose: To evaluate the clinical value of targeted next-generation sequencing (tNGS) using bronchoalveolar lavage fluid (BALF) in diagnosing pulmonary tuberculosis (PTB) and detecting resistance to first-line anti-tuberculosis drugs, and to compare its performance with traditional methods.

Patients and Methods: In this study, BALF samples were collected from 258 patients with suspected PTB and subjected to AFB staining, mycobacterial solid culture, TB-DNA PCR, Xpert MTB/RIF, and tNGS. Using comprehensive clinical diagnosis or phenotypic DST as the reference, we evaluated the sensitivity, specificity, and other metrics of each method and assessed tNGS performance in detecting resistance to rifampicin, isoniazid, streptomycin, and ethambutol.

Results: Overall, tNGS achieved a sensitivity of 91.10%, specificity of 89.70%, and an AUC of 0.904, all significantly higher than traditional methods. It uniquely identified 24 positive cases missed by other methods. In smear-negative patients, sensitivity remained high at 88.10%, with an AUC of 0.891. For drug-resistance detection, tNGS showed the best performance in judging isoniazid resistance (sensitivity 83.10%, specificity 90.30%), and also showed high negative predictive values for rifampicin, streptomycin, and ethambutol (all > 94%).

Conclusion: tNGS provides excellent diagnostic accuracy for PTB and reliable detection of drug-resistant mutations from BALF samples. It is particularly suitable for smear-negative cases and for resistance screening, making it a powerful complement to the existing TB diagnosis system with high clinical application potential.

Keywords: targeted next-generation sequencing, bronchoalveolar lavage, tuberculosis, drug resistance, diagnostic efficacy

Introduction

Pulmonary tuberculosis (PTB) is a chronic infectious lung disease caused by Mycobacterium tuberculosis (Mtb), and remains one of the major challenges to global public health.[1] According to the latest World Health Organization (WHO) statistics, there were an estimated 10.8 million TB cases worldwide in 2023, with 1.25 million deaths, making TB once again the leading cause of mortality from a single infectious disease.[2] Early diagnosis and timely treatment are crucial for reducing transmission and improving survival. Despite advances, PTB diagnosis still faces many bottlenecks. Smear microscopy has low sensitivity, while culture, though highly specific, requires several weeks. Molecular assays such as Xpert MTB/RIF have improved detection speed but are limited in species identification, mixed infections, and

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comprehensive screening of drug-resistant genes.[3,4] Drug-resistant TB (DR-TB) further complicates control efforts. The latest WHO report highlights that multidrug-resistant and rifampicin-resistant TB (MDR/RR-TB) remains a global health crisis. Although the treatment success rate has increased to 68%, only 44% of MDR/RR-TB patients are currently diagnosed and treated.[2] Treatment of DR-TB is prolonged, costly, and often ineffective, making early detection of resistance mutations essential for guiding individualized therapy. Phenotypic drug susceptibility testing (DST), although considered the gold standard, is time-consuming and technically demanding, limiting its clinical application.[5] Rapid molecular methods such as Xpert MTB/RIF can detect some resistance mutations, but have restricted genomic coverage and cannot fully characterize resistance patterns. Therefore, there is an urgent need for rapid and accurate diagnostic techniques that can simultaneously detect Mtb and identify drug-resistance mutations, thereby providing more comprehensive information for clinical management and TB control.

In recent years, with the rapid development of molecular biology techniques, targeted next-generation sequencing (tNGS) has become a research hotspot for pathogen detection and drug-resistance analysis. By efficiently capturing nucleic acid sequences of target pathogens, tNGS combines wide coverage with high sensitivity. The continued evolution of tNGS is closing the clinical gap that conventional methods cannot precisely bridge: in complex infections such as severe pneumonia, a single assay can simultaneously capture the broad spectrum of viral co-infections and secondary bacterial/fungal pathogens,[6] providing an accurate etiologic diagnosis and individualized antimicrobial guidance for the increasingly intricate respiratory infections of the post–COVID-19 era.[7] It can simultaneously detect the Mtb complex and multiple drug-resistance-related genes, while also identifying non-tuberculous mycobacteria (NTM) and co-pathogens, thereby improving the both diagnostic accuracy and breadth.[7–10] Unlike metagenomic sequencing (mNGS), tNGS reduces background interference through enrichment optimization while retaining broad-spectrum detection capability. This makes it particularly suitable for diagnosing TB in difficult, critically ill, and immunocompromised patients, providing a more efficient and comprehensive molecular etiological solution for clinical practice.[11–13] tNGS also offers a favorable balance between specificity and cost. By using pre-designed probes or primers, it can analyze multiple known drug resistance sites in a single sequencing run, significantly shortening the time from sample collection to results and providing timely support for clinical decision-making. This optimization improves detection efficiency, reduces operational complexity, and lowers cost, thereby increasing its potential for widespread clinical application.[14] In drug-resistance analysis, by focusing on known resistance-associated loci, tNGS provides accurate and reliable results that support the design of individualized treatment strategies. Bronchoalveolar lavage fluid (BALF), obtained through bronchoscopy, directly reflects lower respiratory tract infections and is considered an ideal specimen for TB diagnosis. Compared with sputum, BALF demonstrates higher detection rates of Mtb and is especially valuable in smear-negative cases or when sputum samples are difficult to obtain.[15,16]

Based on this background, this present study aimed to explore the application of BALF-based tNGS in diagnosing PTB and detecting drug resistance.

Specifically, we assessed diagnostic indicators such as the sensitivity, specificity, and the area under the receiver operating characteristic curve (AUC), compared tNGS with traditional diagnostic methods, and analyzed drug-resistance genes profiles in BALF samples. In this study, we provided accurate molecular evidence to guide clinical treatment and technical support for the precise prevention and control of TB and infection-control protocols.

Materials and Methods

Subject Enrollment

This retrospective study included 258 patients admitted to the Tuberculosis Department of Nanning Fourth People's Hospital between October 2021 to May 2025 with suspected PTB. ALF samples were collected, and duplicate strains were excluded to ensure the independence and accuracy of the data. The study protocol complied with the Declaration of Helsinki. All data were anonymized to protect patient privacy. Because of the retrospective design, informed consent was waived. Ethical approval was obtained from the Medical Ethics Committee of Nanning Fourth People's Hospital (Approval No.: [2023]24).

Inclusion criteria were as follows: First, typical PTB-related symptoms such as chronic cough, sputum production, fatigue, low-grade fever, night sweats, and weight loss, with abnormal pulmonary findings on physical examination (eg, diminished breath sounds, abnormal percussion dullness). Second, chest imaging (X-ray or CT scan) showing characteristic TB lesions, such as pulmonary nodules, infiltrates, or cavities. Finally, inadequate or absent sputum samples, making effective testing with traditional sputum-based methods impractical.

Diagnostic classification followed the Diagnosis of Pulmonary Tuberculosis (WS 288–2017) standard issued by the Chinese Center for Disease Control and Prevention. Patients were diagnosed with PTB if they met one of the following criteria. Positive etiological results for Mtb by Xpert MTB/RIF test, mycobacterial solid culture, or smear microscopy.

In the absence of molecular or microbiological evidence, were considered PTB cases if they fulfilled clinical diagnostic criteria, including: epidemiological contact with confirmed TB cases; persistent cough for more than 2 weeks, blood in sputum or hemoptysis, or other suspicious symptoms (eg, night sweats, fatigue, intermittent or persistent afternoon low-grade fever, loss of appetite, or weight loss).; chest imaging showing lesions consistent with TB; pathological evidence of epithelioid granulomatous inflammation, with necrotic and non-necrotic granulomas observed microscopically; immunological examination, such as a positive tuberculin skin test, interferon-γ release assay, or Mtb antibody test; clinical improvement after empirical anti-TB therapy, defined as significant lesions reduction or disappearance on imaging after 3 months.

BALF Sample Collection

Before BALF collection, all patients underwent comprehensive preoperative evaluation, including medical history, physical examination, laboratory tests (blood routine, coagulation function, electrocardiogram, etc), and chest imaging examinations (X-ray or CT) to exclude contraindications for bronchoscopy (eg, severe cardiopulmonary insufficiency, coagulation dysfunction, or inability to tolerate the procedure). Patients fasted for 6 h and abstained from water for 2 h prior to the procedure. The purpose, process, and potential risks were explained in detail to all participants. Local anesthesia with 2% lidocaine (nasal and pharyngeal spray, with aerosolized airway anesthesia if needed) was combined with sedation using midazolam or fentanyl, adjusted for body weight and tolerance. Patients were positioned supine with the head slightly extended. Continuous monitoring of heart rate, blood pressure, and oxygen saturation was maintained throughout the procedure.

A flexible bronchoscope was introduced transnasally or orally and advanced into the tracheobronchial tree under direct visualization. Lesion sites were identified according to imaging and endoscopic findings, with lavage typically performed in affected sub-segments of the right or left lower lobe. The bronchoscope tip was wedged into the target bronchus, and pre-warmed sterile saline (37°C, 50–100 mL each, total 100–200 mL) was instilled via the working channel. The fluid was immediately aspirated under negative pressure into a sterile container, with a typical recovery rate of 40%-60%. When recovery was inadequate, lavage site or volume was adjusted. Samples were immediately stored at 4°C and processed within 24 hours. For long-term storage, a protective agent was added and samples were stored at −80°C. All procedures were performed by experienced respiratory physicians following aseptic technique and infection-control protocols. Detailed records were maintained, including lavage site, lavage volume, recovery volume, and sample processing.

Acid-Fast Bacilli Staining

In this experiment, the fluorescent auramine O staining method was used to detection of acid-fast bacilli. Samples were prepared using standard smear techniques, and the resulting slides were heat-fixed by flame. The staining reagents were obtained from a commercial kit provided by Zhuhai Bioso Biotechnology Co., Ltd., which included auramine O staining solution, acid-ethanol decolorizing solution, and a 5% potassium permanganate counterstain. During staining, the auramine O solution was evenly applied to the surface of each slide, and left to incubate for 30 minutes. The slides were then gently with running water. For decolorization, the acid-ethanol solution was applied to completely covered the slide, allowed to act for 3 minutes (or until visible discoloration was complete), and rinsed again with running water. In the counterstaining stage, the potassium permanganate solution was applied for 1 minute. Excess liquid was removed, and the slides were again rinsed with water and left to air-dry at room temperature. Microscopic examination was

performed under dark-field fluorescence conditions. Acid-fast bacilli exhibited characteristic bright yellow fluorescence and appeared as slightly curved, rod-shaped organisms.

TB-DNA Detection

For each patient, 0.5–3 mL of BALF was collected for TB-DNA detection. TB-DNA detection in this study was conducted using dual PCR and Taqman probe technology. Specific primers and probes were designed and optimized to target specific gene sequences of Mtb. Different fluorescent labels were used to distinguish the signal channels, allowing for the simultaneous and accurate identification of the Mycobacterium tuberculosis complex and nontuberculous mycobacteria. Three high-performance real-time PCR instruments were used in the experiment by the 7500 Real-Time PCR System (Applied Biosystems, USA), and the SLAN-96S and SLAN-96P systems (Shanghai Hongshi Biotechnology Co., China). The detection reagents were sourced from a dedicated PCR-fluorescent probe kit provided by Chengdu Biochip Biotech Co., Ltd., which was specially optimized for high accuracy and specificity in the molecular identification of mycobacteria.[4]

Xpert MTB/RIF

The Xpert MTB/RIF assay was performed strictly in accordance with standardized protocols. During sample preparation, 2 mL of the pre-treated liquefied sample was accurately aspirated using a dedicated pipette. Care was taken to control the liquid flow rate during transfer to the test cartridge to minimize the risk of aerosol generation. Following sample loading, the detection program was initiated within 30 minutes to ensure the accuracy and reliability of the test results.

Mycobacterial Solid Culture and Phenotypic Drug-Susceptibility Testing

A 4% (w/v) NaOH solution was prepared by dissolving 4 g of NaOH in 100 mL of distilled water and sterilized by autoclaving for subsequent use. A 5 mL aliquot of the test sample was mixed with an equal volume of 4% NaOH, vortexed for 20 seconds, and allowed to stand at room temperature for 15 minutes. The alkaline mixture was then neutralized with phosphate-buffered saline (PBS, pH 7.2), and the suspension was centrifuged to collect the precipitate. All processing steps were completed within 20 minutes from the initial addition of NaOH. A 0.1 mL aliquot of the processed sample was aseptically inoculated onto the surface of modified Lowenstein–Jensen medium (Zhuhai Bioso Technology Co., Ltd.; 50 tubes/box). The cultures were observed on the 3rd and 7th days post-inoculation and then weekly thereafter. Positive results confirmed by smear microscopy were reported immediately. Isolates showing positive growth within 7 days were classified as rapidly growing mycobacteria, whereas those requiring more than 7 days were classified as slowly growing mycobacteria. Cultures showing no growth by the end of 8 weeks were considered negative. Phenotypic drug susceptibility testing was conducted following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and the WHO, using the proportion method on the Lowenstein-Jensen medium (provided by Zhuhai Baso Biotechnology Co., Ltd). For bacterial suspension preparation, isolates were harvested from the culture medium and centrifuged at 4000×g for 15 minutes. The resulting pellet was resuspended in sterile Middlebrook 7H9 medium containing 0.5% polysorbate 80. The suspension was then adjusted to match a McFarland No. 1 turbidity standard, resulting in a final bacterial concentration of 1 mg/mL. The tested drugs and their concentrations were isoniazid (0.2 mg/L), rifampicin (40.0 mg/L), ethambutol (2.0 mg/L), and streptomycin (4.0 mg/L). Using a standard inoculation loop (0.01 mL) to streak the 10^{-2} and 10^{-4} mg/mL dilutions onto both drug-containing and control media. Using a pipette to aspirate 0.1 mL of the 10^{-3} and 10^{-5} mg/mL bacterial suspensions onto the media, followed by gentle rotation to ensure even distribution. Both inoculation methods ensured final inoculum densities of 10^{-4} and 10^{-6} mg per tube. Inoculated media were incubated at 35–37°C, and results were recorded after 4 weeks. Drug resistance was determined based on the resistance rate, calculated as follow: Drug-resistance rate = (number of growing colonies/number of growing colonies on the control medium) × 100%. A resistance rate >1% was interpreted as drug-resistant, whereas ≤1% was considered drug-sensitive.

Targeted Next-Generation Sequencing Analysis

tNGS in this study was performed in collaboration with Hangzhou Shengting Medical Technology Co., Ltd. Samples were first liquefied and pre-treated, then lysed with 2% NALC-NaOH at 37 °C for 15 min. After adding 20 mg/mL proteinase K, 20 mg/mL lysozyme and plasmid-extraction reagents, the mixture was incubated at 56 °C for 30 min. The

processed sample was transferred into the reaction system, incubated, mixed with 2 volumes of absolute ethanol, vortexed and centrifuged; the pellet was moved to an EP tube. Magnetic beads were added, vortexed and left standing for 10 min, the supernatant was discarded, and 200 μL of 1×WB buffer plus an equal volume of ethanol were added. Following another supernatant removal, the beads were activated and incubated for 5 min. The extracted nucleic acids were subjected to quality controlled using a Qubit 4.0 fluorometer to ensure they met the criteria for library construction. A PCR reaction system was prepared using the extracted template DNA, and the reaction was carried out using an ABI/Bio-Rad PCR instrument (USA). Following PCR amplification, the products were purified and used for library construction. The multiplex PCR products were then barcoded, stored, purified, and subjected to quality control. Sequencing was performed using the Illumina MiseqDX high-throughput platform. Raw sequencing data were filtered and aligned to reference databases for the identification of pathogenic microorganisms and their drug-resistance genes. A result was considered positive if at least one read could be accurately mapped at the species or genus level. The drug-resistance-related mutations analyzed in this study were obtained from published literature. By specifically capturing the drug-resistance-associated loci of Mycobacterium tuberculosis, subjecting them to high-throughput sequencing, and comparing the data with established resistance-associated variants, we can predict phenotypic resistance. The targeted regions included the isoniazid-resistance genes inhA, katG, and ahpC; the rifampicin-resistance genes rpoB and rpoC; the ethambutol-resistance genes embA, embB, and embC; and the streptomycin-resistance genes rpsL and rrs.[17–19]

Statistical Analysis

Statistical analysis was performed using SPSS 20.0. The following diagnostic performance metrics were calculated sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), Kappa coefficient, and the area under the receiver operating characteristic curve (AUC). The McNemar chi-square test was used to compare the diagnostic efficacy of tNGS with other methods including AFB staining, TB-DNA detection, Xpert MTB/RIF, and mycobacterial solid culture. Continuous variables with a normal distribution were expressed as the mean ± standard deviation (SD), and categorical variables were expressed as frequencies and percentages (n [%]). The McNemar χ^2-test was used to compare group differences. For the evaluation of tNGS in detecting drug-resistant TB, phenotypic drug-susceptibility testing served as the reference standard. Relevant statistical indicators were computed accordingly. A two-sided P-value of less than 0.05 was considered statistically significant.

Results

Basic Information of Patients

A total of 258 patients were included in this study and divided into the TB group (n = 180) and the Non-TB group (n = 78) according to diagnostic criteria. Among the participants, 173 were male and 85 were female. The mean age was 51.03±15.83 years. The mean age in the TB group was 49.01±16.00 years, and that in the Non-TB group was 55.71±14.48 years. Details regarding comorbidities and clinical characteristics are summarized in Table 1.

Comparison of Diagnostic Performance Among tNGS and Other Detection Methods

As shown in Table 2, five diagnostic methods were compared. tNGS exhibited the highest overall diagnostic performance, with a sensitivity of 91.10%, specificity of 89.70%, an AUC of 0.904 (95% CI: 0.858–0.950), a Kappa coefficient of 0.786, indicating excellent agreement with the final diagnosis. In contrast, AFB staining showed the lowest sensitivity (39.40%), and an AUC of 0.652 (95% CI: 0.584~0.720). The AUC values of culture, TB-DNA, and Xpert MTB/RIF were similar, which were 0.824, 0.823, and 0.840, respectively. Among these three, Xpert MTB/RIF had slightly better sensitivity (70.60%) and a Kappa coefficient of 0.574. Importantly, tNGS independently identified 24 additional TB-positive cases that were missed by other methods, demonstrating its strong supplementary value in comprehensive diagnostic workflows (Figure 1).

Table 1 Patient Characteristics

Clinical Characteristics	Total (n=258)	TB (n=180)	Non-TB (n=78)
Gender			
Male	173	123	50
Female	85	57	28
Age	51.03 ± 15.83	49.01 ± 16.00	55.71 ± 14.48
Comorbidities			
NTM	9	1	8
Diabetes	39	35	4
HIV	43	15	28

Notes: "TB" refers to the number of cases confirmed as tuberculosis after comprehensive clinical evaluation according to the diagnostic criteria listed in the "Methods" section of this article.
Abbreviations: TB, tuberculosis; NTM, nontuberculous mycobacteria; HIV, human immunodeficiency virus.

Table 2 Comparison of Diagnostic Efficacy Among Different Detection Methods

Detection Method	Final Diagnosis		Sensitivity	Specificity	PPV	NPV	χ^2	Kappa	AUC (95% CI)
	Negative	Positive							
tNGS									
Negative	70	16	91.10%	89.70%	95.30%	81.40%	160.092	0.786	0.904(0.858~0.950)
Positive	8	164							
AFB									
Negative	71	109	39.40%	91.00%	91.00%	39.40%	23.953	0.222	0.652(0.584~0.720)
Positive	7	71							
Culture									
Negative	77	61	66.10%	98.70%	99.20%	55.80%	91.932	0.532	0.824(0.775~0.874)
Positive	1	119							
DNA									
Negative	76	59	67.20%	97.40%	98.40%	56.30%	91.2	0.536	0.823(0.773~0.874)
Positive	2	121							
Xpert									
Negative	76	53	70.60%	97.40%	98.40%	58.90%	100.627	0.574	0.840(0.792~0.888)
Positive	2	127							

Abbreviations: tNGS: targeted next-generation sequencing; AFB: acid-fast bacilli; PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve.

Comparison of Diagnostic Performance Between tNGS and Other Detection Methods in Smear - Negative Patients

In smear-negative patients, tNGS demonstrated superior diagnostic performance. It achieved a sensitivity of 88.10%, specificity of 90.10%, AUC of 0.891 (95% CI: 0.838–0.945), and a Kappa coefficient of 0.771, indicating high concordance

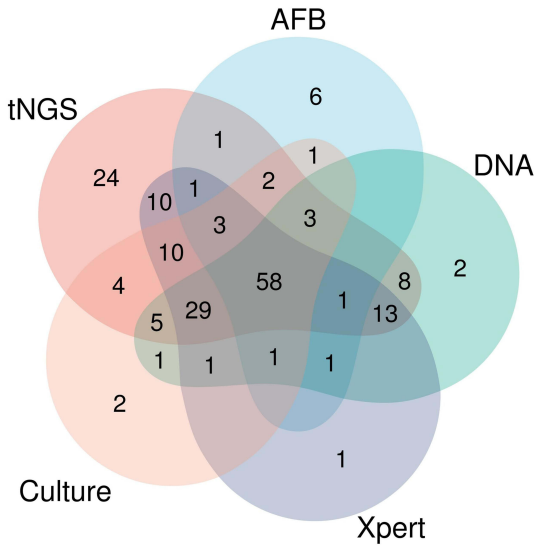

Figure 1 Venn diagram of positive cases.
Notes: Each value indicates the number of individuals who met the corresponding detection criterion and tested positive.

with final clinical diagnoses (Table 3). While culture, TB-DNA, and Xpert showed perfect specificity and PPV (100%), their sensitivities were generally lower, 47.70%, 54.10%, and 58.70%, respectively, and their AUC values were also lower than that of tNGS (0.739, 0.771, and 0.794, respectively). These findings suggest that tNGS offers a more reliable diagnostic solution in smear-negative patients, especially for detecting low bacterial loads or latent TB infections.

Performance of tNGS in Detecting Resistance to Rifampicin, Isoniazid, Streptomycin, and Ethambutol in Tuberculosis Patients

This diagnostic efficacy of tNGS for resistance to four first-line anti-TB drugs is summarized in Table 4. For isoniazid, tNGS showed high sensitivity (83.10%) and specificity (90.30%), with a Youden index of 73.40%. In contrast, although

Table 3 Comparison of Diagnostic Efficacy in Smear-Negative Cases

Detection method	Final Diagnosis		Sensitivity	Specificity	PPV	NPV	χ^2	Kappa	AUC (95% CI)
	Negative	Positive							
tNGS									
Negative	64	13	88.10%	90.10%	93.20%	83.10%	107.449	0.771	0.891(0.838~0.945)
Positive	7	96							
Culture									
Negative	71	57	47.70%	100.00%	100.00%	55.50%	47.632	0.418	0.739(0.668~0.809)
Positive	0	52							
DNA									
Negative	71	50	54.10%	100.00%	100.00%	58.70%	57.17	0.482	0.771(0.703~0.838)
Positive	0	59							
Xpert									
Negative	71	45	58.70%	100.00%	100.00%	61.20%	64.688	0.529	0.794(0.729~0.858)
Positive	0	64							

Abbreviations: tNGS, targeted next-generation sequencing; AFB, acid-fast bacilli; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

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Table 4 The Diagnostic Efficacy of tNGS for Drug Resistance to Four First-Line Anti-Tuberculosis Drugs

Medication	Mutation Type	Drug Susceptibility Test/Strain		Sensitivity	Specificity	PPV	NPV	Youden Index
		Resistant	Susceptible					
Rifampicin	Wild-type	36	14	82.10%	67.90%	79.00%	72.00%	50.00%
	Mutant	17	64					
Isoniazid	Wild-type	65	10	83.10%	90.30%	87.50%	86.70%	73.40%
	Mutant	7	49					
Streptomycin	Wild-type	93	5	78.30%	86.10%	54.50%	94.90%	64.40%
	Mutant	15	18					
Ethambutol	Wild-type	91	4	75.00%	79.10%	33.30%	95.80%	54.10%
	Mutant	24	12					

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

the sensitivity of rifampicin was relatively high (82.10%), but its specificity was lower (67.90%), resulting in a reduced Youden index (50.00%) and a higher likelihood of false positives. The sensitivities of streptomycin and ethambutol were 78.30% and 75.00%, respectively, and the specificities were 86.10% and 79.10% respectively. Although the NPV for all four drugs exceeded 94%, indicating strong ability to exclude resistance, the PPV were relatively low, especially for ethambutol (33.30%). These results suggest that while tNGS is highly reliable for ruling out resistance, it may have limitations in confirming resistance, depending on the drug.

The distribution of drug-resistance mutations is detailed in Table 5. Rifampicin resistance was predominantly associated with mutations in the rpoB gene (102 cases), with Ser531Leu being the most frequent (55.88%), followed by Leu511Pro (7.84%) and Leu533Pro (4.90%). Isoniazid resistance was mainly associated with katG mutations (65

Table 5 Distribution of Drug Resistance Genes in Drug-Resistant Strains

Medication	n	Gene	Mutation Site	n	Proportion
Rifampicin	102	rpoB	Ser531Leu	57	55.88%
			Leu511Pro	8	7.84%
			Leu533Pro	5	4.90%
			His526Asp	3	2.94%
			Asp516Val	2	1.96%
			His526Arg	2	1.96%
			His526Leu	2	1.96%
			Ser450Leu	2	1.96%
			Ser531Trp	2	1.96%
			Asp516Gly	1	0.98%
			Asp516Tyr	1	0.98%
			Glu460Gly	1	0.98%
			His526Asn	1	0.98%

(*Continued*)

Table 5 (Continued).

Medication	n	Gene	Mutation Site	n	Proportion
			His526Tyr	1	0.98%
			Leu457Pro	1	0.98%
			Phe505Leu	1	0.98%
			Ser512Gly	1	0.98%
			His526Asn+Asp516Tyr	1	0.98%
			His526Gln+Leu511Pro	1	0.98%
			Leu511Pro+Asp516Gly	1	0.98%
			Leu511Pro+His526Asn	1	0.98%
			Leu511Pro+His526Gln	1	0.98%
			Leu511Pro+Ser531Leu	1	0.98%
			Ser512Gly+His526Tyr	1	0.98%
			Ser531Leu+Ile480Val	1	0.98%
			Ser531Leu+Phe503Ser	1	0.98%
			Asp516Tyr+Leu511Arg	1	0.98%
			Asp516Val+Gln513Glu	1	0.98%
Isoniazid	65	katG	Ser315Thr	40	61.54%
			Ser315Asn	5	7.69%
			Ser303Leu	1	1.54%
			Trp300Gly	1	1.54%
			Trp321	1	1.54%
			Ser315Thr+Asp381Gly	1	1.54%
			Phe252Leu+Ser315Thr	1	1.54%
		inhA	/	9	13.85%
		ahpC	/	4	6.15%
		ahpC+katG	Ser315Asn	1	1.54%
		katG+inhA+ahpC	Ser315Gly	1	1.54%
Streptomycin	37	rpsL	Lys43Arg	29	78.38%
			Lys88Arg	7	18.92%
		rrs	/	1	2.70%
Ethambutol	37	embA	/	1	2.70%
		embB	Asp328Gly	1	2.70%

(*Continued*)

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Table 5 (Continued).

Medication	n	Gene	Mutation Site	n	Proportion
			Asp354Ala	1	2.70%
			Gln497Lys	2	5.41%
			Gly406Ala	3	8.11%
			Gly406Asp	2	5.41%
			Met306Ile	8	21.62%
			Met306Leu	1	2.70%
			Met306Val	16	43.24%
		embB+embA	Met306Val	1	2.70%
			Met306Val	1	2.70%

Note: "/" indicates an undefined mutation site.

cases), especially the Ser315Thr mutation (61.54%). Streptomycin resistance mainly involved rpsL mutations (37 cases), with Lys43Arg accounting for 78.38%, and rrs gene mutations accounting for only 2.70%. Ethambutol resistance was closely related to the embB mutations (37 cases), especially Met306Val (43.24%) and Met306Ile (21.62%). Other observed mutations included Gly406Ala (8.11%) and Met306Ile (21.62%). These molecular findings provide valuable insights into the current landscape of drug-resistant TB and offer a scientific basis for epidemiological surveillance and personalized treatment strategies.

Discussion

It is estimated that in 2023 there were 10.8 million new TB cases worldwide, including 400,000 cases (3.7%) of MDR/RR-TB.[2] Traditional diagnostic methods suffer from inherent limitations in both sensitivity and speed, resulting in a large number of patients failing to receive timely diagnosis. With recent advances in molecular biology, tNGS has emerged as a powerful tool for genotypic drug resistance testing in pathogenic microorganisms. Compared with the latest nanopore sequencing technology, tNGS demonstrates significant advantages in cost-effectiveness and clinical maturity. Its detection cost has approached that of conventional molecular diagnostic methods, making it more conducive to clinical promotion and application. Additionally, tNGS utilizes the well-established Illumina short-read platform, with standardized bioinformatics workflows and clear quality control indicators. Our previous research has explored the diagnostic performance of nanopore sequencing in cerebrospinal fluid for tuberculous meningitis.[20] Building on this foundation, the current study further focuses on the application of tNGS in bronchoalveolar lavage fluid samples, systematically evaluating its diagnostic efficacy in pathogen detection and resistance gene mutation identification, and conducting a comprehensive comparison with mainstream diagnostic methods. This study represents the large sample size, surpassing previous comparable investigations.[21]

Our results demonstrated that tNGS achieved superior diagnostic performance, with sensitivity of 91.10%, specificity of 89.70%, the AUC of 0.904, and a Kappa coefficient of 0.786, indicating excellent consistency with the final clinical diagnosis. In contrast, the sensitivity of AFB staining was only 39.40%, reaffirming previous findings of its low detection rate.[22] Although the AUC values of culture, TB-DNA, and Xpert were all above 0.82, their sensitivity and consistency were still inferior to those of tNGS. The low PPV of tNGS and Xpert MTB/RIF also warrant discussion. TB-DNA can persist in sputum for weeks; remnants from prior infections, environmental mycobacterial fragments, or laboratory cross-contamination are readily detected by the highly sensitive tNGS and Xpert MTB/RIF. When such cases are culture-negative and the patient's symptoms have resolved, they are often classified as "false positives" in clinical practice,

further depressing the PPV. Notably, tNGS independently detected 24 TB-positive cases, indicating its unique advantage in detecting low-burden MTB or atypical infections. These findings suggest that, in clinical practice, the combined application of tNGS with traditional methods can further enhance diagnostic accuracy and reliability, particularly in complex or diagnostically challenging cases.

This study innovatively compared the diagnostic performance of tNGS with various traditional detection methods in smear-negative PTB. The diagnosis of smear-negative PTB has always been a major challenge in TB prevention, control, and clinical practice.[23,24] Due to the low Mtb burden, insufficient bacterial activity, uneven distribution in the body, and often atypical clinical manifestations, many patients cannot be diagnosed in a timely manner.[25] Behind a negative smear lies the hidden risk of delayed treatment, disease progression, and continued transmission resulting from diagnostic delay. Although these patients are not "visible" under microscopy, they may still contribute to disease spread and are at risk of developing more severe or drug-resistant forms of TB if left untreated.[26] It is precisely in this difficult clinical context that tNGS demonstrates substantial application potential. In smear-negative patients, tNGS demonstrated significantly better comprehensive diagnostic efficacy than traditional and molecular detection methods. Specifically, tNGS achieved a sensitivity of 88.10%, specificity of 90.10%, an AUC of 0.891, and a Kappa coefficient of 0.771, indicating a high degree of concordance with the final clinical diagnosis. In contrast, the sensitivities of culture, TB-DNA, and Xpert ranged only from 47.70% to 58.70%, and their corresponding AUC values were also lower than that of tNGS. These limitations in conventional methods may contribute to missed diagnoses, delayed treatment, and heightened transmission risk. Our findings are consistent with the previous report by Yan et al, which also reported a low sensitivity for culture (36.11%) and limited AUC value (AUC = 0.565). Although Xpert MTB/RIF demonstrated higher sensitivity (40.28%) and AUC value (0.689) than culture, its overall performance remained unsatisfactory and insufficient meet clinical needs.[27] By contrast, the high NPV of tNGS (83.10%) underscores its reliability in ruling out TB infection in smear-negative patients. Taken together, these results highlight the excellent diagnostic value of tNGS in smear-negative PTB and support its role as an important supplementary tool in precision TB prevention and control systems. For cases that are AFB-positive but molecularly negative, the possibility of NTM infection or laboratory contamination should be highly suspected, and further NTM species identification together with comprehensive clinical evaluation is recommended.

This study systematically evaluated the diagnostic performance of tNGS in detecting resistance to four first-line anti-TB drugs (rifampicin, isoniazid, streptomycin, and ethambutol) and analyzed the mutation spectrum of drug resistance-related genes. The results showed that the efficacy tNGS varied across different drugs, reflecting both its overall high application potential and the distinct genetic mechanisms underlying resistance. Among the four drugs, tNGS performed best in detecting isoniazid resistance, with a sensitivity of 83.10%, specificity of 90.30%, and a Youden index of 73.40%, indicating high diagnostic. This finding is consistent with the relatively concentrated resistance mechanism of isoniazid. Both this study and previous reports confirm that the Ser315Thr mutation represents the predominant molecular basis of isoniazid resistance.[28–30] The high prevalence of this single mutation reduces genetic complexity and facilitates precise detection using targeted approaches such as tNGS. In contrast, although rifampicin resistance is largely mediated by mutations in the rpoB gene (over 90%), the mutation sites are more dispersed.[31,32] In addition to the common Ser531Leu mutation (55.88%), numerous low-frequency mutations (eg, Leu511Pro, His526Asp), and even complex mutations (eg, co-occurrence of Leu511Pro and Ser531Leu), were observed. This heterogeneity likely contributes to the lower specificity (67.90%) and Youden index (50.00%) of tNGS for rifampicin compared with isoniazid. Some mutations may lie outside the targeted detection range, while the phenotypic relevance of others remains uncertain, potentially leading to false-positive results. For streptomycin and ethambutol, tNGS demonstrated relatively high negative predictive values (NPVs, both above 94%), underscoring its reliability in excluding resistance to these drugs. This capacity is clinically valuable, as it helps avoid unnecessary drug substitutions or treatment intensification. The mutation spectrum identified in this study was broadly consistent with global and regional drug resistance surveillance data.[33–36] For example, rpoB Ser531Leu, katG Ser315Thr, and rpsL Lys43Arg were the most prevalent mutations conferring resistance to rifampicin, isoniazid, and streptomycin, respectively. These high-frequency mutations provide clear targets for primer design and probe optimization in tNGS. However, multiple low-frequency and complex mutations were also detected, suggesting the heterogeneity of TB resistance mechanisms. Thus, when designing tNGS panels, it is essential not only to

include common mutations but also to account for regional patterns and rare variants to maximize both comprehensiveness and accuracy.

This study confirmed the significant advantages of tNGS in the diagnosis of PTB and the detection of drug resistance, particularly in smear-negative patients, where conventional methods remain inadequate. These findings provide strong evidence supporting the broader clinical implementation of tNGS, which could improve the timeliness and accuracy of TB diagnosis and guide individualized treatment strategies. However, this study has certain limitations. The tNGS resistance panel only covers first-line drugs; second-line resistance was not assessed. Future studies should expand the sample population, include diverse patient subgroups, and integrate resistance testing for second-line drugs as well as other emerging agents currently on the market, in order to comprehensively evaluate the clinical utility of tNGS.

Such efforts will further promote the adoption of tNGS as a powerful tool for precise TB diagnosis, resistance monitoring, and personalized treatment planning.

Conclusion

tNGS has significant advantages over traditional methods for the diagnosis of PTB and the detection of drug resistance, especially showing excellent supplementary diagnostic value in smear-negative patients. It offers high sensitivity and specificity, and performs robust performance in detecting resistance mutations for four first-line anti-TB drugs. Among these, tNGS achieved the highest accuracy for isoniazid resistance and showed extremely high reliability in excluding resistance to streptomycin and ethambutol. Importantly, tNGS enables comprehensive and unbiased detection of drug resistance-related mutations, including low-frequency and complex mutations, providing molecular-level drug resistance profiling. Future research should focus on second-line resistance mechanisms and multicenter validation, concurrently expanding the landscape of resistance targets and deeply integrating tNGS into clinical workflows, thereby driving the implementation of personalized therapy and enabling real-time resistance surveillance.

Data Sharing Statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request (Qing-Dong Zhu: zhuqingdong2003@163.com; Chang-Yue Jiang: 651642097@qq.com).

Ethical Statement

The study protocol was approved by the Ethics Committee of The Fourth People's Hospital of Nanning (Ethical Approval Number: [2023]24). The requirement for informed consent to study inclusion and the need for consent to participate were waived by the Ethics Committee of The Fourth People's Hospital of Nanning because of the lack of study intervention in patient diagnosis and treatment and the retrospective nature of the study. This study is in accordance with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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

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