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ORIGINAL RESEARCH

Application of Metagenomic and Targeted Next-Generation Sequencing in Diagnosis of Pulmonary Tuberculosis in Bronchoalveolar Lavage Fluid

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Purpose: To explore the application value of metagenomic next-generation sequencing (mNGS) and targeted next-generation sequencing (tNGS) in the diagnosis of pulmonary tuberculosis (PTB) in bronchoalveolar lavage fluid (BALF).

Patients and Methods: Data from 202 patients with suspected PTB at Wuhan Central Hospital (Jan 2022 - Jan 2024) were retrospectively analyzed. BALF samples were collected and examined using mNGS and tNGS, comparing their sensitivity to traditional methods like acid-fast staining, TB culture, and TB-DNA. Mixed microbial species were identified from the BALF using mNGS and tNGS, and the pros and cons of tNGS were evaluated against mNGS.

Results: Of the 202 patients evaluated, 94 were diagnosed with PTB. The BALF mNGS and tNGS exhibited a sensitivity of 77.66% and a specificity of 100%, with positive and negative predictive values of 100% and 83.72%, respectively, outperforming conventional diagnostic methods. It was possible to compare the AUC values of the ROC curves of the BALF mNGS and tNGS with the corresponding values of the other three assay methods (0.89 vs 0.56, $p < 0.05$), MTB culture (0.89 vs 0.71, $p < 0.05$), and TB-DNA (0.89 vs 0.68, $p < 0.05$). Additionally, these techniques identified mixed microbial species in 52.13% of the BALF samples. Although both mNGS and tNGS demonstrated similar diagnostic rates, tNGS proved to be faster, more cost-effective, and incorporated a tuberculosis-specific wall-breaking technology, thereby suggesting greater clinical utility.

Conclusion: BALF mNGS and tNGS technologies quickly and accurately detect PTB patients with greater sensitivity and specificity than traditional MTB methods. While both mNGS and tNGS demonstrate enhanced capacity for polymicrobial detection, the clinical significance of co-detected microorganisms requires integration with clinical context to differentiate colonization from active infection. Compared to mNGS, tNGS provides distinct advantages in clinical utility.

Keywords: metagenomic next-generation sequencing, targeted next-generation sequencing, pulmonary tuberculosis, bronchoalveolar lavage fluid, diagnosis

Introduction

Pulmonary tuberculosis (PTB) is a respiratory infectious disease caused by Mycobacterium tuberculosis (MTB) infection. In 2023, approximately 10.8 million cases were confirmed, with an incidence rate of 134 per 100,000 individuals, and 8.2 million confirmed cases, marking the highest level since surveillance began in 1995 and reaffirming its status as the world's deadliest infectious disease. In China, an estimated 741,000 new cases were reported in 2023, with an incidence rate of 52 per 100,000, ranking third globally, and a mortality rate of 2.0 per 100,000.[1] Given its high

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transmissibility, severity, and complex treatment regimens, PTB poses a significant threat to patient safety, emphasizing the critical need for early detection, diagnosis, and treatment to control the spread of the disease.

Traditional diagnostic methods, including acid-fast bacillus (AFB) smear and Mycobacterium tuberculosis (MTB) culture, have limitations. While AFB smears are simple and cost-effective, they suffer from low sensitivity. MTB cultures, with a long turnaround time of 4–8 weeks and low positivity rates, fail to meet the clinical demand for rapid diagnosis. There is an urgent need for the development of rapid and accurate molecular diagnostic techniques. Since December 2010, the World Health Organization has recommended the use of the more sensitive and specific GeneXpert Mycobacterium tuberculosis/Rifampicin (GeneXpert MTB/RIF) assay which, despite its higher sensitivity and specificity, is costly and dependent on complex equipment, with coverage for MTB detection still needing improvement.[2] The "new standards" for tuberculosis diagnosis implemented in 2018 included tuberculosis deoxyribonucleic acid (TB-DNA) testing detection as a novel diagnostic indicator; however, this method cannot distinguish between viable bacteria and the progression of tuberculosis.[3] Compared to conventional diagnostic methods, mNGS offers high-throughput, unbiased pathogen detection with broad microbial coverage and enhanced sensitivity, making it particularly valuable for identifying emerging or rare pathogens.[4] However, mNGS faces notable limitations, including prolonged turnaround times, elevated costs, intricate bioinformatics requirements, and challenges in standardization, which collectively hinder its widespread clinical adoption.[5] To address these challenges, tNGS has emerged as a complementary approach. By focusing on predefined genomic regions of interest, tNGS achieves higher specificity, reduced sample input requirements, and simplified laboratory workflows. Additionally, its minimal susceptibility to human DNA interference and standardized bioinformatics pipelines facilitate robust reproducibility across diverse clinical settings.[6] In recent years, high-throughput sequencing technologies, including meta-genomics next-generation sequencing (mNGS) and targeted next-generation sequencing (tNGS), have garnered significant attention as rapid detection methods for a variety of pathogens, including MTB. Numerous studies have explored the advantages of mNGS in MTB diagnosis, with a meta-analysis of 17 studies showing a pooled sensitivity and specificity of 0.69 [0.58–0.79] and 1.00 [0.99–1.00], respectively.[7] However, there are fewer reports on the newly developed tNGS technology for MTB detection, and its diagnostic value requires to be further clarification. This study aims to assess the clinical application value of mNGS and tNGS in the diagnosis of PTB by comparing them with traditional methods for detecting MTB in bronchoalveolar lavage fluid (BALF).

Materials and Methods

Study Subjects and Design

A retrospective study was performed to gather data from 202 patients with suspected pulmonary tuberculosis who underwent bronchoalveolar lavage fluid (BALF) analysis at Wuhan Central Hospital, affiliated with Tongji Medical College of Huazhong University of Science and Technology, between January 2022 and January 2024. This study involved the collection and analysis of mNGS, tNGS, AFB staining, MTB culture, TB-DNA testing, Xpert MTB/RIF assay results, and patient-related clinical medical records.

Inclusion criteria: (1) patients with a clinical suspicion of pulmonary tuberculosis (PTB) presenting with subacute cough, fever, night sweats, weight loss, and other symptoms associated with tuberculosis infection; (2) evidence from chest computed tomography (CT) or X-ray imaging indicating miliary pulmonary nodules, patchy shadows, or lung cavities; (3) all patients underwent BALF mNGS or tNGS detection.

Diagnostic criteria: Pulmonary tuberculosis was diagnosed according to the Chinese National Health Industry Standard WS 288–2017: Diagnostic Criteria for Pulmonary Tuberculosis.[3] The diagnosis is confirmed if one or more of the following criteria are met: (1) a positive mycobacterial culture from BALF or a positive molecular biology test; (2) lung tissue biopsy findings consistent with the pathological characteristics of tuberculosis; (3) in patients negative for Mycobacterium tuberculosis but with a clinical diagnosis of pulmonary tuberculosis, a definitive diagnosis can be made if anti-tuberculosis treatment for three months results in a reduction or resolution of lung lesions.

BALF Specimen Collection

All patients underwent bronchoscopy in accordance with established standard operating procedures (SOP) for BALF collection.[8] The bronchoscope was positioned at the entrance of the designated bronchial segment or subsegment. For

patients with localized lesions, the lesion segment was targeted, whereas for those with diffuse lesions, either the middle lobe of the right lung or the lingual lobe of the left lung was selected. Room temperature normal saline was then rapidly introduced through the operating channel in aliquots of 20–50 mL, totaling 60–120 mL. The procedure was performed a total of three to four times using an appropriate negative pressure for suction, ensuring that the pressure did not exceed 100 mmHg, and achieving a recovery rate greater than 30%.

AFB Smear and MTB Culture Assays

According to the lab SOP, the collected BALF samples were separated with centrifugation to remove cells and other impurities. The supernatant was taken and using a sterile glass slide, the liquid was dropped on the slide and spread evenly to form a thin smear. After smearing, the slides were left to dry naturally at room temperature. Staining was performed using the Ziehl-Neelsen stain. The dried smear is gently heated over a flame, followed by drop-wise addition of a staining solution, usually alkaline formalin stain, for about 5 minutes. This is followed by rinsing with water and finally contrast staining with toluene stain. The resuspended sediment was inoculated into Löwenstein–Jensen medium (Baso Di-agnostics Inc Zhuhai, China) and incubated for 8 weeks.

TB-DNA Testing

In line with the lab SOP and operation guidelines, the turbidity of the collected BALF was used to determine the appropriate amount of 4% NaOH solution to add for liquefaction and decontamination, and the DNA in the samples was extracted using the steps in the instruction manual of the TB-DNA kit (Shanghai Rendu Biotechnology Co., Ltd., China), and the extracted DNA was used in the real-time fluorescence quantitative PCR (qPCR) for detection of the amplification products.

Xpert MTB/RIF Assay

Nucleic acid extraction was performed with the specific reagents in the BALF and then transferred to the Xpert MTB/RIF test card according to the instructions and laboratory SOP of the Xpert MTB/RIF kit (Cepheid AB, Sweden). The card contains all the reagents required for the PCR reaction, including primers and probes. The PCR reaction occurs in the device and is usually completed within 45 minutes. After PCR amplification, the Xpert MTB/RIF system automatically analyzes the results and is capable of simultaneously detecting the presence of Mycobacterium tuberculosis and its resistance to rifampicin. The GeneXpert instrument produced the final MTB detection results within two hours.

mNGS

BALF was sealed in a sterile manner and either stored at –20 °C or quickly sent to UW Genetics in Shenzhen on dry ice for mNGS analysis (the collection, preservation, and transportation of mNGS samples adhered strictly to the company's standardized guidelines). (1) DNA was isolated from 0.5 to 3 mL of bronchoalveolar lavage fluid (BALF) using the TIANamp Micro DNA kit (DP316, Tiangen Biotech, Beijing, China). (2) Library construction and sequencing: Approximately 50 ng of DNA from each sample was utilized for library construction. Approximately 50 ng of DNA from each sample was utilized for library creation, involving DNA fragmentation, end repair, splice ligation, and PCR amplification. Quality control utilized an Agilent 2100, while sequencing was conducted on the BGISEQ-500 platform by the Beijing Genomics Institute in Wuhan, China. (3) Sequencing tags were used to partition the data, junctions were removed, and sequences of low quality and those that were repetitive were filtered, and the repetitive and low-quality sequences were excluded, and the sequencing data were accessed from the National Center for Biotechnology Information. (NCBI ftp://ftp.ncbi.nlm.nih.gov//genomes/) Using GRCH38 genomes, the pathogen database, which contains the 16S and ITS gene database from NCBI, was downloaded as a reference genome to exclude human host DNA, which included 19,088 bacterial, 8,082 fun-gal, and 231 non-bacterial pathogen reference genes. (4) Reporting criteria: As described before, sequencing data were stratified into four pathogen-specific categories (bacteria [non-mycobacterial], fungi, viruses, and parasites), followed by consolidation into a unified taxonomic database. For bacterial (excluding mycobacteria), viral, fungal, and parasitic pathogens, species-level microorganisms were classified as clinically significant if their sequence reads ranked among the top 10 most abundant taxa in the consolidated dataset.[9]

For MTB, a unique read aligning to genomic regions specific to MTB was considered a positive indication. Causative pathogens were ultimately adjudicated through our validated bioinformatics pipeline, which integrates microbial abundance with clinical relevance. Three experienced laboratory clinicians analyzed the mNGS results, factoring in clinical features, other microbiological assessments, and lung imaging.

tNGS

The BALF was preserved on dry ice and sent to Adicon Medical Laboratory for tNGS testing (tNGS specimens were collected, preserved, and transported in accordance with the company's standardized specifications) for tNGS testing. (1) BALF samples were centrifuged (20,000×g, 10 min), and 500 μL of supernatant was used for nucleic acid extraction. Nucleic acids were extracted using a ZymoBiomics DNA/RNA MiniprepKit (Adicon). (2) Library preparation and quality control: Library construction reagents were used with a Pathogeno One MTB&NTM Library Construction Kit (Adicon). (3) High-throughput sequencing: An Illumina MiSeq Reagent Nano Kit (Adicon test) was used for high-throughput sequencing, and the sequencing platform was an Illumina MiSeq/ KM MiniseqDx-CN gene sequencer. (4) Bioinformatics analysis: Bioinformatics software was used to process the sequencing data and identify high-quality data, and the retained reads were compared with the sequences of the pathogens in the databases downloaded from NCBI, such as RefSeq, GenBank, and the Pathogenic Microorganisms Data Analysis Management System (PMDAMS), to read out the pathogen detection results with the following quality requirements: Q30 ≥ 75%, the minimum number of original reads ≥ 50k, and the amplification of internal reference genes ≥ 50k. Quality requirements were as follows: Q30≥75%, minimum number of raw reads≥50k, number of amplified reads≥200. (5) Reporting criteria: This testing included 198 clinically important or relevant pathogens, based on the aforementioned official information and research articles (Supplementary Table 1). Following the methodology described in this article, we set up a series of interpretation rules for the tNGS assay, mainly achieved through metrics like microbial specificity reads per million (RPM) and RPMsample/RPMNTC (RPM-r).[10] The tNGS results were also analyzed by three experienced lab clinicians, taking into account clinical features, other microbiological assessments, and lung imaging.

Statistical Analysis

Data analyses were performed using SPSS (version 22; IBM Corp., Armonk, NY, USA). Figures were constructed with GraphPad Prism (version 9; GraphPad Software, La Jolla, CA, USA) and R (version 4.2.3, New Zealand). Non-normally distributed measurement data were expressed as the median [interquartile range (IQR)]. Non-parametric *U*-tests were used for comparison between two independent groups as follows: Count data are expressed as (%), the two groups were compared using the Chi-squared ($\chi 2$) test (all expected counts ≥5), Yates' corrected $\chi 2$ test (if any expected count 1–5) or Fisher's exact test (if any expected count <1 or total sample size <40). Combining with etiology, pathology and clinical features as the comprehensive diagnostic criteria, the sensitivity and specificity of BALF mNGS and tNGS, AFB smear, MTB and TB-DNA in the diagnosis of pulmonary tuberculosis were calculated. A receiver operating characteristic (ROC) curve was plotted among different groups, and the area under the curve (AUC) was calculated. $P < 0.05$ was considered statistically significant.

Results

General Clinical Data of Study Participants

Among 202 patients with suspected PTB, 113 (55.9%) were male and 89 (44.1%) were female, with a median age of 61.5 years [interquartile range (IQR): 51.0–70.0]. According to the comprehensive diagnostic criteria of etiology, pathology, and clinical features, 94 cases of pulmonary tuberculosis were finally diagnosed, comprising 86 cases of etiological diagnosis (Etiological diagnosis refers to the confirmation of MTB through microbiological or molecular methods, including AFB staining, MTB culture, TB-DNA, or mNGS/tNGS), 5 cases of pathological diagnosis (3 cases of percutaneous lung biopsy, 1 case of mediastinal right upper paratracheal lymph node biopsy based on ultrasound bronchoscope, and 1 case of lung biopsy based on ultrasound bronchoscope), and 3 cases of clinical diagnosis. There were 108 cases of non-pulmonary tuberculosis, comprising 89 cases of infection with other pathogenic bacteria infection, 4 cases of lung cancer, 3 cases of non-tuberculous mycobacteria, and 12 cases of other infections (Figure 1).

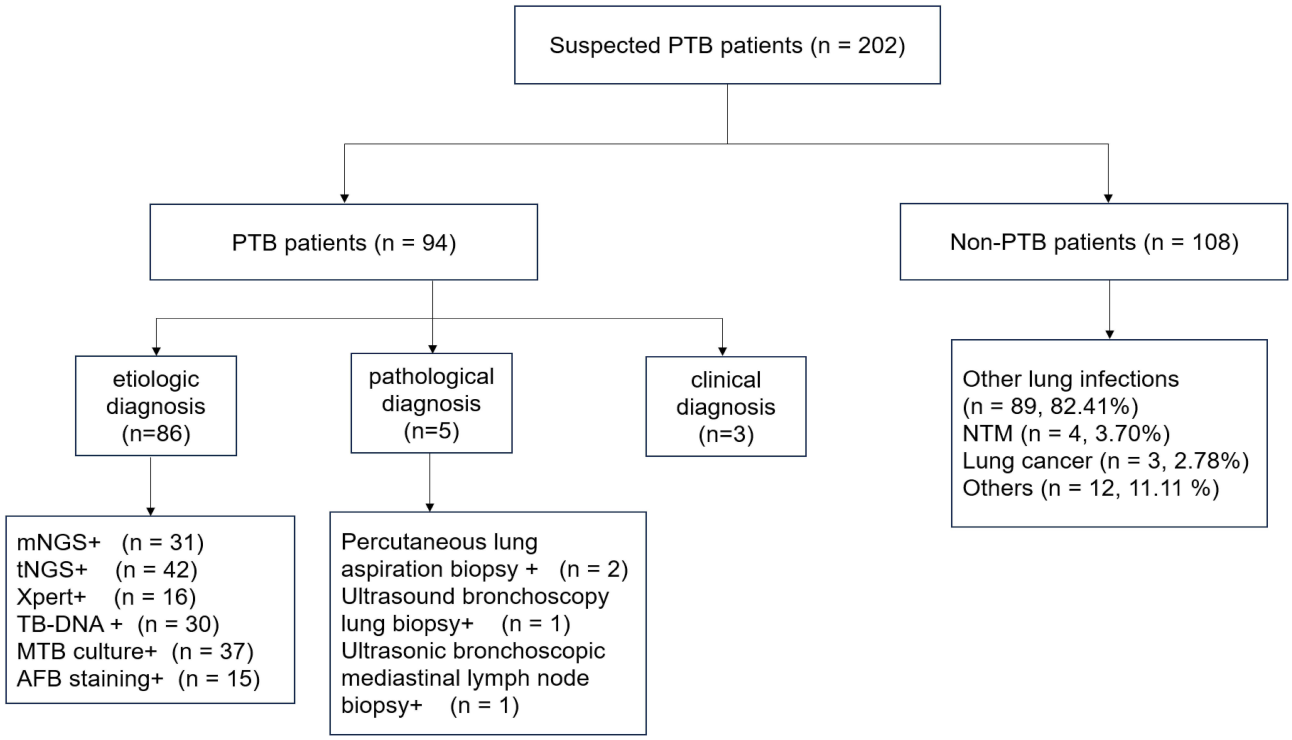

Figure 1 Flow chart of patient enrollment and test results.

The median age of PTB patients was 63.5 years [IQR: 54.0–70.3], with 60.6% (57/94) male; non-PTB patients had a median age of 60.0 years [IQR: 46.5–69.8], of whom 51.9% (56/108) were male (Table 1). There was no difference in the proportion of patients with previous tuberculosis history between PTB and non-PTB patients. There was no statistical significance in basic lung diseases (chronic obstructive pulmonary disease, chronic bronchitis, bronchiectasis or lung cancer) between the two groups. Therefore, it is necessary to carry out routine screening of active tuberculosis in patients with a history of tuberculosis.

Table 1 Characteristics of Patients with PTB and Non-PTB

Group/Project	PTB (n=94)	Non-PTB (n=108)	P value
Age/[(M [IQR]), years]	63.5 [54.0,70.3]	60.0 [46.5,69.8]	0.057
Male/Female	57/37	56/52	0.211
Previous PTB	7 (7.4)	4 (3.7)	0.243
Pulmonary complications (%)			
Chronic obstructive pulmonary disease	16 (17.0)	11 (10.2)	0.155
Bronchiectasis	10 (10.6)	4 (3.7)	0.053
Pulmonary malignant tumor	3 (3.2)	0 (0)	0.062
Chronic bronchitis	2 (2.1)	2 (1.9)	0.889
Extrapulmonary complications (%)			
Hypertension	29 (30.9)	21 (6.5)	0.062
Coronary heart disease	7 (7.4)	5 (4.6)	0.399
Type 2 diabetes	18 (19.1)	15 (13.9)	0.314
Chronic kidney disease	3 (3.2)	1 (0.9)	0.250
Chronic liver disease	5 (5.3)	3 (2.8)	0.357

Abbreviations: PTB, pulmonary tuberculosis; Non-PTB, Other infectious diseases of the lungs other than tuberculosis.

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PTB-Detection Rates of Smear, Culture, TB-DNA, and mNGS and tNGS Assays

Among the 94 confirmed PTB cases, the positivity rates of various diagnostic modalities were as follows: mNGS detected 31 positive cases, tNGS identified 42 positives, AFB staining revealed 15 positives, mycobacterial culture confirmed 37 positives, TB-DNA testing detected 30 positives, and Xpert MTB/RIF assay detected 16 positives. Histopathological examination of lung biopsies confirmed 5 cases, while clinical diagnosis accounted for 3 cases. One positive antacid staining smear was finally diagnosed as Mycobacterium avium intracellulare, a non-tuberculous mycobacterium, and one positive TB-DNA result was obtained, but tuberculosis was eventually excluded for the latter. As there were only 16 clinically valid BALF X-pert cases and all of them were positive, the detection rate was not calculated. The results of AFB staining, MTB culture, TB-DNA, mNGS, and tNGS for the detection of MTBC in BALF were obtained in 94 patients with pulmonary tuberculosis (Figure 2A), and the detection efficiency of the four methods was analyzed (Figure 2B). Further analysis revealed the following: 12 cases were positive according to all four tests; 7 cases were positive according to all three methods (NGS, MTB culture and TB-DNA); 3 cases were positive according to all three tests (NGS, ABF staining and MTB culture); 12 cases were positive according to both NGS and ABF staining; and 5 cases were positive according to both NGS and TB-DNA. However, 31 cases were positive according to mNGS or tNGS only, 8 patients were positive according to Xpert MTB/RIF only, and only 5 patients were positive according to TB-DNA alone (Figure 2C).

Comparison of Diagnostic Efficacies of Smear, Culture, TB-DNA, and mNGS and tNGS Assays

In our sample, the diagnostic sensitivity of BALF mNGS and tNGS for TB was 77.66% (73/94), with a specificity of 100% (108/108), a positive predictive value (PPV) of 100% (73/73), and negative predictive value (NPV) of 83.72% (108/129).

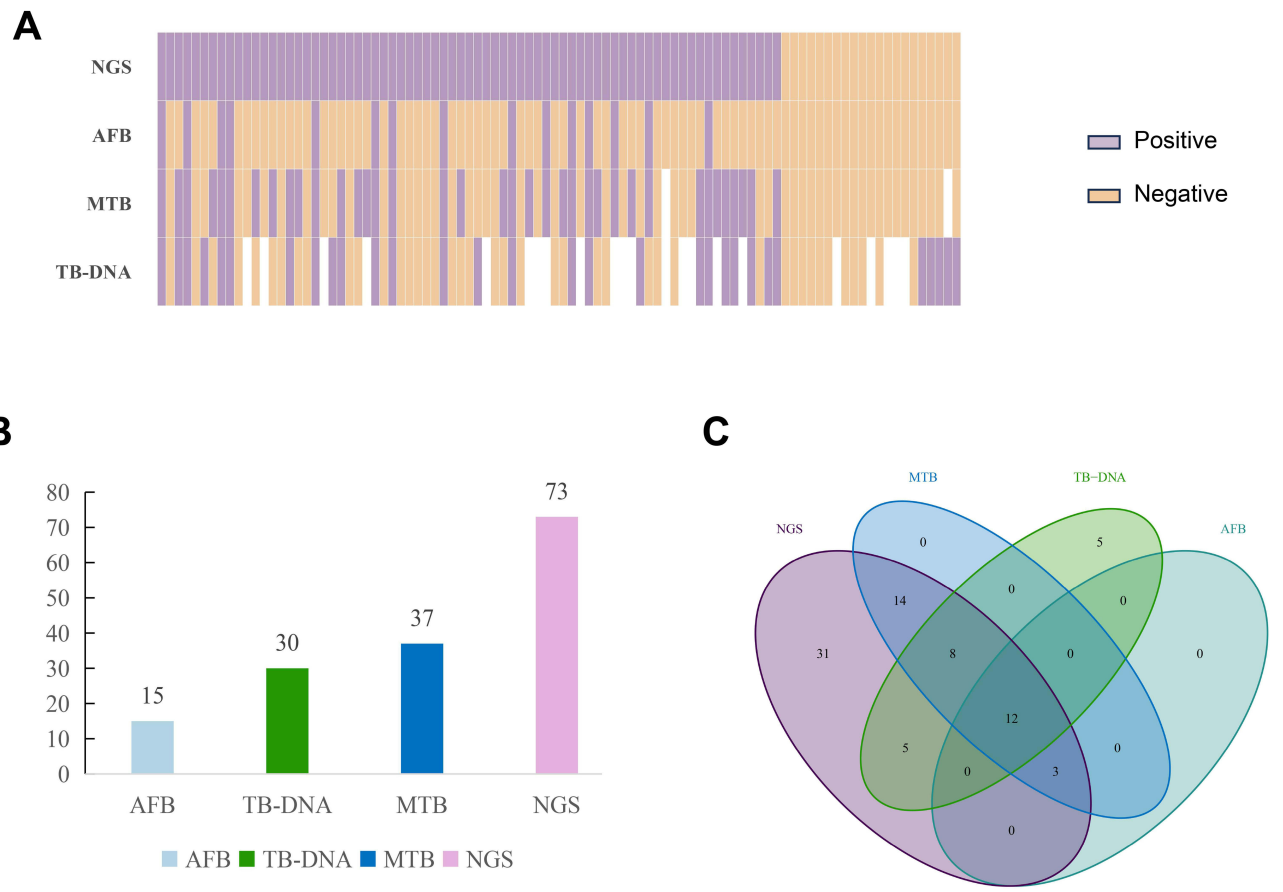

Figure 2 Positive PTB-detection rates obtained for AFB smear, MTB culture, TB-DNA, and NGS. (**A**) Heatmap depicting the identification of PTB by the 4 methods in BALF samples. (**B**) Number of samples yielding positive PTB-detection results for the four assays. (**C**) Venn diagram of PTB-positive results obtained for the four assays.

Table 2 The Diagnostic Ability of the Four Tests for Pulmonary Tuberculosis

Test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	AUC (95% CI)
NGS	77.66% (0.68–0.85)	100% (0.96–1.00)	100% (0.94–1.00)	83.72% (0.76–0.89)	0.89 (0.83–0.96)
AFB staining	15.96% (0.10–0.25)	99.07% (0.94–0.99)	93.75% (0.68–0.99)	57.53% (0.50–0.64)	0.56 (0.42–0.71)
MTB culture	40.21% (0.30–0.51)	100% (0.85–1.00)	100% (0.88–1.00)	33.73% (0.24–0.45)	0.71 (0.60–0.82)
TB-DNA	41.67% (0.30–0.54)	98.21% (0.89–0.99)	96.77% (0.81–0.99)	56.70% (0.46–0.67)	0.68 (0.55–0.80)

Abbreviations: NGS, next-generation sequencing; AFB, acid-fast bacillus; MTB, mycobacterium tuberculosis; TB-DNA, tuberculosis DNA; PPV, positive predictive value; NPV, negative predictive value; AUC, area under curve.

mNGS and tNGS were significantly more sensitive than the AFB smear method (15.96%, 15/94) ($\chi 2 = 71.867$, $P < 0.001$), MTB culture method (40.22%, 37/92) ($\chi 2 = 26.974$, $P < 0.001$), and TB-DNA (41.67%, 30/72) ($\chi 2 = 22.43$, $P < 0.001$), and the differences were statistically significant (Table 2). No significant difference in sensitivity was found between MTB culture and TB-DNA ($\chi 2 = 0.035$, $P = 0.874$). By generating ROC curves for further analysis (Figure 3), it was possible to compare the AUC values of the ROC curves of BALF mNGS and tNGS with the corresponding values of the other three assay methods (0.89 vs 0.56, $p < 0.05$), MTB culture (0.89 vs 0.71, $p < 0.05$), and TB-DNA (0.89 vs 0.68, $p < 0.05$).

BALF mNGS and tNGS Detected Mixed Microbial Species

In this study, both the mNGS and tNGS identified mixed microbial species in 52.13% (49/94) of BALF samples. mNGS detected mixed microbial species in 22 cases, and tNGS in 27 cases detected mixed microbial species (Figure 4A). Beyond Mycobacterium tuberculosis detection, mNGS revealed 8 instances of single bacterial species co-detection, 2 cases with two species, 5 cases with three species, and 57 cases with four or more species. In comparison, tNGS detected bacterial species co-occurrences in 10, 4, 9, and 4 cases, respectively (Figure 4B). Both methods demonstrated capacity to identify specific microorganisms in polymicrobial findings (Figure 5A and B). Viral and fungal species were co-detected in 9 and 5 instances, respectively, while there were 15 bacterial co-detections for mNGS and 16 for tNGS (Figure 5C and D). The most frequently observed bacterial species included Haemophilus influenzae (11 cases) and

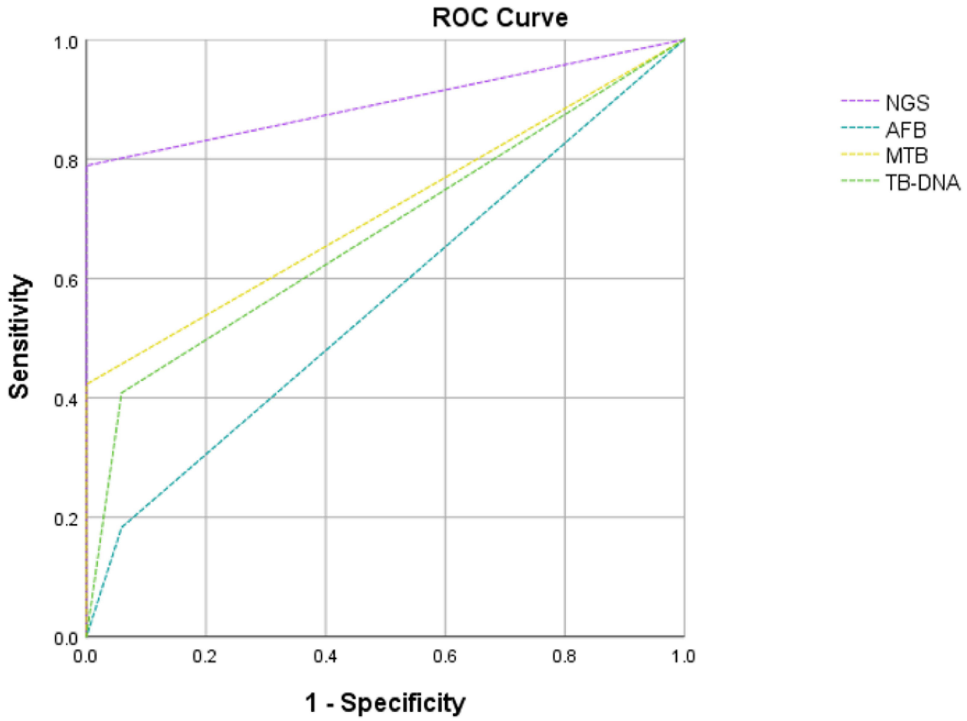

Figure 3 Receiver operating characteristic (ROC) curve-based PTB-detection results obtained using the four diagnostic assays.

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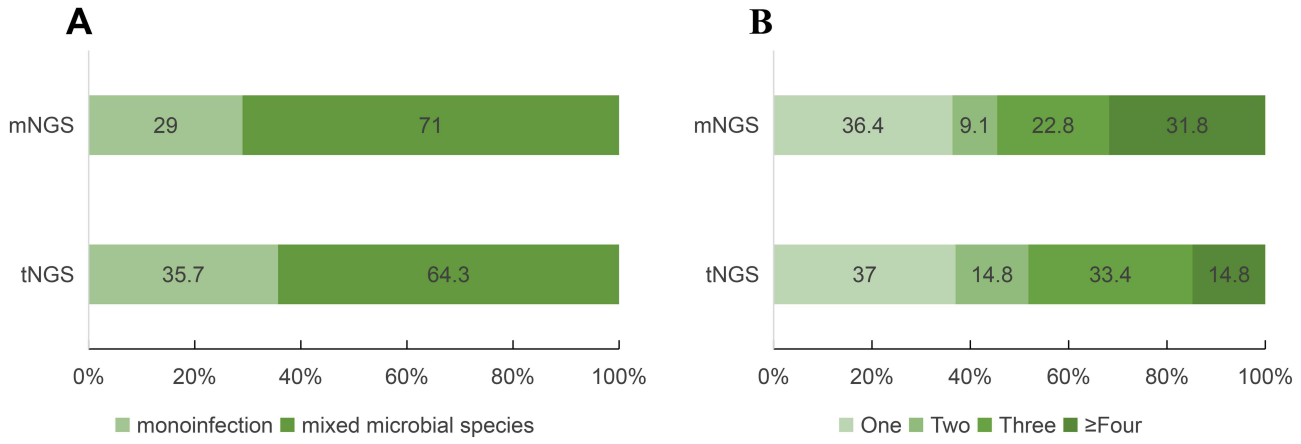

Figure 4 mNGS and tNGS detection of monoinfections and mixed microbial species. (**A**) Stacked bar graphs show the proportion of monoinfections and mixed microbial species in the mNGS and tNGS groups. (**B**) Stacked bar graphs show the proportion of the mNGS and tNGS groups that had microbial species other than Mycobacterium tuberculosis.

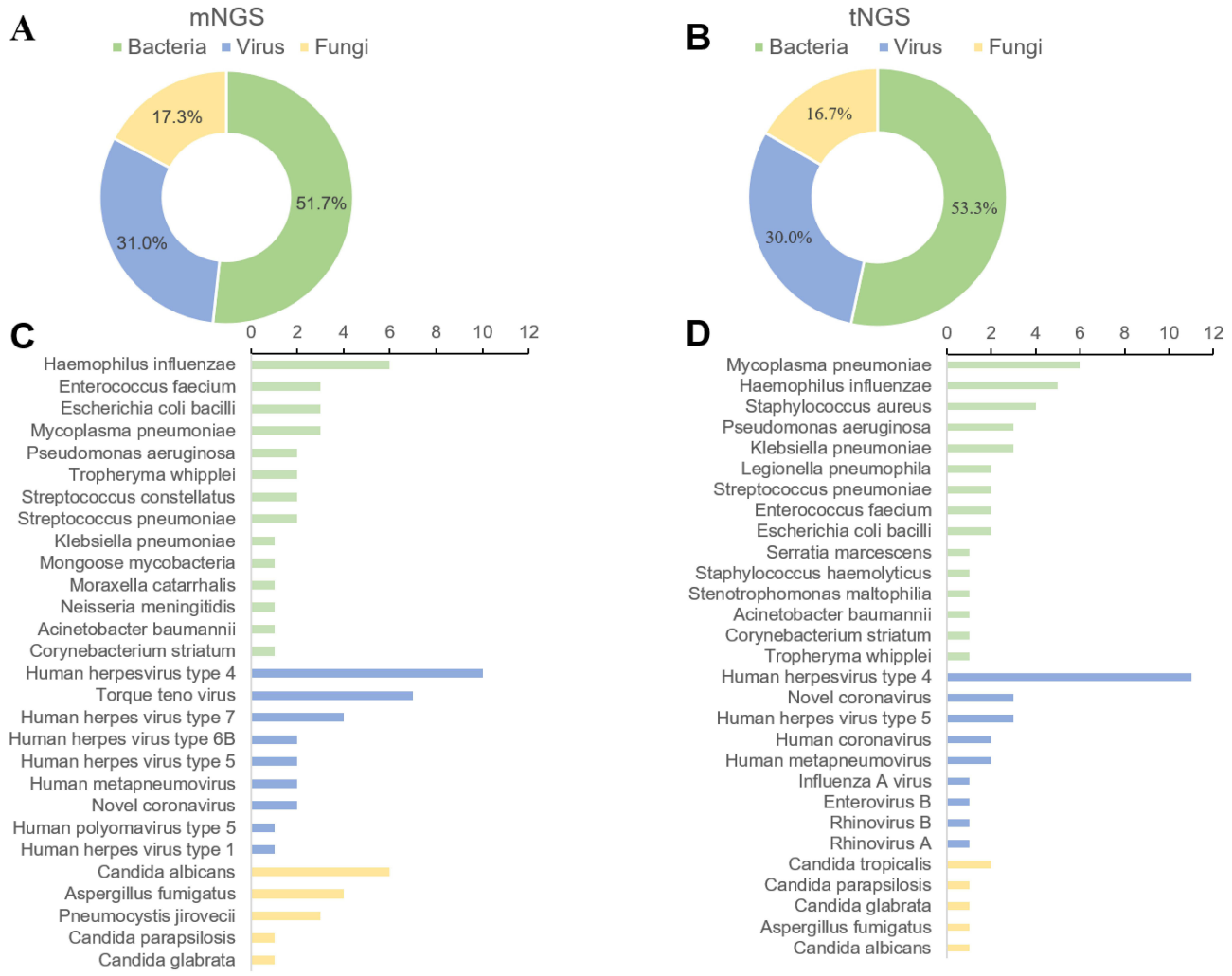

Figure 5 Pathogen identification by mNGS and tNGS workflows. (**A** and **B**) show the distribution of microbial species detected by mNGS and tNGS assays by categories, respectively. (**C** and **D**) show the microbial species detection results of mNGS and tNGS assays, respectively.

Mycoplasma pneumoniae (9 cases). The most common associated viruses were human gammaherpesvirus 4 (21 cases) and papillomavirus (7 cases). Among the fungi detected, Candida albicans (7 cases), Aspergillus fumigatus (5 cases), and Pneumocystis japonicus (3 cases) had the highest detection rates, though clinical correlation is required to differentiate colonization from active infection.

Comparison of BALF mNGS and BALF tNGS

Of the 94 PTB patients, 42 underwent mNGS testing with 31 positives, and 52 underwent tNGS testing with 42 positives. The positivity rates for the two tests did not differ significantly (73.81% vs 80.77%, P=0.463). However, mNGS has some limitations, including the detection of many microorganisms that make it difficult to distinguish between colonization and infection, interference from host background information, a higher detection cost, a longer detection time (about 24–36 h), and a lack of uniform standards for experimental procedures and the interpretation of results.[11] Compared with mNGS, tNGS has a lower detection cost, achieved rapid detection (about 8~16 h), and provided improved detection of intracellular pathogens and fungi, especially for RNA viruses (eg, neo-coronaviruses), which is advantageous as it helps in the detection and prevention of emergency public health events, providing greater potential for clinical application.[12] tNGS has also demonstrated its effectiveness in detecting drug-resistant Mycobacterium tuberculosis strains, identifying drug-resistant genes, and guiding clinical therapeutic decisions, which are difficult to achieve with mNGS. In addition, operational costs for tNGS in China are approximately 800 Chinese Yuan (CNY; ~100 United States Dollars, USD)—one-fourth the expense of mNGS (~400 USD). Clearly, tNGS has the ability to help diagnose TB early at an accelerated rate and a low cost.

Discussion

Early diagnosis and prompt treatment are crucial strategies for controlling the transmission of tuberculosis. However, the World Health Organization reports that approximately 30% of tuberculosis cases remain undiagnosed or are incorrectly diagnosed.[13] Conventional methods for detecting pathogenic micro-organisms remain prevalent in the clinical diagnosis of pulmonary tuberculosis, yet they exhibit limitations in terms of sensitivity, specificity, and timeliness. For instance, the acid-fast staining smear method demonstrates a sensitivity of only 30% and is unable to distinguish between Mycobacterium tuberculosis (MTB) and non-tuberculous mycobacteria.[14,15] MTB culture is the "gold standard" for diagnosis; however, although its sensitivity has been improved, it takes a long time and the positive rate is low. Compared with the culture method, TB-DNA is a PCR cycle product detected using fluorescent chemical reagents, and the undetermined DNA sequence of the specimen is quantitatively analyzed with internal and external reference. It is simple to use and can quickly and effectively diagnose tuberculosis. However, it is not suitable for tuberculosis patients from whom it is not easy to obtain test specimens, and because DNA from dead bacteria can remain in the body for a long time, it is impossible to identify whether the bacteria are alive or not. Cross-contamination during testing and false positives can easily occur, resulting in reduced diagnostic value.[16] The Xpert MTB/RIF assay simplifies rifampicin resistance detection within 2 hours but suffers from high reagent costs and an inability to differentiate MTB from NTM, hindering its widespread adoption in resource-limited settings.[17] NGS, including mNGS and tNGS, is a new method developed for pathogenic detection. Several studies have shown that mNGS has high sensitivity and specificity in the diagnosis of PTB.[7] However, there are few reports on tNGS in PTB, and its diagnostic efficacy is not clear. This study aimed to investigate the clinical value of BALF mNGS and tNGS in the diagnosis of PTB.

In this study, only ideal BALF samples were selected for mNGS or tNGS detection. Compared with other detection methods, it can provide more valuable diagnostic in-formation, because compared with conventional sputum samples that are easily contaminated by bacteria in the oropharynx, while BALF is taken from the site of pulmonary infection. The possibility of obtaining pathogens is higher, there is less microbial contamination of the upper respiratory tract is less, and the content of human-derived nucleic acids is low, resulting in little interference with the results.[18] Our study demonstrates that BALF mNGS and tNGS achieve superior diagnostic performance compared to conventional methods, with a sensitivity of 77.66% and a specificity of 100% (PPV=100%, NPV=83.72%). The sensitivity of mNGS and tNGS in the diagnosis of PTB was significantly higher than that of AFB (15.96%), MTB culture (40.21%), and TB-DNA (41.67%), and the difference was statistically significant ($P < 0.001$). Notably, our ROC analysis revealed an AUC of 0.89 for BALF mNGS/tNGS, surpassing other modalities and supporting their clinical adoption. The results were

consistent with previous studies.[7,19,20] Gao et al[19] included 186 patients with suspected pulmonary tuberculosis, and 38 of them were finally diagnosed with PTB. The sensitivity of BALF mNGS was 78.95%, which was higher than that of AFB (27.59%) and MTB culture (44.12%), but similar to X-pert (72.73%). Another prospective single-center study showed that the sensitivity of BALF mNGS in the diagnosis of PTB was 47.92%, which was similar to Xpert (45.83%) and MTB culture (46.81%), but much higher than AFB (29.17%). However, there are few reports on the efficacy of BALF tNGS in the diagnosis of pulmonary tuberculosis. Studies have shown that tNGS can quickly identify Mycobacterium tuberculosis complex and drug resistance directly from sputum samples, and shorten the detection time of drug resistance from 4~6 weeks to about 3 days.[21] In another study, the positive rate of MTB detected using tNGS was 68%, and 74% of the positive samples obtained a complete MTB drug resistance test report, and the consistency with the results of sputum culture drug sensitivity was high ($\kappa = 0.82$).[22] The enhanced sensitivity of tNGS (82% vs 58% in standard protocols) is attributed to its dual lysis methodology, combining the enzymatic disruption of mycobacterial mycolic acids combined with mechanical bead-beating to fracture the lipid-rich cell wall. This approach increases the DNA yield by 3.5-fold, enabling robust sequencing even in low-bacterial-load specimens.[23]

This study showed that there was no significant difference in the sensitivity of MTB culture and TB-DNA ($P > 0.05$). In a similar retrospective study, using a positive culture as the gold standard, the sensitivity of TB-DNA was 69.33% and the specificity was 96.61%, values which were in good agreement with the culture method. While TB-DNA PCR and culture showed comparable accuracy, PCR's rapid turnaround (hours vs weeks) offers critical advantages for early therapy initiation. The AUC is 0.830, which is also close to the critical AUC value of 0.9 with high diagnostic value, suggesting that TB-DNA detection has relatively high diagnostic value for pulmonary tuberculosis.[24] In theory, combining mNGS or tNGS with Xpert can be considered to further improve the diagnostic rate of clinical PTB. However, considering the cost to patients, the clinical feasibility of both tests must be considered. In this study, only 16 of the 92 patients diagnosed with PTB were tested with NGS and Xpert at the same time. Because of the small sample size, the diagnostic efficacy of NGS and Xpert was not compared in this study. However, some studies have shown that among 67 cases with extremely low detection via BALF-Xpert, which were used as the standard for suspected pulmonary tuberculosis, 43 were finally diagnosed as pulmonary tuberculosis, and 24 were non-pulmonary tuberculosis. The misdiagnosis rate was as high as 35.82%. There was a high false positive rate in the diagnosis of pulmonary tuberculosis, with extremely low MTB detected using BALF-Xpert.[25]

The false positive rate of Xpert was mainly related to the extremely low copy number of MTB, a previous history of tuberculosis, and inactive pulmonary tuberculosis imaging. Among the 16 cases of Xpert positive results in this study, there were 7 cases with extremely low MTB, 4 cases with low and high MTB, and 1 case with medium MTB. The final diagnosis was consistent with pulmonary tuberculosis.

Existing diagnostic methods for tuberculosis struggle to provide both rapid diagnosis and highly accurate, and lack the ability to diagnose mixed infections. mNGS and tNGS can improve the diagnostic accuracy of mixed infections by detecting not only Mycobacterium tuberculosis but also other potential pathogens rapidly and with high accuracy. Our data revealed mixed microbial species in 52.13% of PTB patients, with bacterial co-detections dominated by *Haemophilus influenzae* and *Mycoplasma pneumoniae*. The prevalence of the latter may reflect seasonal Mycoplasma epidemics occurring during the study period. Among fungal co-detections, *Candida albicans* was detected the most frequently (n=7), though its detection in BALF likely represents colonization rather than invasive infection in immunocompetent hosts. Conversely, the *Aspergillus fumigatus* (n=5) and *Pneumocystis jirovecii* (n=3) findings warrant attention—all *Aspergillus*-positive cases presented cavitary lesions, underscoring the clinical relevance of fungal co-infections in TB progression.[26] Our study revealed that human *gammaherpesvirus 4 (Epstein-Barr virus, EBV)* was the viral species most frequently co-detected via NGS, having been identified in 21 cases. mNGS further resolved herpesvirus diversity, detecting subtypes including *Human herpesvirus 6* (HHV-6), *HHV-6B, HHV-7*, and *Herpes simplex virus 1* (HSV-1). These findings align with existing evidence suggesting that herpesvirus co-detection (eg, EBV, cytomegalovirus [CMV]) correlates with elevated serum inflammatory markers (C-reactive protein, Interleukin-6) and radiographic cavitary lesions, implying potential synergistic interactions in PTB progression.[27,28] Combined fine cyclic viruses were detected in seven patients, but the number of sequences detected was not high in any of them, fluctuating in the range of 1–25. As they are human-coexisting viruses, none were considered pathogenic or specifically considered in clinical diagnosis and treatment.[29] Moreover, their etiological pathogenic mechanism remains unclear, and the clinical significance of these detections needs to be further tracked for clinical progress. The ability of NGS to recognize

multiple pathogens using a single assay proves its ability to detect mixed infections, which can particularly benefit patients with low specimen volumes and severe infections.

PTB is an intracellular pathogen characterized by the release of fewer nucleic acid sequences to the outside of the cell. Therefore, mNGS can provide a positive result by detecting only one MTB nucleic acid sequence.[30] The tNGS detection of specific sequences is as follows: with >100 sequences, it can be used as a positive standard; with specific data <100 sequences, to the result must be confirmed with real-time fluorescence quantitative PCR. When there are fewer than 10 sequences of specific data, careful interpretation is needed, and it is recommended to consider testing another sample to support the test results.[31] In this study, three patients with only one mNGS sequence were finally diagnosed with PTB, and the number of tNGS detection series was greater than 10, and four patients with less than 100 were finally diagnosed with PTB. This indicates that BALF mNGS and tNGS can be used to diagnose pulmonary tuberculosis with a low mycobacterial load. Therefore, the 100% specificity calculated for BALF mNGS and tNGS in our study is credible.

This study showed that there was no significant difference in the positive detection rate of BALF mNGS and tNGS in the diagnosis of pulmonary tuberculosis (73.81% vs 80.77%, P=0.463). However, tNGS has many advantages over mNGS, including sensitivity, timeliness and cost. tNGS is especially suitable for the detection of common, infectious diseases. In addition, tNGS may be a good tool for *Aspergillus fumigatus, Mycobacterium tuberculosis, Cryptococcal meningitis*, or low-copy pathogens, and it affords the use of related technologies, such as wall-breaking technology for myco-bacteria, mycobacterial nucleic acid extraction technology, and human reference genome sequence data removal, which improves its positive rate of PTB detection.[32] tNGS is highly sensitive and specific in detecting drug resistance across panels of tuberculosis drugs and can be performed directly on clinical samples, and tNGS can also be applied to the detection of influenza A virus and cytomegalovirus pathogens and drug resistance assessment.[33–35] Like mNGS, tNGS also has the disadvantage of lacking a uniform interpretation standard; it also lacks a large-scale clinical validation study conducted with traditional diagnostic methods. The clinical interpretation of the test results is controversial. Therefore, further clinical research is needed to analyze the results of tNGS detection and explore the efficacy of its etiological detection so that its advantages of accuracy and speed can be fully utilized in clinical practice.

The limitation of this study is the fact that it is a single-center, retrospective clinical study. The sample size is relatively small, and the data may be biased. Missing data, particularly for Xpert comparisons (n=16), were addressed through strict exclusion criteria but may affect generalizability. Therefore, it is necessary to further use a multi-center, prospective, large-sample randomized con-trolled study to evaluate the efficiency of BALF mNGS or tNGS in the diagnosis of pulmonary tuberculosis and to explore the diagnostic efficacy of BALF mNGS or tNGS compared with X-pert to distinguish whether the positive Mycobacterium tuberculosis is colonized or active.

Conclusion

In conclusion, our study demonstrates that BALF mNGS and tNGS significantly enhance the diagnosis of pulmonary tuberculosis, achieving 77.66% sensitivity and 100% specificity with an AUC of 0.89, outperforming conventional methods such as acid-fast staining (15.96% sensitivity), mycobacterial culture (40.21%), and TB-DNA PCR (41.67%). These assays excel not only in detecting Mycobacterium tuberculosis but also in identifying co-occurring pathogens, including bacteria (*Haemophilus influenzae, Mycoplasma pneumoniae*), fungi (*Candida albicans, Aspergillus fumigatus*), and viruses (*Epstein–Barr virus, cytomegalovirus*). However, distinguishing clinically relevant infections from colonization—particularly for fungi like Candida and viruses such as papillomaviruses—requires integration with host immune status and radiographic findings. Compared to mNGS, tNGS offers comparable diagnostic accuracy (80.77% vs 73.81%, P=0.463) with accelerated turnaround time (8–16 hours vs 24–36 hours) and reduced costs, positioning it as a pragmatic alternative for resource constrained settings. Further validation is needed to standardize interpretation criteria and clarify the clinical significance of low-abundance microbial signals.

Ethics Approval and Informed Consent

This study was conducted in accordance with the Declaration of Helsinki. This study was ap-proved by the Ethics Committee of Wuhan Central Hospital (Permit numbers: WHZXKYL2024-187), and the need for obtaining written

informed consent was waived due to the retrospective nature of this study and the fact that the data did not contain personal information about the patients. Patient data was used only for this study and was kept strictly confidential.

Author Contributions

Zhen Yang and Qian Liu contributed equally to this work and share first authorship. 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

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