Infection and Drug Resistance

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ORIGINAL RESEARCH

Evaluating a Dual-Gene qPCR Melting Curve Assay for Rapid Detection of Tuberculosis in Suspected Pulmonary Cases

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Purpose: New economic, rapid, and efficient diagnostic methods are desirable for the control of tuberculosis. This study aimed to evaluate the performance of a dual-gene qPCR melting curve assay (DGPMC) in detecting tuberculosis among patients with suspected pulmonary tuberculosis.

Patients and Methods: The DGPMC assay based on *rpoB* and *IS6110* gene sequences has been established for detection of *Mycobacterium tuberculosis*. A prospective study was conducted among adult patients with suspected pulmonary tuberculosis from June 2021 to September 2023 at Shanghai Pulmonary Hospital, China. All patients received symptom assessment, high-resolution chest CT scan, and bronchoscopy. Bronchoalveolar lavage fluid was collected for mycobacterial culture and acid-fast staining, GeneXpert MTB/RIF, and DGPMC assay. The diagnostic performance of DGPMC assay was evaluated against the composite reference standard.

Results: Overall, 240 patients were included in this trial, including 80 (33.3%) asymptomatic patients. Clinical diagnosis of tuberculosis was confirmed in 191 (79.6%) patients and 49 (20.4%) patients were confirmed without tuberculosis. The overall sensitivity of the DGPMC assay was 55.0% (95% CI: 47.6–62.1%), and the corresponding specificity was 85.7% (95% CI: 72.1–93.6%) in the diagnosis of tuberculosis. The sensitivity of DGPMC assay was higher than that of GeneXpert test (55.0% vs 47.1%, P = 0.038). The Youden index and weighted Youden index of the DGPMC assay were 40.7% and 28.4%, respectively. Subgroup analyses demonstrated that the sensitivity was 32.4% (95% CI: 22.3–44.4%) in the individuals with negative results for both culture and GeneXpert test. The DGPMC assay performed significantly better than the melting curves based on *rpoB* gene or *IS6110* gene alone (P = 0.0000; P = 0.0020).

Conclusion: The DGPMC assay is an alternative tool favorable for the detection of tuberculosis in patients with suspected pulmonary tuberculosis, especially in the patients with low bacterial load.

Keywords: pulmonary tuberculosis, polymerase chain reaction, melting curve, diagnostic performance

Introduction

It is estimated that 10.8 million people worldwide suffer from tuberculosis in 2023, but only 8.2 million were officially notified, with a large gap of missed cases.[1] One of the most serious challenges in the prevention and control of tuberculosis is early, efficient, and accurate diagnosis of the disease.[2] However, the relatively low positive rate and the time-consuming nature prevent it from fulfilling the need for early diagnosis.[3,4] In 2013, WHO began recommending the use of molecular tests such as the GeneXpert MTB/RIF based on assay of MTB and rifampicin resistance (RIF).[5] Such new techniques demonstrated high sensitivity in the detection of tuberculosis in the sputum-smear-positive specimens, but low sensitivity in sputum-smear-negative specimens and the specimens of low bacterial load.[6,7] Therefore, it is still required to develop more

Received: 26 October 2024
Accepted: 8 February 2025
Published: 25 February 2025

rapid, efficient, and low-cost methods for diagnosis of tuberculosis. Particularly, efficient diagnosis of subclinical pulmonary tuberculosis with low bacterial load has emerged as a new direction for tuberculosis control.[8]

The melting curve-based assays involve the incorporation of fluorescence dye into double-strand DNA fragments which are generated during quantitative polymerase chain reaction (qPCR) using target gene-specific primers, followed by reading the fluorescent signal by PCR apparatus. After PCR ends, the melting curves of fluorescence-labeled PCR fragments are analyzed by a PCR machine with a program for target-specific identification.[9] Most of the previous reports have focused on the detection of drug resistance genes in tuberculosis.[10,11] In contrast, relatively few reports are available regarding the detection of MTB-negative pulmonary tuberculosis. The DGPMC method offers advantages such as multi - target detection, high - resolution melting curve analysis for accurate identification, and a wide dynamic range for detecting targets. Owing to these strengths, the DGPMC method demonstrates high sensitivity, low cost, acceptable specificity, and a rapid processing time of merely 2 hours.[12] Particularly, DGPMC is especially suitable for application in PCR detection of low copy templates. Our study aimed to evaluate the diagnostic performance of the DGPMC method in the diagnosis of tuberculosis.

Materials and Methods

Patients

The patients with imaging findings indicative of probable pulmonary tuberculosis were prospectively recruited from June 1, 2021, to September 30, 2022. All the study participants meet the following inclusion criteria: imaging findings suggest probable pulmonary tuberculosis; they are at least 15 years old; and their HIV test results are negative. No high-quality sputum sample was available or the sputum acid-fast bacilli (AFB) staining, and smear tests were negative for all patients before hospital admission. The study protocol was approved by the Institutional Review Board of Shanghai Pulmonary Hospital (No. K21-257). Written informed consent was obtained from all participants. The approving ethics committee judged that the participants under the age of 18 were able to provide their own informed consent. Our study complied with the Declaration of Helsinki. The diagnosis of tuberculosis was based on the Chinese Clinical Guideline for Diagnosis of Pulmonary Tuberculosis (WS 288–2017)[13] and the WHO tuberculosis treatment guidelines (2010 Edition).[14]

Collection and Processing of Clinical Samples

The patients underwent fiberoptic bronchoscopy for collection of bronchoalveolar lavage fluid (BALF) after signing their informed consent form. The patients who could not tolerate bronchoscopy were withdrawn from study. For patients with diffuse lesions in both lungs, the posterior segment of the upper lobe or the dorsal segment of the lower lobe was selected for collection of BALF. For patients with limited lesions, BALF could be collected from the bronchial lobes corresponding to the limited lung lesions. The patients whose bronchial stenosis in the lesion segment prevented effective bronchial irrigation were withdrawn from study. If bleeding occurred during lavage, the patient was excluded to avoid errors in the test results caused by blood contamination of BALF.

DNA Extraction and Purification

At least 10 mL BALF was used for DNA extraction in the Biosafety Level 2 Laboratory (BSL-2) of Shanghai Pulmonary Hospital. The DNA samples were subjected to double-gene PCR melting curve (DGPMC) analysis, which is based on the detection of both *rpoB* and *IS6110* genes. At the same time, AFB stain and smear, mycobacterial culture (BACTEC MGIT 960 system), and GeneXpert MTB/RIF were also performed on the BALF samples.

DNA purification was performed at room temperature using an automatic nucleic acid purification instrument – GenePure Pro Nucleic Acid Purification System (Hangzhou BIOER Technology Co., Ltd., China). BALF sample (200 μL) was mixed with 500 μL of cell lytic solution (Shanghai Liquidbio Biotechnology Co., Ltd., China) containing magnetic beads. The mixture was vortexed for 3 minutes. The DNA on magnetic beads was subsequently washed two times (3 minutes each) with 500 μL of 75% ethanol. The DNA was then eluted into 40 μL TE buffer.

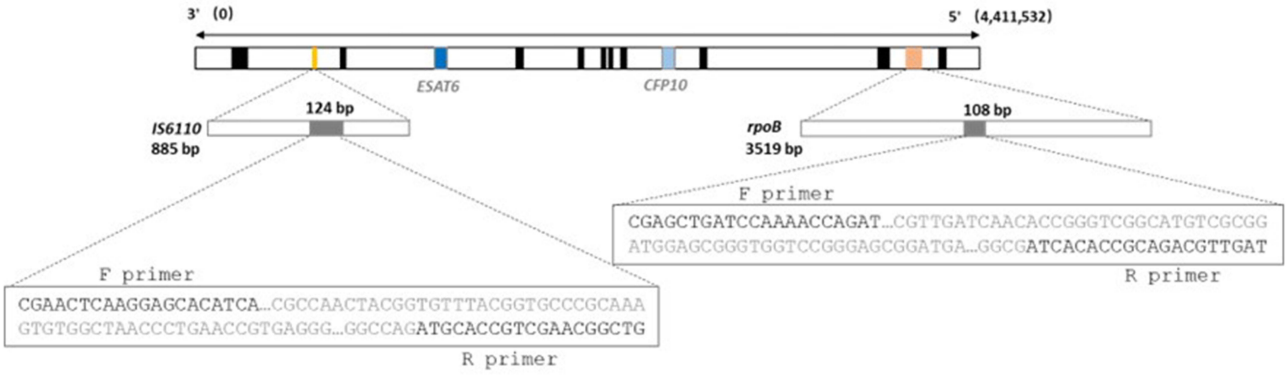

Figure 1 The oligonucleotide sequences of forward and reverse primers specific for PCR amplification of *rpoB* and *IS6110* genes. Primers were designed based on two genes, *IS6110* and *rpoB*, from the reference MTB strain H37Rv. The lengths of the PCR products obtained were 124 bp and 108 bp, respectively.

Fluorescent qPCR

The DNA oligonucleotide primers for PCR amplification were based on the genome sequence of MTB strain H37Rv. Two pairs of primers specific for the detection of MTB *rpoB* and *IS6110* genes were designed independently and prepared by a DNA synthesizer (Figure 1).

A fluorescent qPCR protocol was used for the detection of both *rpoB* and *IS6110* genes. A 25 μL PCR reaction volume consisted of forward and reverse primers (5 pM each), 0.5 unit UDGase, 5 μL DNA temperate, and 15 μL of Hieff qPCR SYBR Green Master Mix (containing 1 x buffer, dNTP, Taq DNA polymerase, SYBR). The PCR cycle consisted of 25°C for 5 min, 95°C for 5 min, followed by 45 cycles of 95°C for 10 sec, 56°C for 10 sec, and 60°C for 20 sec. Both PCR cycle threshold (CT) value and melting curve data were used for the resulting interpretation.

Two tubes of reagents are used to detect the *rpoB* and *IS6110* genes, respectively. If the CT value is ≤40 and the Tm value of the melting curve of either gene falls between 86.5°C and 88.2°C, it is judged as "positive". When there is no CT value or the Tm value is not within the range of 86.5°C and 88.2°C, it is judged as "negative".

Reference Strains

The national reference strains for PCR detection of MTB were used, including *Mycobacterium avium, Mycobacterium terrae, Mycobacterium stutzeri, Mycobacterium kansasii, Mycobacterium asiaticum*, and MTB H37Rv (batch number 230030–201703).

Assessment of Melting Curve and CT Value

The value of the fluorescent qPCR melting curve is related to the length of the amplified PCR fragments and the composition of nucleotide bases (G, A, T, C) in the PCR fragment. Longer PCR fragment with higher percentage of G and C nucleotide bases requires higher temperature to break the hydrogen bonds of PCR fragment and so higher melting curve. The CT value is directly related to the concentration of the target template in the samples and the efficiency of the PCR reaction. The MTB strain H37Rv was used as a standard template to generate the standard melting curve value. The high-frequency base polymorphism of the MTB target gene in the sample and the unavoidable slight shift rate of each reading of the fluorescent PCR instrument will lead to slight changes of melting curve value of the target gene to be detected. At the same time, other nontuberculous mycobacteria and pathogenic microorganisms, as well as primer dimers, will also express CT values and melting curves in PCR detection. To eliminate these non-specific interferences, we screened and confirmed the characteristic tuberculosis-specific melting curves by correlating to the clinical features, mycobacterium smear and culture, and radiographic findings. The recognized results were confirmed by sequencing analysis (Figures S1 and S2).

https://doi.org/10.2147/IDR.S498180

Statistical Analysis

A specific database was created to record all the clinical information and laboratory test results of patients. SPSS 26.0 software for Windows was used for data analysis. Measurement data were expressed as mean ± standard deviation. One-way analysis of variance and two independent sample *t*-test were used for comparison between groups. Enumeration data were expressed as a rate (%) and compared by chi-square test. The diagnostic performance was evaluated by Youden's index (J = sensitivity + specificity −1) and weighted Youden's index (Jω = 2 × [ω × sensitivity + (1-ω) ×specificity] −1, ω = 0.7). Youden's index provides a comprehensive measure of the test's ability to correctly identify both positive and negative cases. A higher Youden index indicates better overall diagnostic accuracy. The weighted Youden index considers the relative significance of sensitivity and specificity using the predefined weight ω. It helps in making a more refined assessment of the assay's diagnostic value, especially when the balance between them matters in specific clinical or research scenarios. Incorporating it enables a more accurate evaluation of the dual - gene qPCR melting curve assay's performance in tuberculosis detection. The primary outcome was the test performance (sensitivity and specificity) of DGPMC against composite diagnostic criteria. The secondary outcomes included the comparative diagnostic performance of DGPMC in subgroups in terms of bacterial loads.

Results

DGPMC Assay

The DGPMC method was established based on the melting curves of both *IS6110* and *rpoB* genes for detection of MTB. The *IS6110* and *rpoB* dual-gene melting curve based on the national reference MTB strain was achieved as follows (Figures 2 and 3).

Detection Limit of the DGPMC Assay

Sensitivity of melting curve method was analyzed in terms of diluted MTB. The positive control (PC) was 1000 copies/mL national reference strain. The negative control (NC) was DNase-free water. Both the positive and negative controls were tested in duplicates. The limit of detection was 16 copies/mL for *IS6110* and 32 copies/mL for *rpoB* (Table 1, Figures 4 and 5).

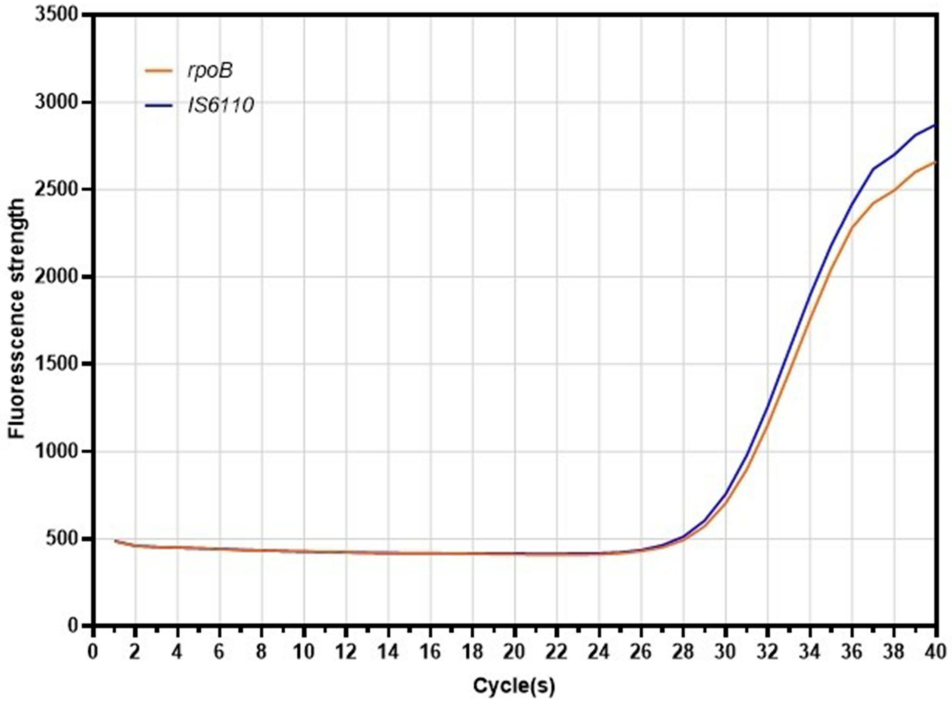

Figure 2 The cycle thresholds (CT) of *rpoB* and *IS6110* genes are based on the reference strain H37Rv. *IS6110* showed a higher copy number than *rpoB*. When the fluorescence reaches a specific threshold, the CT value is inversely proportional to the initial amount of the target DNA sequence (gene copy number).

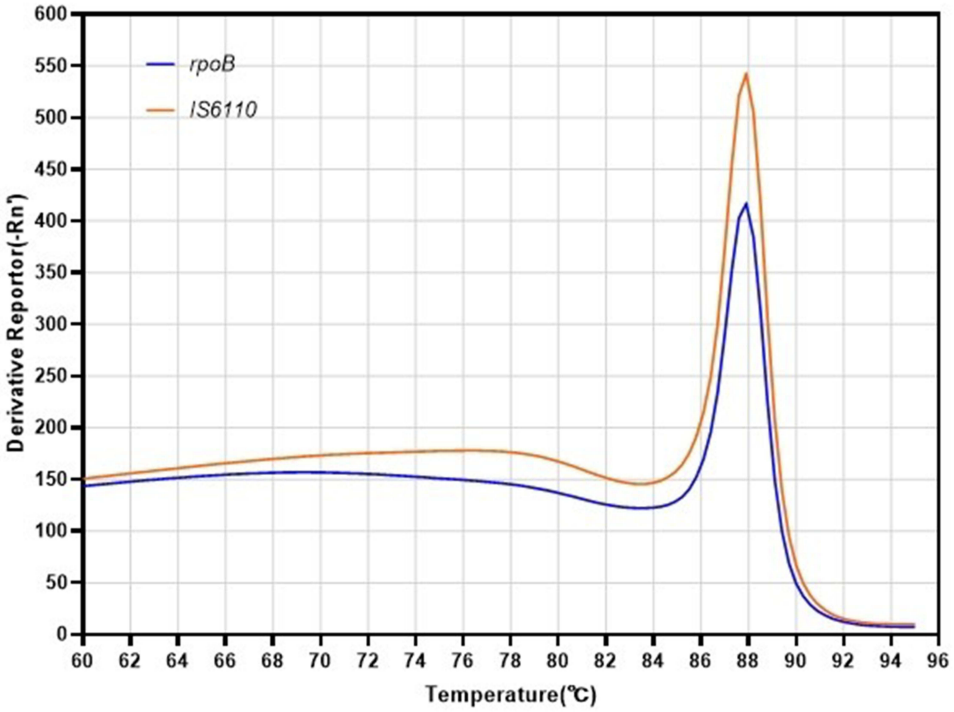

Figure 3 Both *rpoB* and *IS6110* amplicons showed similar melting curves. The melting temperatures of the dual-genes are between 86.5°C and 88.2°C.

Validation of the DGPMC Assay

MTB reference strain (batch number 230030–201703) and one clinical strain of MTB isolated from patient in our hospital were used in the test to confirm the specificity of the melting curve-based assay. The fluorescent signal was detected by real-time PCR in MTB (P01) only. None of the other NTM bacterial strain (N01 to N05) showed the specific melting curves (86°C–88°C) of MTB *IS6110* and *rpoB* amplicons (Table 2, Figures 6 and 7). Figures 6 and 7 show the melting curves of MTB *IS6110* and *rpoB* amplicons, respectively.

Interpretation of DGPMC Assay Results

The melting curves for either MTB *rpoB* or *IS6110* were between 86.5°C and 88.2°C. In addition, the melting curves of both *rpoB* and *IS6110* were greater than 83.5°C and less than 86.4°C or greater than 88.3°C and less than 88.6°C. The CT value of *IS6110* gene was 1.5 greater than that of *rpoB* gene.

Table 1 Sensitivity of PCR Amplification of rpoB and IS6110 Genes in the Reference MTB Strain H37Rv

Concentration	*rpoB*		*IS6110*	
	CT Value	Melting Curve Temperature (°C)	CT Value	Melting Curve Temperature (°C)
4 copies/mL	37.21	–	36.63	78.55
16 copies/mL	37.98	–	34.88	87.42
32 copies/mL	35.22	86.62	35.29	86.92
100 copies/mL	34.04	87.62	23.8	86.72
500 copies/mL	35.28	87.02	22.71	86.82

Abbreviation: CT, cycle threshold.

https://doi.org/10.2147/IDR.S498180

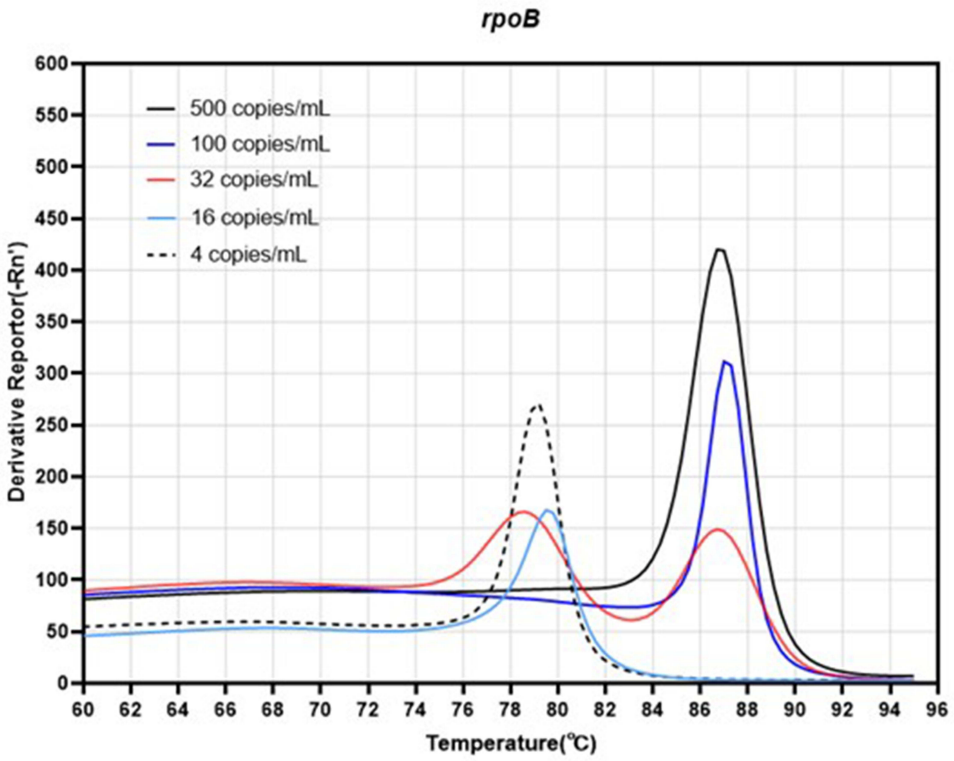

Figure 4 The melting curves (86.5°C and 88.2°C) for amplification of *rpoB* with different numbers of the reference MTB strain H37Rv. The melting curves lower than 80°C were due to primer dimers. In this study, 500, 100, 32, 16, and 4 copies/mL of *M. tuberculosis* were detected. The results showed the minimum detection limit of *rpoB* was 32 copies/mL.

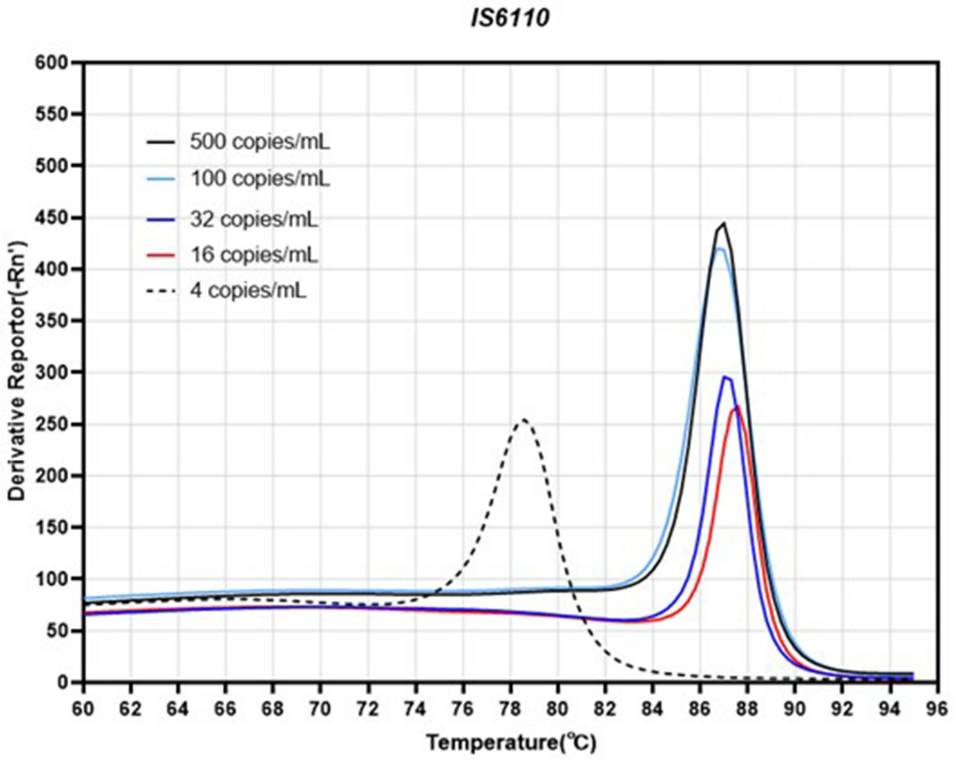

Figure 5 The melting curves 86.5°C and 88.2°C) for amplification of *IS6110* with different numbers of the reference MTB strain H37Rv. The melting curves lower than 80°C were due to primer dimers. In this study, 500, 100, 32, 16, and 4 copies/mL of *M. tuberculosis* were detected. The results showed the minimum detection limit of *IS6110* was 16 copies/mL.

Table 2 Specificity of PCR Amplification of MTB IS6110 and rpoB Genes

Control	Code	Strain	*rpoB*		*IS6110*	
			CT Value	Melting Curve Temperature (°C)	CT Value	Melting Curve Temperature (°C)
NC	N01	*Mycobacterium avium*	34.49	79.05	33.81	76.65
	N02	*Mycobacterium terrae*	35.17	79.54	33.31	76.95
	N03	*Mycobacterium stutzeri*	34.70	79.94	33.95	76.75
	N04	*Mycobacterium kansasii*	34.95	78.95	33.97	76.85
	N05	*Mycobacterium asiatica*	34.28	79.15	33.55	76.65
PC	P01	*Mycobacterium tuberculosis*	29.38	87.42	29.06	87.12

Abbreviations: CT, cycle threshold; NC, negative control; PC, positive control.

Clinical Details

Of the 283 patients screened, 43 patients were excluded, including 5 patients who did not give his/her initial consent or withdrew consent, 15 patients who failed to complete the bronchoscopy, 4 patients who withdrew from the study due to severe bronchial stenosis or bronchoatresia, and 19 patients who had nontuberculous mycobacteria as the culture results. Finally, 240 patients were included in this study (Figure 8).

The symptoms of suspected tuberculosis, high-resolution chest CT scan findings, immunological and microbiological test results are presented in Table 3. The median age was 43.1 (interquartile range [IQR]: 25.2–61.0) years. About 64.2% of the patients were males. Overall, 80 (33.3%) patients did not have any symptom. The median number of lung fields infected was 2 (IQR: 1–3). BALF acid-fast smear, GeneXpert MTB/RIF, Mycobacterium Growth Indicator Tube (MGIT) culture, and DGPMC assay were positive in 51 (21.3%), 90 (37.5%), 101 (42.1%), and 112 (46.7%) cases, respectively.

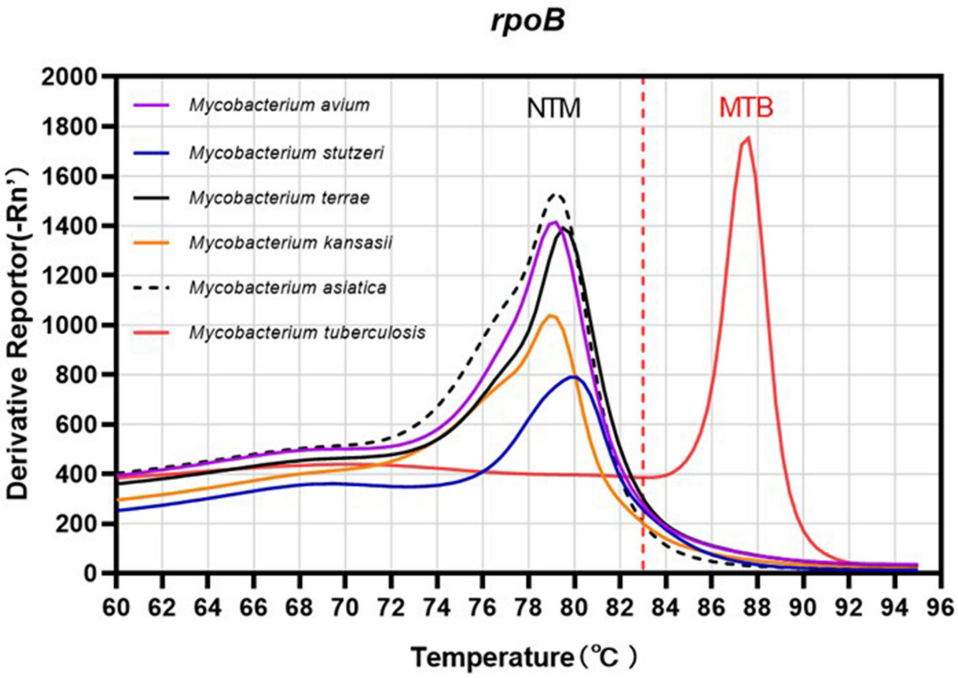

Figure 6 Specificity of PCR amplification of MTB *rpoB* gene. In the detection of *rpoB* gene, *Mycobacterium tuberculosis* can be specifically detected, and the melting curve shows a positive peak (86.5°C and 88.2°C), while other non-tuberculosis mycobacteria only show primer dimers' peak, such as *M. avium, M. stutzeri, M. terrae, M. kansasii, M. asiatica*.

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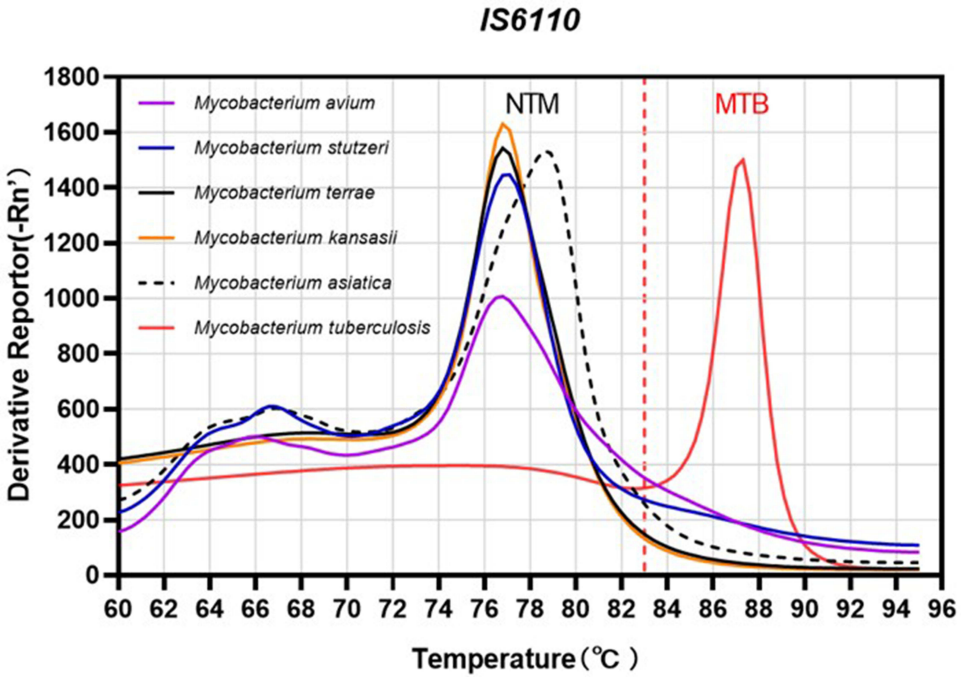

Figure 7 Specificity of PCR amplification of MTB *IS6110* gene. In the detection of *IS6110* gene, *Mycobacterium tuberculosis* can be specifically detected, and the melting curve shows a positive peak (86.5°C and 88.2°C), while other non-tuberculosis mycobacteria only show primer dimers' peak, such as *M. avium, M. stutzeri, M. terrae, M. kansasii, M. asiatica.*

Against the composite reference standard, 191 (79.6%) patients were clinically diagnosed with tuberculosis, including 101 (42.1%) patients who had a positive culture of MTB, 16 (6.7%) patients who had a negative MTB culture but a positive GeneXpert result, and 74 (30.8%) patients who had negative results for both MTB culture and GeneXpert test. Overall, non-tuberculosis was identified in 49 (20.4%) cases (Table 3).

Diagnostic Performance of DGPMC Assay in Terms of Bacterial Load

Compared with the composite reference standard, the sensitivity and specificity of the DGMCD assay was 55.0% (95% CI: 47.6–62.1%) and 85.7% (95% CI: 72.1–93.6%), respectively, in the entire study cohort. The sensitivity of DGPMC assay was higher than that of GeneXpert test (55.0% vs 47.1%, P = 0.038). The Youden index and weighted Youden index of the DGPMC method were 40.7% and 28.4%, respectively. Subgroup analyses demonstrated that the sensitivity of the DGPMC assay was 73.3% in the individuals with positive culture. Moreover, the sensitivity of the DGPMC assay was 32.4% in the individuals with negative results for both culture and GeneXpert (Table 4).

The DGPMC assay showed higher diagnostic efficacy than the Xpert method in the entire study cohort ($P = 0.0038$). Moreover, the DGPMC assay provided higher diagnostic efficacy than the Xpert method in the patients whose MTB culture was negative ($P = 0.0166$). The DGPMC assay also showed higher diagnostic efficacy than the melting curves of either *rpoB* gene or *IS6110* gene alone ($P = 0.0000$; $P = 0.0020$) (Table 5).

Discussion

This study offers the first prospective evaluation of the DGPMC assay in the patients with suspected tuberculosis, particularly the asymptomatic patients and the population with minimal imaging findings or negative bacteriological smears. It may provide one of the valuable methods for the early diagnosis of tuberculosis. In the present study, the sensitivity of the DGPMC assay in the detection of pulmonary tuberculosis ranged from 32.4% to 73.3%, and the corresponding specificity was 85.7%. Rapid and accurate detection methods are crucial for early diagnosis and effective treatment of pulmonary tuberculosis. Therefore, molecular diagnosis has been employed widely in clinical settings thanks to its high sensitivity and specificity.[15] Nevertheless, in patients with smear-negative pulmonary tuberculosis, the low bacterial load in respiratory samples and the possibility of mutations within target gene loci which can affect the binding sites of

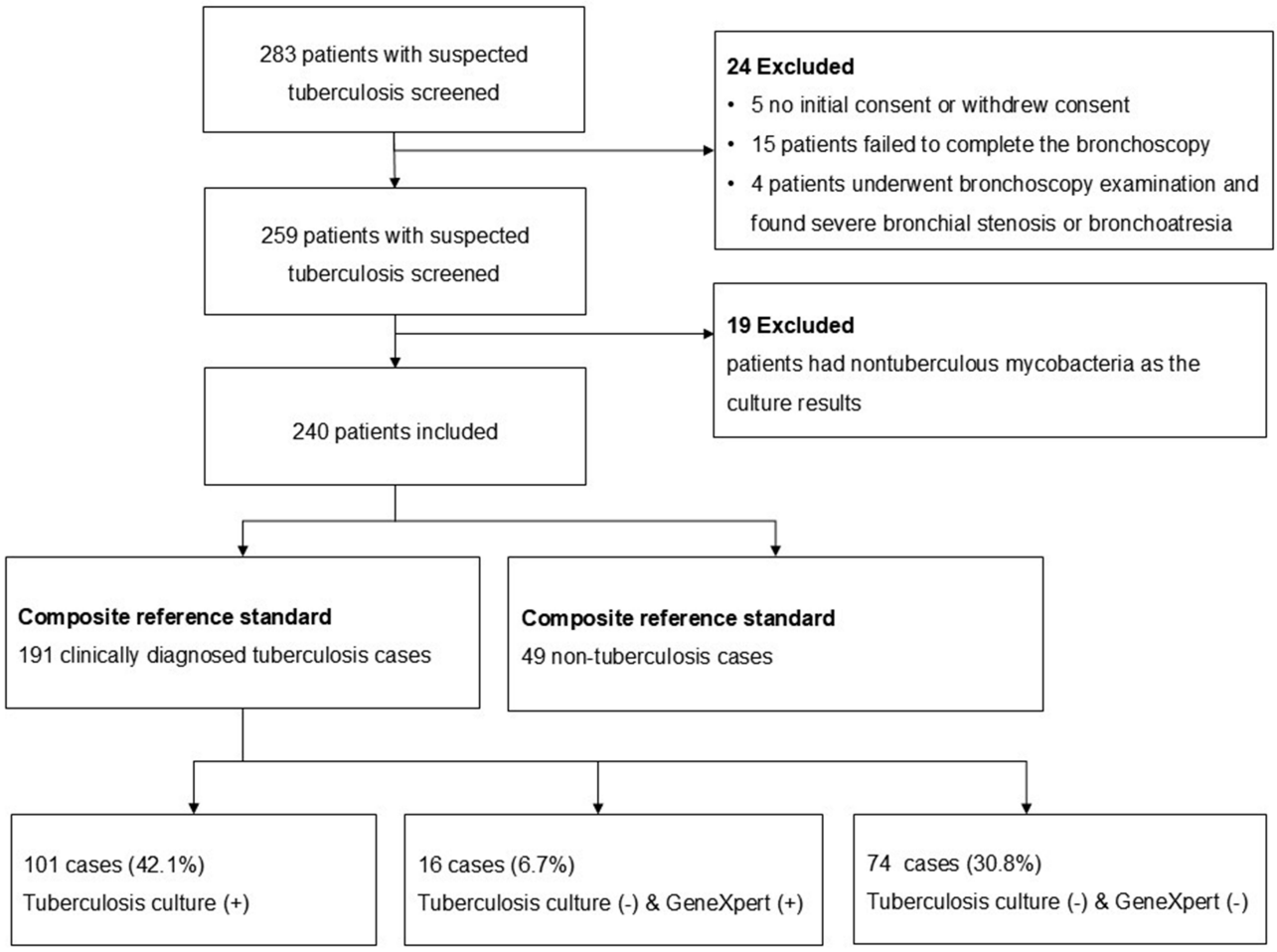

Figure 8 Flowchart showing patients disposition in the study.

primers and probes may lead to false-negative results in molecular assays and failure of diagnosis.[16] Asymptomatic patients accounted for 33.3% of the patients in this study, and 71.3% of them had lung lesions only involving 1–2 lung fields. The low bacterial load in these patients probably caused the failure of diagnosis. BALF was tested instead of sputum in our research because it was difficult to obtain sputum samples in the patient cohort. According to previous reports, the use of BALF obtained from the site of bronchial lesions could effectively enhance the detection of tuberculosis.[17]

Table 3 Patient Characteristics and Diagnostic Test Results

Characteristic	Value (n=240 Patients)
Age, years	43.1 ± 17.9
Sex	
Male	154 (64.2)
Female	86 (35.8)
Symptoms suggestive of tuberculosis	
Cough	112 (46.7)
Expectoration	88 (36.7)
Fever	24 (10.0)
Haemoptysis	22 (9.2)
Chest pain	27 (11.3)
Chest tightness	34 (14.2)

(*Continued*)

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Table 3 (Continued).

Characteristic	Value (n=240 Patients)
No symptoms	80 (33.3)
Comorbidity	
COPD	3 (1.3)
Diabetes mellitus	16 (6.7)
Tumor	4 (1.7)
Autoimmune disease	2 (0.8)
High-resolution chest CT scan	
No. of pulmonary fields infected	
1–2	171 (71.3)
3–4	51 (21.3)
5–6	18 (7.5)
Pulmonary cavity	
Yes	31 (12.9)
No	209 (87.1)
QuantiFERON-TB	
Positive	172 (71.7)
Negative	66 (27.5)
Not done	2 (0.8)
DGPMC (BALF)	
Positive	112 (46.7)
Negative	128 (53.3)
GeneXpert (BALF)	
Positive	90 (37.5)
Negative	150 (62.5)
Acid-fast smear (BALF)	
Positive	51 (21.3)
Negative	189 (78.7)
MGIT culture (BALF)	
Positive	101 (42.1)
Negative	139 (57.9)
Composite reference standard	
Clinically diagnosed tuberculosis	191 (79.6)
Culture positive	101 (42.1)
Culture negative but GeneXpert positive	16 (6.7)
Both culture and GeneXpert negative	74 (30.8)
Non-tuberculosis	49 (20.4)

Notes: Data are presented as mean ± SD or number (%) unless otherwise specified.
Abbreviations: COPD, chronic obstructive pulmonary disease; DGPMC, dual-gene PCR melting curve; BALF, bronchoalveolar lavage fluid; MGIT, *Mycobacterium* Growth Indicator Tube.

Molecular testing has shown robust diagnostic capability for sputum smear-positive pulmonary tuberculosis, but not so good for smear-negative pulmonary tuberculosis. A multi-center study that enrolled a total of 6648 patients reported that based on the sputum culture-positive results, the sensitivity of Xpert test with a single gene target was 90.3%, but the sensitivity dropped to 76.9% for bacterium-negative pulmonary tuberculosis.[18] The genes of MTB are inclined to mutate and adapt to the environment, particularly under the pressure of antibiotic treatment, expressing nucleotide changes/polymorphisms in certain antibiotic-target genes and causing structural changes in related proteins.[19,20] For example, *RpoB*, the target of rifampicin, has been found to have more than 100 types of gene mutations.[21] These mutations not only affect effective treatment but also lead to the failure of detection probes to bind to the relevant sites, resulting in false-negative results in molecular diagnosis. The high polymorphism rate of genes may also cause deviations in melting

Table 4 The Diagnostic Efficacy of DGPMC Assay in Different Groups of Patients

	Number of Cases	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Overall	240	76	7	115	42	55.0% (47.6–62.1%)	85.7% (72.1–93.6%)
Subgroup analysis							
Culture positive	101	74	0	27	0	73.3% (63.4–81.4%)	
Culture negative + GeneXpert negative	74	24	0	50	0	32.4% (22.3–44.4%)	
Non-Tuberculosis	49	0	7	0	42		85.7% (72.1–93.6%)

Abbreviations: DGPMC, dual-gene PCR melting curve; TP, true positive; FP, false positive; FN, false negative; TN, true negative; CI, confidence interval.

Table 5 Diagnostic Efficacy of DGPMC Assay and Xpert Test Compared With PCR Melting Curve Assays Based on rpoB or IS6110 Gene Alone

	Sensitivity (95% CI)	Specificity (95% CI)	*P* value	Youden Index	Weighted Youden Index
DGPMC assay	55.0% (47.6–62.1%)	85.7% (72.1–93.6%)		0.407	0.284
Xpert test	47.1% (39.9–54.4%)	100% (90.9–100%)	0.0038	0.471	0.260
PMC (*rpoB*)	39.8% (32.9–47.1%)	85.7% (72.1–93.6%)	0.0000	0.255	0.071
PMC (*IS6110*)	50.3% (43.0–57.5%)	87.8% (74.5–94.9%)	0.0020	0.380	0.230
Subgroup analysis					
DGPMC (culture negative)	27.8% (19.1–38.4%)	85.7% (72.1–93.6%)		0.135	−0.097
Xpert (culture negative)	17.8% (10.8–27.6%)	100% (90.9–100%)	0.0166	0.178	−0.151

Abbreviations: CI, confidence interval; DGPMC, dual-gene PCR melting curve; PMC, PCR melting curve.

curve assay.[22] However, the impact is much smaller compared to the probe method, mainly manifested as slight changes in melting temperature and the shift of melting point in melting curve assays. In such a cohort with low bacterial load in our study, the sensitivity of *RpoB* in the PMC assay and the Xpert test all showed a downward trend, which is consistent with the previously mentioned study.

To enhance the sensitivity of DGPMC assay, we incorporated the specific gene *IS6110* and established a method with dual target genes. *IS6110* plays a crucial role in the transmission dynamics and pathogenicity of MTB[23] and is absent in most nontuberculous mycobacteria.[24] In our study, *IS6110* is used as the second target to increase the sensitivity and specificity of the assay. The limit of detection might be as low as 16 and 32 copies/mL for *IS6110* and *rpoB,* respectively. This might be the reason why the diagnostic efficacy of the DGPMC assay is better than that of the Xpert test in both the entire study cohort and the culture-negative cohort. The higher diagnostic efficacy of the DGPMC assay compared to the melting curves based on either *rpoB* gene or *IS6110* gene alone also reflects the unique advantages of our dual-gene method. The same findings also apply to the Xpert MTB/RIF Ultra (abbreviated as Ultra) technology, which uses high-resolution melting curve technology and takes less time. At the same time, based on *rpoB* gene, the detection of *IS6110* and *IS1081* has added two multi-copy target genes. The limit of detection of Ultra is significantly lower than that of Xpert test in H37Rv strain.[25] The improved sensitivity of Ultra supports its application in various case finding scenarios.[26]

The weighted Youden´s index of the DGPMC assay was higher than that of Xpert test. This finding also supports the diagnostic utility of DGPMC assay in patients with active pulmonary tuberculosis. More importantly, the weighted Youden's index of the DGPMC assay was higher than that of the Xpert test in cases diagnosed as active tuberculosis in the culture-negative group. Therefore, the DGPMC assay may be a useful tool in clinical practice for identifying patients with negative TB test results. Actually, the assay is more sensitive in diagnosing culture negative TB.

Despite the promising performance of the DGPMC assay in several aspects, it is essential to also consider its potential drawbacks. First of all, the small number of controls may result in a certain bias in specificity. Secondly, as a single-center study in a country with high burden of TB, the patients enrolled in this study are predominantly those with low

bacterial load and associated with mild symptoms, thus the conclusion from this study cannot be generalized to the whole TB population. Our preliminary findings should be tested and confirmed in more diverse types of TB patients in subsequent multicentre studies. Thirdly, since the Xpert MTB/RIF Ultra reagent has not been widely used in China at the time of our research, we were unable to compare the melting curve with the Ultra method. To overcome the current limitations, future research should focus on conducting large-scale multicenter studies to validate the DGPMC assay across diverse patient populations. Additionally, comparative studies with other emerging diagnostic technologies, such as the Xpert MTB/RIF Ultra, should be carried out as soon as the technology becomes more widely available. These studies will provide a more comprehensive understanding of the DGPMC assay's position in the diagnostic landscape and help optimize its application.

Our research findings indicate that the DGPMC assay demonstrates remarkable potential as an accessible and efficient diagnostic instrument in resource-limited regions. Facilitating the early identification of tuberculosis, it can substantially decrease the transmission rate and enhance patient outcomes. In such areas, healthcare providers could potentially embrace this assay, which would not only augment their diagnostic proficiency but also curtail the diagnostic duration. Subsequently, this would contribute to the comprehensive management of tuberculosis and exert a significant influence on public health. Additionally, the establishment of this methodology offers a useful tool for detection of tuberculosis from other samples, such as exosomes and tongue swabs, and lays the foundation for further screening out tuberculosis patients or latent infections.

Conclusion

In summary, the DGPMC assay provides a practical alternative tool for detection of suspected or subclinical pulmonary tuberculosis patients. The DGPMC assay has unique advantages, especially in the patients with low bacterial load. It is expected to see the application of this economic, efficient, and accurate test in resource-limited regions to help find out and control tuberculosis.

Acknowledgments

We acknowledge the support of Shanghai Liquidbio Biotechnology Co., Ltd. for providing support in experimental technologies for this study. We also acknowledge Xuewen Tang, Yuanyuan Huang, and Dr. Xing Tang from Liquidbio Biotech for their scientific guidances and suggestions on nucleic acid extraction and detection reagent validations.

Funding

This study was supported by the grants from the National Key R&D Program of China (grant No.: 2023YFC2307300, 2023YFC2307305); Shanghai Science and Technology Commission Project (grant No.: 22Y11900900); and Shanghai Three-Year (2023–2025) Action Plan to strengthen the public health system (grant No.: GWVI-11.2-YQ06, GWVI-11.1-05); Shanghai's 2023 "Science and Technology Innovation Action Plan", special general project of medical innovation research (Grant No.23Y11900300).

Disclosure

The authors report no conflicts of interest in this work.

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