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Research article

Establishment of cross-priming amplification for point-of-care detection of *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria

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A R T I C L E I N F O

Keywords:
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Mycobacterium tuberculosis
Non-tuberculosis mycobacteria
Point-of-care testing

A B S T R A C T

Objective: To simplify sputum sample preparation steps and achieve point-of-care testing (POCT) of *Mycobacterium tuberculosis* (MTB) and non-tuberculosis mycobacteria (NTM) using Cross-Priming Amplification (CPA) technology on portable devices, overcoming the challenges of existing nucleic acid detection technologies that cannot be widely promoted in grassroots settings in China.

Methods: Evaluate the liquefying ability of high-concentration guanidine thiocyanate (GTC) for sputum and the effectiveness of MTB inactivation; establish a rapid detection system for MTB and NTM based on CPA technology using EasyNAT integrated detection tubes, with the left amplification zone specific to MTB CPA amplification and the right amplification zone specific to both MTB and NTM CPA amplification. Suspected pulmonary tuberculosis (PTB) patients or patients diagnosed as suspected NTM pulmonary infections specimens collected from the Second Hospital of Longyan, Fujian Province, from September 2022 to September 2023, acid-fast bacilli (AFB) smear, quantitative real-time PCR (RT-PCR) and CPA-POCT were performed. The kappa coefficients was used to evaluate the consistency between the RT-PCR and CPA-POCT.

Results: The liquefaction effect of 6M GTC on sputum was equivalent to 4 % NaOH, and no MTB growth was observed in the Lowenstein-Jensen medium of sputum samples treated with 6M GTC incorporating the H37Rv strain. The newly established CPA-POCT method showed good agreement with RT-PCR with a positive compliance rate of 86.27 %, a negative compliance rate of 89.36 %, an overall compliance rate of 87.75 %, and a Kappa coefficients of 0.786 ($P < 0.05$).

Conclusion: 6M GTC can liquefy sputum and render MTB non-viable, eliminating the need for Mycobacterium nucleic acid testing in BSL-2 laboratories; the newly established CPA method can rapidly and accurately distinguish MTB and NTM in the form of POCT, with simple and fast operation, suitable for promotion and application in grassroots medical institutions and remote rural areas.

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Abbreviations

POCT	point-of-care testing
CPA	cross-priming amplification
MTB	mycobacterium tuberculosis
NTM	non-tuberculous mycobacteria
PTB	pulmonary tuberculosis
GTC	guanidine thiocyanate
RT-PCR	quantitative real-time polymerase chain reaction
NTM-PD	nontuberculous mycobacteria-pulmonary disease
WHO	World Health Organization
NAATs	Nucleic acid amplification tests
AFB	acid-fast bacilli
EDTA	ethylenediaminetetraacetic acid
BSL	biosafety level
NaOH	sodium hydroxide
CT	Cycle threshold
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

1. Introduction

Mycobacteria include three major types: *Mycobacterium tuberculosis* (MTB), non-tuberculosis mycobacteria (NTM), and leprosy mycobacteria [1]. Currently, tuberculosis remains one of the major infectious diseases posing a serious threat to global public health, with approximately 10 million new cases and around 1.5 million deaths reported annually. In recent years, there has been a rapid increase in NTM diseases caused by NTM infections, particularly among immunocompromised patients, posing a significant public health challenge [2,3]. Both MTB and NTM primarily affect the pulmonary. In 2022, among the 7.5 million registered cases of tuberculosis globally, 83 % were pulmonary tuberculosis (PTB) cases (6.2 million cases), with NTM pulmonary diseases accounting for approximately 70%–80 % of NTM diseases [4]. Moreover, the clinical manifestations and radiographic features of NTM pulmonary diseases closely resemble those of PTB, often leading to misdiagnosis of NTM pulmonary diseases as PTB in clinical practice [5,6]. Given that the treatment regimens for PTB and NTM pulmonary diseases are entirely different, accurate discrimination of NTM during the initial screening of suspected tuberculosis patients is crucial [7].

Due to the positive acid-fast bacilli (AFB) smear results for both MTB and NTM, culture is typically required for differentiation, but this method is time-consuming and has a low positivity rate [8]. Nucleic acid amplification tests (NAATs) recommended by the WHO, such as Xpert MTB/RIF, have significantly improved the early diagnosis rate of PTB [9,10]. However, Xpert MTB/RIF detection is only targeted at MTB and cannot directly diagnose NTM infections. Moreover, the high cost of detection equipment and reagents has resulted in low accessibility at the grassroots level in China, where the incidence of tuberculosis is high in rural and grassroots areas [11,12]. In China, there are very few rapid detection methods available for simultaneous detection of MTB and NTM, all of which require facilities with nucleic acid testing capabilities found mainly in large hospitals, resulting in low accessibility at the grassroots level [13,14]. Therefore, there is an urgent need in China for a rapid, efficient, and cost-effective point-of-care testing (POCT) method to detect both MTB and NTM in grassroots and remote rural areas.

Cross-Priming Amplification (CPA) technology is the first nucleic acid amplification technique in China with independent intellectual property rights. CPA has been shown to rapidly and effectively detect MTB in sputum samples under isothermal conditions [15,16]. Its fundamental principle involves the design of 4 or 5 specific primers targeting 4 or 5 regions of the target gene. It utilizes Bst DNA polymerase with strand displacement specificity and betaine to efficiently, rapidly, and specifically amplify the target sequence at around 63 °C. Depending on the number of cross primers, CPA can be classified into double cross-amplification and single cross-amplification [17]. The EasyNAT MTC assay kit based on CPA technology integrates DNA extraction, purification, target gene amplification, and detection into three independent chambers within the same assay kit, completing the test within 90 min. The kit utilizes vitrification technology for convenient storage and transportation at room temperature, reducing reagent costs, and has successfully achieved POCT for MTB [18]. Currently, POCT testing for MTB in sputum specimens based on CPA technology, like many NAATs, still requires several manual steps for sputum processing. Specifically, sputum specimens must first be liquefied with 4 % NaOH, followed by high-speed centrifugation to remove the supernatant. After resuspension in saline, the sample can then be introduced into a fully automated integrated nucleic acid detection tube for analysis. These procedures still need to be performed in a strictly controlled biosafety level 2 (BSL-2) laboratory, which limits their application in primary care settings and remote rural areas [18].

Highly infectious and viscous sputum specimens used for tuberculosis diagnosis require complex and labor-intensive sample preparation steps, which have long been a bottleneck in establishing POCT molecular diagnostics for MTB [19]. To address this challenge, numerous researchers have explored various solutions. Bariki Mtafya and colleagues identified a practical method for safely applying TB molecular bacterial load quantification in clinical settings without the need for a biosafety level 3 laboratory [20]. However, their study only demonstrated the effectiveness of guanidine thiocyanate (GTC) in inactivating MTB cultures and did not address the issue of sputum viscosity. Before testing, viscous sputum samples still require liquefaction, which poses ongoing biosafety risks. Interestingly, research by Nasir Ali et al. indicates that high concentrations of GTC can liquefy sputum matrices composed of porcine mucin [21]. However, their preliminary study only utilized porcine mucin without validating the approach with clinical

sputum samples. Given that in molecular biology, GTC rapidly disrupts cells or viruses to release nucleic acids and inhibits nucleases released by cells, thereby maintaining the integrity of nucleic acid primary structures, it is a crucial component in nucleic acid extraction [21]. We hypothesized that using high concentrations of GTC as a liquefaction agent for viscous sputum specimens could simplify molecular biological testing by eliminating the need for complex buffer exchanges and high-speed centrifugation steps. This could make POCT for MTB feasible. Therefore, this study directly applied high concentrations of GTC to liquefy highly viscous sputum specimens, evaluating the effectiveness of GTC in liquefying sputum and inactivating MTB in viscous sputum specimens. The aim was to simplify sputum sample preparation steps, enabling MTB nucleic acid testing to be conducted without a BSL-2 laboratory, and to achieve POC testing for MTB and NTM using CPA technology on portable devices, thus addressing the challenges of implementing existing nucleic acid detection technologies in primary care settings in China.

2. Materials and methods

2.1. Sample collection

In this study, three sputum samples (MTB negative) (≥15 ml) were collected to evaluate the liquefaction effect of 6M GTC on sputum. Additionally, sixteen sputum samples (negative for MTB) were collected and mixed with H37Rv strains to create simulated MTB-positive sputum specimens. These specimens were used to test the effectiveness of 6M GTC in inactivating MTB as part of the biosafety risk assessment for conducting mycobacterial nucleic acid testing in grassroots laboratories without BSL-2 laboratory infrastructure.

Prospective collection of sputum or bronchoalveolar lavage specimens from suspected PTB patients or cases of suspected NTM pulmonary infections in the Second Hospital of Longyan Fujian Province from September 2022 to September 2023 for clinical validation experiments. Inclusion criteria: All of the following criteria must be met: (1) Age and gender are not limited; (2) Sputum or bronchoalveolar lavage sample volume is not less than 3 ml; (3) Suspected PTB patients with PTB symptoms or signs; (4) Cases suspected NTM pulmonary infections. Exclusion criteria: Any case meeting any of the following criteria must be excluded: (1) Sample contamination or ineffective detection due to any factors; (2) Inability to trace case information in hospital medical records. All specimens underwent AFB smear, quantitative real-time polymerase chain reaction (RT-PCR) (Mycobacterium nucleic acid detection kit, CapitalBio, Chengdu), and CPA-POCT mycobacterial nucleic acid testing.

2.2. Evaluation of sputum liquefaction with 6M GTC

A total of 146 g ethylenediaminetetraacetic acid (EDTA) [Product Number: A600107, Sangon Biotech (Shanghai) Co., Ltd., Shanghai] was dissolved in 900 ml double-distilled water, and the pH was adjusted to 8.0 with NaOH (Xilong Scientific, Guangdong). The solution was then brought to a volume of 1000 ml with double-distilled water to prepare a 0.5M stock solution. Additionally,

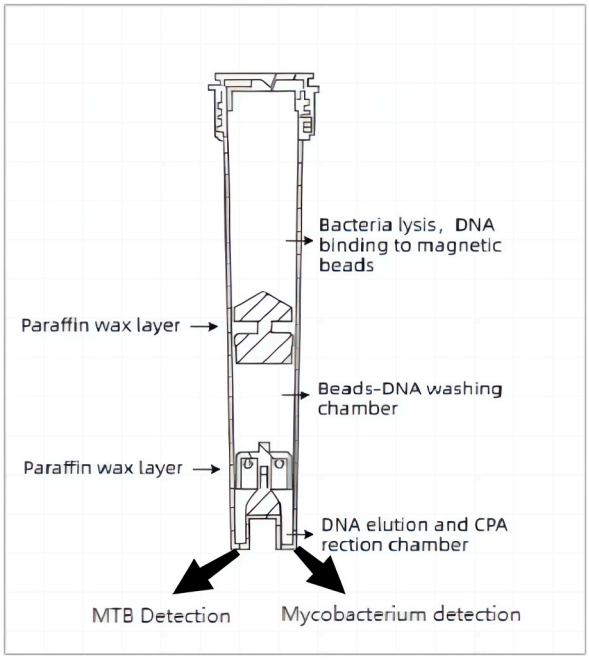

Fig. 1. Fully automated integrated nucleic acid test tubes. DNA: deoxyribonucleic acid; CPA: cross-priming amplification; MTB: mycobacterium tuberculosis.

354.4 g GTC [Product Number: A610224, Sangon Biotech (Shanghai) Co., Ltd., Shanghai] was dissolved in 400 ml 0.5M EDTA (pH 8.0) and then supplemented with 0.5M EDTA (pH 8.0) to a final volume of 500 ml.

Three sputum samples (A, B, C) were each divided into 8 portions of 1 ml. Two researchers separately added 3 ml of 6M GTC liquefaction reagent, 5 ml of 6M GTC liquefaction reagent, 3 ml of 4 % NaOH solution, and 3 ml of physiological saline to each portion of the sputum sample. All samples were shaken for 1 min and then stood at room temperature. The initial viscosity of the sputum was categorized as ++++, and its evaluation was based on the change from ++++ (most viscous or unchanged) to + (least viscous or completely liquefied) after treatment with different chemical substances.

The samples were gently mixed, and liquefied samples were aspirated using a 10 μl pipette for observation of aspiration. Simultaneously, the viscosity was subjectively assessed by visual observation and measured. Relative viscosity was evaluated independently by two researchers, with each researcher performing three independent experiments and recording the results.

2.3. Evaluation of the effectiveness of MTB inactivation by 6M GTC

Sixteen 5 ml sputum specimens (MTB negative) were collected, and each sputum specimen was spiked with 5 μl of 0.5 McFarland turbidity (1.5 × 10^8 CFU/mL) standard H37Rv strain suspension to simulate MTB-positive sputum specimens. Among these, eight specimens were treated with 2–4 times 4 % NaOH based on sample viscosity, and the remaining eight were processed with 2–4 times 6M GTC based on sample viscosity. All samples were mixed for 1 min, left to stand at room temperature for 15 min, and then 0.1ml–0.3 ml of the pretreated specimens were taken and aseptically inoculated onto the slant of the Lowenstein-Jensen medium, with two culture tubes inoculated simultaneously for each sample. The inoculated culture tubes were incubated at 35°C–37 °C with 5%–10 % CO2. After 24 h of incubation with the caps loosened, the culture tubes were placed loosely capped at an angle for 24 h before the caps were locked and placed vertically. Colony growth was observed on the 3rd and 7th days after inoculation and incubation. If colonies were observed and confirmed as mycobacteria by AFB smear, rapid-growing mycobacterial culture positivity was reported. Subsequently, observations were made weekly to record colony growth and contamination.

2.4. Establishment of CPA-POCT method for detecting MTB and NTM

The fully automated integrated nucleic acid detection tube (Ustar Biotechnologies Co., Ltd., Hangzhou, China) comprises a main chamber and two side chambers located below it (Fig. 1), with hydrophobic separation layers within the tube that isolate the lysate, washing solution, and reaction solution. The main chamber is used for the addition of samples, lysates, nanomagnetic beads, and internal control templates. Under the control of external heating instruments, the extraction solution chemically lyses the sample at high temperatures and releases nucleic acids. Through the magnetic guiding effect of the external instrument, the nucleic acids in the sample traverse different liquid layers within the tube. Ultimately, the nucleic acids are eluted in the tube's leg, where amplification reactions occur. This design achieves a "one-tube" fully automated nucleic acid analysis, performing lysis, binding, washing, elution, and amplification reactions within a single sealed detection tube [22]. The left amplification region of the detection tube qualitatively detects the MTB-specific *IS6110* insertion sequence [22]. In contrast, the right amplification region of the detection tube simultaneously detects the specific sequences of *16S rRNA* genes of both MTB and NTM [23]. The *rpoB* sequence of the conservative RNA

Table 1
Primer and probe sequences for the EasyNAT integrated detection cartridge.

Primers	Primer and Probe Sequence (5′-3′)
EasyNAT Integrated Detection Cartridge - Right Amplification Region Primer and Probe Sequence	
MTB/NTM-BF	5′-GGAATTCCTGGTGTAGCGG-3′
MTB/NTM-BR	5′-CAGGCGGGGTACTTAATGC-3′
MTB/NTM-CPF	5′-GCGTCAGTTACTGCCCAGAGAC-AATGCGCAGATATCAGGAGG-3′
MTB/NTM-CPR	5′-CTGGTAGTCCACGCCGTAAACG-GTTAGCTACGGCACGGATC-3′
MTB/NTM-LF	5′-CGCCTTCGCCACCGGTGT-3′
MTB/NTM-LR-FAM	5′-6-FAM-CCGAGCGATTCGCTCGG-(Int BHQ1 dT)- GTGGG TACTAGGTGTGGGTT-3′
EasyNAT Integrated Detection Cartridge - Left Amplification Region Primer and Probe Sequence	
MTB-BF	5′-TCAACCGGGAGCCCAG-3′
MTB-BR	5′-TTTGCCGCGGGTGGTC-3′
MTB-CPF	5′-CGTAAACACCGTACTTGGCCGCCCGCCATCGCGAACTCA-3′
MTB-CFR	5′-GTGCCCGCAAAGTGTGGCTAACAGTTTGGTCATCAGCCGTTC-3′
MTB-LF	5′-TGGACGCGGCTGATGTGC-3′
MTB-LR-FAM	5′-6-FAM-CCGAGCGATTCGCTCGG (Int BHQ1 dT)TGAACCGTGAGGGCATCGAG-3′
Reference Primer and Probe Sequence	
Bsu-ropB-BF	5′-GTGTTAGAATTACCAAATCTCATTG-3′
Bsu-ropB-BR	5′-AATTAAACGAACCTTCACTCTT-3′
Bsu-ropB-CPF	5′-CCAGTGAAATCCTCAATTGGTGATAACCTCTTCTTATCAGTGGTTTC-3′
Bsu-ropB-CPR	5′-AGTTTAGGTGAGCCTAAATATCCTGGGAGCTGAGTAAGTCACATC-3′
Bsu-ropB-LF	5′-CCAGTGAAATCCTCAATTGGTGAT-3′
Bsu-ropB-LR	5′-AGTTTAGGTGAGCCTAAATATCCTG-3′
Bsu-ropB-LR-CY5	5′-6-Cy5-CCGAGCGATTCGCTCGG-(Int BHQ2 dT)-CCTCTCTCTTGAGTTCATTGA-3′

Note: MTB, *Mycobacterium tuberculosis*; NTM, Non-tuberculous mycobacteria; Bsu, *Bacillus subtilis*.

polymerase β subunit coding gene of *Bacillus subtilis* (Bsu) was used as the target sequence for the internal control system, and an internal control system was designed accordingly. The primer-probe sequences for the left and right amplification regions and the internal control system of the EasyNAT detection tube are listed in Table 1. No requirement is imposed on the internal control detection result when the test result is positive. However, when the test result is negative, the internal control detection result must be positive; otherwise, the test result is considered invalid. If both the left and right amplification regions are positive, it is considered MTB; if the left is negative and the right is positive, it is considered NTM; if both are negative, it is considered a non-mycobacterial infection. The interpretation of results is detailed in Table 2 and Fig. 2.

2.5. Sensitivity and specificity assessment of CPA-POCT

Using the National reference for PCR-based detection of *Mycobacterium tuberculosis* (Batch Number: 230030-202205) provided by the National Institutes for Food and Drug Control of China. The reference include positive and negative controls (MTB, P1-P15, 1 × 10^3 bacteria/mL; NTM, N1-N10, 1 × 10^4 bacteria/mL; other respiratory pathogens, N11-N15, 1 × 10^4 bacteria/mL), as well as minimum detectable quantity reference [MTB (CMCC 93009) single-cell suspension, MTB-S1, 1 × 10^3 bacteria/mL, MTB-S2, 1 × 10^2 bacteria/mL, MTB-S3, 1 × 10^1 bacteria/mL, MTB-S4, 1 × 10^0 bacteria/mL]. Each component of the reference was mixed with 1 ml of DNA extraction solution (containing magnetic beads and internal standard template) and transferred into EasyNAT integrated detection tubes. The tubes were sealed, shaken, and used as the test detection tubes. The test sample detection tubes were placed into the sample chamber of the fully automated nucleic acid detection instrument (UC0108) for detection, and experimental results were recorded.

2.6. Clinical validation of CPA-POCT method

2.6.1. Flowchart of clinical validation (Fig. 3)

The flowchart of clinical validation of this study is shown in Fig. 3.

2.6.2. AFB smear

Sputum smear microscopy was carried out with the modified Ziehl-Neelsen staining method (cold staining method), strictly performed according to the manufacturer's instructions. The brief procedure is as follows: approximately 0.05 ml of sputum sample containing caseous or purulent material, or suspicious areas, is picked and evenly spread on the front side of a slide to form an oval sputum film measuring 10mm × 20 mm, which is then air-dried. Before staining, the slide is heat-fixed (passing it through a flame four times within 5 s). After fixation, the slide is placed on a staining rack with a spacing of at least 10 mm between slides. Carbonate fuchsin stain is added dropwise to cover the slide completely, followed by staining at room temperature for 10 min. The slide is then gently rinsed with running water from one end to remove excess stain, drained, and covered with an acidic alcohol solution for 1–2 min to decolorize. Decolorization is continued until the sputum film is no longer visibly red. The slide is again rinsed gently under running water for 10–20 s to remove the acidic alcohol solution, drained, and covered with methylene blue solution for 30–60 s. After another gentle rinse under running water from one end to remove excess stain, the slide is drained and ready for microscopic examination.

2.6.3. CapitalBio mycobacterium RT-PCR detection

Detection was strictly performed according to the manufacturer's instructions. Depending on the viscosity of the sputum, add 2–4 times 4 % NaOH solution to liquefaction. 1 ml of the liquefied specimen was transferred to a 1.5 ml centrifuge tube, centrifuged at 12000×*g* for 5 min, and discard the supernatant. Then, 1 ml of physiological saline was added, and the mixture was vortexed thoroughly. After centrifugation at 12000×*g* for 5 min, the precipitate was obtained. Subsequently, the precipitate and the nucleic acid extract were added to the nucleic acid extraction tube and vortexed for 10 min. After heating at 95 °C in a metal bath for 10 min, the mixture was centrifuged at 10000×*g* for 2 min to obtain the DNA of mycobacteria. The extracted DNA was amplified according to the manufacturer's instructions for amplification reactions, using a fluorescent quantitative PCR instrument (ABI7500, USA) for amplification and detection. MTB and NTM were amplified and detected within 3 h [13].

2.6.4. Mycobacterium CPA-POCT detection

Based on the viscosity of the sputum, add 2–4 times 6M GTC liquefaction, and the specimen was vortexed for 30 s to homogenize. The mixture was left at room temperature for at least 15 min until the specimen was fully liquefied. One milliliter of liquefied specimen

Table 2
Interpretation of CPA-POCT results.

Results			Interpretation of the results
Left amplification region	Right amplification region	Internal control system	
Positive	Positive	No requirement	MTB positive
Positive	Negative	No requirement	MTB positive
Negative	Positive	No requirement	NTM positive
Negative	Negative	Positive	Mycobacterium Negative

Note: MTB, *Mycobacterium tuberculosis*; NTM, Non-tuberculous mycobacteria.

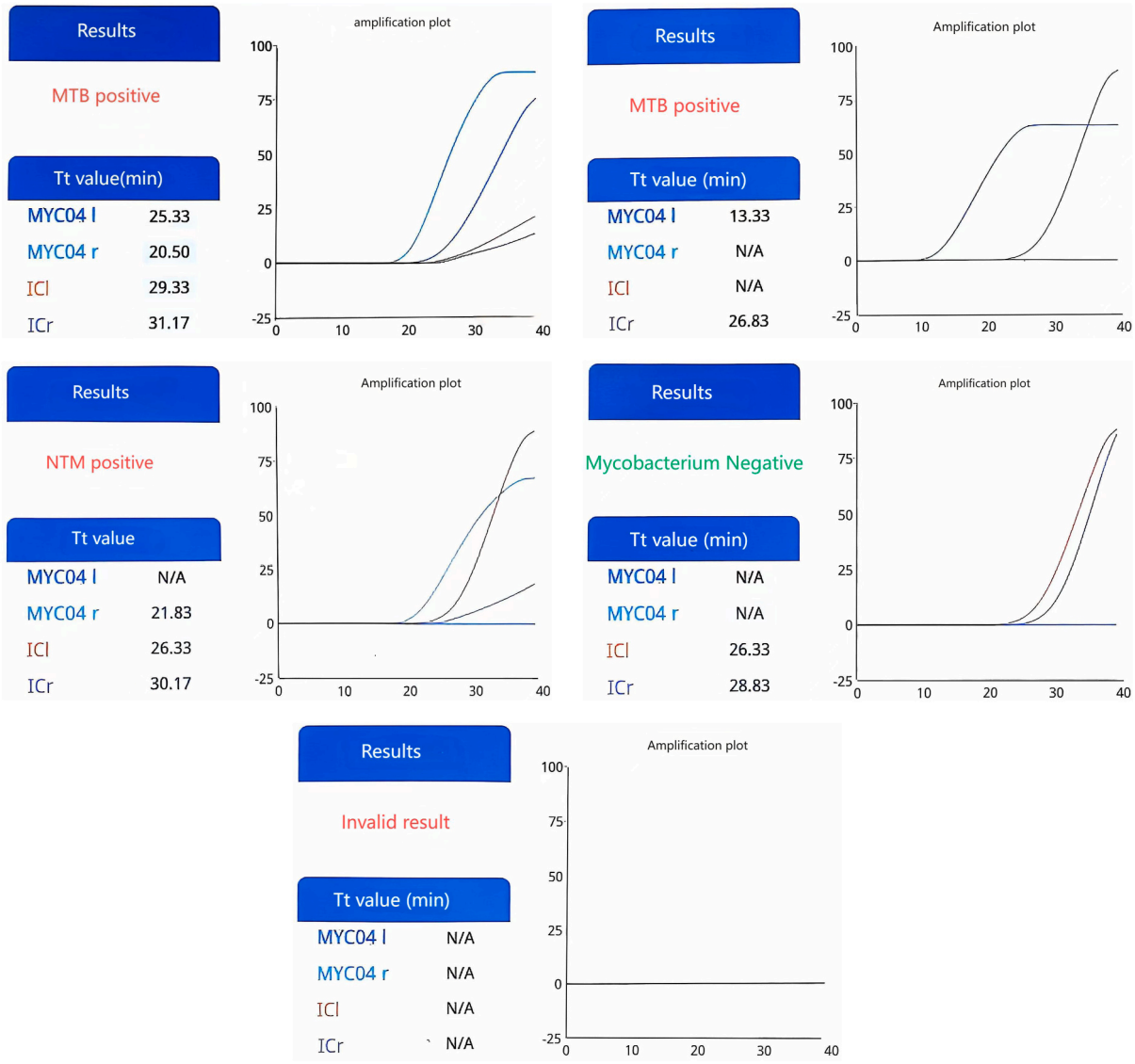

Fig. 2. Example of results. MTB positive (A); MTB positive (B); NTM positive(C); MTB positive; Mycobacterium Negative (D); Invalid result (E). MTB: mycobacterium tuberculosis; NTM: non-tuberculous mycobacteria.

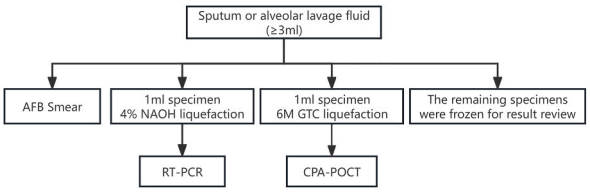

Fig. 3. Schematic diagram of the clinical validation workflow. AFB: acid-fast bacilli; NAOH: sodium hydroxide; GTC: guanidine thiocyanate; RT-PCR: quantitative real-time polymerase chain reaction; CPA: cross-priming amplification; POCT:point-of-care testing.

and 1 ml of DNA extraction solution (containing magnetic beads and internal standard template) were mixed and transferred into EasyNAT integrated detection tubes. The tube was covered, shaken well, and used as the test tube for detection. The test sample tubes were placed into the fully automatic nucleic acid detection instrument (UC0108) (Portable detection device, no special requirements for experimental environment, rapid on-site detection, independent detection module, available for immediate testing) sample chamber for detection, and the experimental results were recorded.

2.6.5. Statistical analysis

IBM SPSS 26 statistical software was used to organize, count, and analyze the data. Categorical data were expressed as proportion, such as the sensitivity, specificity, positive coincidence rate, and negative coincidence rate of the CPA-POCT. The consistency of RT-PCR and CPA-POCT results was assessed by kappa coefficients. Kappa coefficients are measures of correlation between categorical variables often used as reliability or validity coefficients. The calculation of kappa coefficients is detailed in the article published by Helena Chmura Kraemer et al. [24] Kappa coefficients: to judge the consistency of the two assays between 0 and 1. The larger the kappa coefficients, the better the consistency. A kappa coefficients $\geq$0.75 indicates a good degree of consistency. If the Kappa coefficients is $<$ 0.4, the degree of agreement is not good enough.

3. Results

3.1. Evaluation of 6M GTC liquefaction effect

Three sputum specimens A, B, and C were liquefied using different ratios of 4 % NaOH and 6M GTC. The results showed that after 15 min of liquefaction when the ratio of sputum to 6M GTC was 1:5, the liquefaction effect was comparable to that of sputum to 4 % NaOH at a ratio of 1:3, as shown in Table 3.

3.2. Evaluation of the effectiveness of MTB inactivation by 6M GTC

After pretreating eight simulated MTB-positive sputum specimens with 4 % NaOH for 15 min, they were inoculated onto Lowenstein-Jensen medium. In one case, a sample was found on the third day of inoculation to have bacterial growth that was not the typical colony morphology of MTB. Acid-fast staining confirmed the growth was acid-fast negative, indicating contamination. The remaining seven specimens showed typical MTB colony growth on day 21 of incubation. These colonies were confirmed as acid-fast positive and identified as MTB by RT-PCR, thus confirming them as MTB-positive. In contrast, when eight simulated MTB-positive sputum specimens were pretreated with 6M GTC for 15 min and inoculated onto Lowenstein-Jensen medium, no colony growth was observed up to the eighth week of incubation, indicating a negative result for Mycobacterium culture. This demonstrates that MTB cannot survive in sputum specimens treated with 6M GTC for 15 min.

3.3. Sensitivity and specificity of CPA-POCT for MTB and NTM detection

The CPA-POCT method developed in this study was validated for sensitivity and specificity using the National Reference Material for *Mycobacterium tuberculosis* PCR Detection Kit provided by the China National Institute for Food and Drug Control. Fifteen MTB strains from the reference material were tested in triplicate, all results were positive for MTB. Ten different non-tuberculous mycobacteria (NTM) strains from the reference material were also tested in triplicate, with all results positive for NTM. In contrast, five other pathogenic strains from the reference material tested negative. These results demonstrate that the established CPA-POCT method exhibits high specificity for detecting *Mycobacterium tuberculosis*, as shown in Table 4. Additionally, MTB (CMCC 93009) single-cell suspensions at various concentrations were tested in triplicate. MTB concentrations of 100 cells/mL or higher were detectable, with a detection limit of 100 cells/mL, as shown in Table 5.

3.4. Clinical test results

3.4.1. Diagnosis and classification of patients

The diagnosis of PTB refers to “Diagnostic criteria for pulmonary tuberculosis WS288-2017" [25]. The diagnosis of PTB mainly relies on microbiological (including bacteriological and molecular biological) examinations, combined with epidemiological history, clinical manifestations, chest imaging, relevant auxiliary examinations, and differential diagnosis, to make a comprehensive analysis and diagnosis. Microbiological and pathological results serve as the basis for confirmation. Patient categories are defined based on the following criteria: (1) Confirmed PTB: Sputum or bronchoalveolar lavage smear positive and/or culture positive and/or molecular

Table 3
Effectiveness of different chemicals on liquefaction of sputum.

Different chemicals	Sputum: liquefied liquid	Liquefied for 7.5 min			Liquefied for 15 min			Liquefied for 30 min			Liquefied for 60 min		
		A	B	C	A	B	C	A	B	C	A	B	C
4 % NaOH	1:3	++			+			+			+		
6M GTC	1:3	+++			++			+			+		
6M GTC	1:5	++			+			+			+		
Saline	1:3	++++			++++			++++			+++		

Note: NaOH, sodium hydroxide; 6M GTC, 6 mol/L guanidine thiocyanate."++++" indicates that the sample has visible sputum, and the pipette is blocked and cannot operate; "+++" indicates that the sample is partially liquefied, and the pipette is blocked and cannot operate; "++" indicates that the sample is basically liquefied, and the pipette is partially blocked; "+" indicates that the sample is completely liquefied, and a 10 μl pipette can be used for aspiration.

Table 4
Specificity of the CPA-POCT assay.

Pathogen	Positive rate
M.tuberculosis (P1-P15)	45/45
M.avium (N1)	3/3
M.terrae (N2)	3/3
M.shimodii (N3)	3/3
M.Kansasii (N4)	3/3
M.asiaticum (N5)	3/3
M.scrofulaceum (N6)	3/3
M.gordonae (N7)	3/3
M.chelonae/M.abscessus (N8)	3/3
M.fortuitum (N9)	3/3
M.phlei (N10)	3/3
Nocardia brasiliensis (N11)	0/3
corynebacterium pekinense (N12)	0/3
Pneumococcus (N13)	0/3
Legionella pneumophila (N14)	0/3
Bordetella pertussis (N15)	0/3

Table 5
Detection limit of the CPA-POCT assay.

MTB(CMCC 93009) Single cell suspension concentration	Positive rate
1000 bacteria/mL	3/3
100 bacteria/mL	3/3
10 bacteria/mL	0/3
1 bacteria/mL	0/3

biology examination positive, confirming PTB. (2) Suspected PTB: No bacteriological evidence of tuberculosis was obtained, with PTB diagnosis based solely on symptoms, radiographic images, treatment response, and follow-up data. (3) Non-tuberculous diseases: Cancers or other diseases diagnosed by histopathology or other examinations.

Diagnosis of NTM pulmonary Disease is referenced from the "Diagnosis and Treatment Guidelines for Non-tuberculosis Mycobacterial Diseases (2020 Edition)" [26], with diagnostic criteria as follows: Patients presenting with respiratory system symptoms and/or systemic symptoms, with chest imaging revealing findings such as cavitary shadows, bronchiectasis, and multiple nodular lesions, and other pulmonary diseases being excluded, under the premise of ensuring that the specimen is free from exogenous contamination, can be diagnosed with NTM pulmonary disease if they meet one of the following conditions: (1) Two separate sputum specimens sent for examination are positive for NTM culture and identified as the same pathogen, and/or NTM molecular biology testing confirms the same pathogen; (2) Bronchoalveolar lavage fluid or bronchial washing fluid is positive for NTM culture and/or

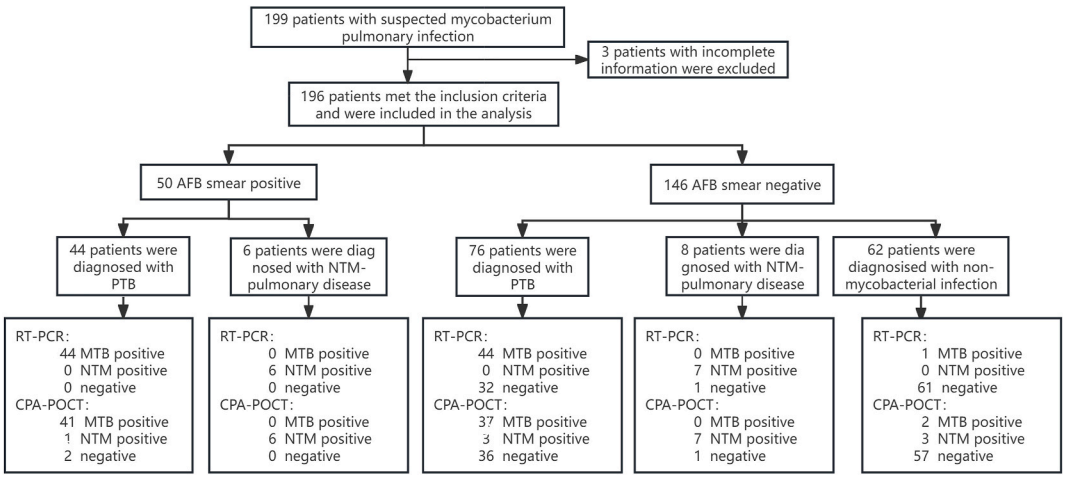

Fig. 4. Diagnostic classification of study participants. AFB: acid-fast bacilli; PTB: pulmonary tuberculosis; NTM: non-tuberculous mycobacteria; MTB: mycobacterium tuberculosis; RT-PCR: quantitative real-time polymerase chain reaction; CPA: cross-priming amplification; POCT:point-of-care testing.

molecular biology testing at least once; (3) Pulmonary histopathological changes characteristic of mycobacterial disease (granulomatous inflammation or AFB positive) are observed through bronchoscopy or other means of pulmonary tissue examination, and NTM culture and/or molecular biology testing is positive; (4) Pulmonary histopathological changes characteristic of mycobacterial disease (granulomatous inflammation or AFB positive) are observed through bronchoscopy or other means of pulmonary tissue examination, and NTM culture and/or molecular biology testing is positive in one or more sputum specimens, bronchoalveolar lavage fluid, or bronchial washing fluid samples.

3.4.2. Demographic and clinical characteristics of study participants

A total of 199 suspected PTB or NTM pulmonary disease patients were enrolled, with 3 patients excluded from the study due to incomplete case information, as shown in Fig. 4. The final sample size for this study was 196 cases, including 120 cases of PTB (61.22 %), 14 cases of NTM pulmonary disease (7.14 %), and 62 cases of non-mycobacterial disease (31.63 %). In this study, we defined patients with suspected PTB or suspected pulmonary disease who did not receive bacteriological evidence, as well as patients diagnosed with cancer or other diseases through histopathological examination or other tests, as non-mycobacterial disease. Among the 14 NTM pulmonary disease patients, 6 were positive on sputum smear microscopy, and 8 were negative; species identification revealed 5 cases of mycobacterium intracellulare, 3 cases of mycobacterium chelonae or mycobacterium abscessus, and 6 cases of *Mycobacterium avium*. The 62 non-mycobacterial disease patients included 5 cases of suspected PTB, 1 case of suspected NTM pulmonary disease, 9 cases of pulmonary cancer, 2 cases of AIDS, and 45 cases of other infectious diseases.

The proportion of males among PTB patients was significantly higher than that among NTM pulmonary disease patients and non-mycobacterial disease patients ($\chi2 = 24.50$, $P < 0.05$), and there was a statistical difference in the presence of diabetes between PTB patients and non-mycobacterial disease patients ($\chi2 = 10.03$, $P < 0.05$). However, there were no statistically significant differences among the three groups of patients in terms of age, history of PTB, or presence of hypertension ($P > 0.05$), as shown in Table 6.

3.4.3. Analysis of the consistency of AFB smear, RT-PCR, and CPA-POCT tests with clinical diagnostic results

The clinical sensitivity, specificity, and accuracy of CPA-POCT were 69.71 %, 91.93 %, and 75.51 %, respectively, which were superior to those of AFB smear (37.31 %, 100 %, and 57.14 %; $\chi2 = 42.53$, $P < 0.05$), but lower than those of RT-PCR (75.37 %, 98.38 %, and 82.65 %; $\chi2 = 90.39$, $P < 0.05$).

Among the three methods, the consistency of CPA-POCT results with clinical confirmation/exclusion results (Kappa coefficients = 0.583, $P > 0.05$) was better than that of AFB smear (Kappa coefficients = 0.274, $P > 0.05$), but inferior to RT-PCR (Kappa coefficients = 0.693, $P > 0.05$), as shown in Table 7.

3.4.4. Detection results of clinical samples of CPA-POCT

Compared to RT-PCR, the positive coincidence rate of CPA-POCT was 86.27 % (88/102), the negative coincidence rate was 89.36 % (84/94), and the total coincidence rate was 87.75 % (172/194). The Kappa coefficients was 0.786, indicating good consistency between the two methods, as shown in Table 8.

4. Discussion

In resource-limited and impoverished areas, and at grassroots healthcare facilities, well-equipped tuberculosis laboratories are extremely limited, making early and accurate diagnosis of pulmonary mycobacterial infections highly challenging [27,28]. Currently, most molecular tests for pulmonary mycobacterial diseases may generate aerosols during the manual or automated extraction of nucleic acids from sputum or bronchoalveolar lavage fluid, posing a biosafety risk to laboratory personnel [10]. Bariki Mtafya et al. [20,21] found that GTC, as an important component of nucleic acid extraction, can effectively inactivate MTB. Therefore, GTC was selected as the liquefaction in this study, and it was found that after liquefaction for 15 min, the liquefaction effect was comparable

Table 6
Demographic and clinical characteristics of study participants.

Characteristics	PTB (N = 120)	NTM-Pulmonary disease (N = 14)	Non-mycobacterial disease (N = 62)
Median age (years)	60 (16–88)	66 (51–85)	61 (18–84)
Male	105 (87.5 %)	6 (42.8 %)	38 (61.3 %)
Treatment status			
First treatment	107 (89.17 %)	/	/
Retreatment	13 (10.83 %)	/	/
History of tuberculosis	21 (17.50 %)	2 (14.29 %)	15 (24.19 %)
Combined extrapulmonary infection			
Tuberculous pleurisy	14 (11.67 %)	/	/
Others	5 (4.17 %)	/	/
Basic illness			
Diabetes	36 (30.0 %)	0	9 (14.5 %)
Hypertension	26 (21.7 %)	1 (7.14 %)	16 (25.8 %)
AIDS	0	0	2

Note: PTB, pulmonary tuberculosis; NTM, Non-tuberculous mycobacteria; AIDS, acquired immunodeficiency syndrome. Others refer to one case of extrapulmonary combined lymph node tuberculosis, two cases of spinal tuberculosis, and two cases of tuberculous pericarditis.

Table 7
Consistency analysis of three detection methods and clinical diagnosis/exclusion results.

		Clinical confirmation/exclusion results			Total	Clinical sensitivity	Clinical specificity	Accuracy	Kappa coefficients
		PTB	NTM-Pulmonary disease	Non-mycobacterial disease					
AFB smear	Positive	44	6	0	50	37.31 %	100 %	57.14 %	0.274
	Negative	76	8	62	146				
	Total	120	14	62	196				
RT-PCR	MTB	88	0	1	89	75.37 %	98.38 %	82.65 %	0.693
	NTM	0	13	0	13				
	Negative	32	1	61	94				
	Total	120	14	62	196				
CPA-POCT	MTB	78	0	2	80	67.91 %	91.93 %	75.51 %	0.583
	NTM	4	13	3	20				
	Negative	38	1	57	96				
	Total	120	14	62	196				

Note: PTB, pulmonary tuberculosis; NTM, Non-tuberculous mycobacteria; AFB, acid fast bacteria; RT-PCR,quantitative real-time polymerase chain reaction; MTB, mycobacterium tuberculosis; NTM, non-tuberculous mycobacteria; CPA-POCT, cross-priming amplification-point-of-care testing.

Table 8
Detection results of clinical samples of CPA-POCT.

Test results		RT-PCR			Total
		MTB	NTM	Negative	
CPA-POCT	MTB	75	0	5	80
	NTM	2	13	5	20
	Negative	12	0	84	96
Total		89	13	94	196
positive coincidence rate		86.27 %			
negative coincidence rate		89.36 %			
total coincidence rate		87.75 %			
Kappa coefficients		0.786			

Note:RT-PCR, quantitative real-time polymerase chain reaction; MTB, mycobacterium tuberculosis; NTM, non-tuberculous mycobacteria; CPA-POCT, cross-priming amplification-point-of-care testing.

when the ratio of sputum to 6M GTC was 1:5, and when the ratio of sputum to 4 % NaOH was 1:3. Moreover, after 15 min of liquefaction with 6M GTC, MTB could not survive, whereas MTB could be cultured after liquefaction with 4 % NaOH. Indicated that as a pretreatment fluid for respiratory specimens, 6M GTC is more effective than 4 % NaOH in reducing the risk of MTB transmission in healthcare facilities and communities, while also providing hope for the establishment of molecular POCT methods for mycobacterial diseases.

Zhang et al. [18] demonstrated that for smear-negative and smear-positive tuberculosis patient samples, the positive detection rate of CPA was significantly higher than that of the Xpert MTB/RIF method. For special populations, Quan et al. [29] simultaneously tested gastric fluid samples from 239 pediatric patients using CPA and Xpert MTB/RIF, finding similar sensitivity of the two methods in total active TB patients. You et al. [22] successfully established an MTB-POCT based on CPA technology, but like Xpert MTB/RIF, it can only detect MTB and cannot differentiate between MTB and NTM. Therefore, CPA is a sensitive and easily implementable POCT technology. Mycobacterium nucleic acid detection kit (RT-PCR) from CapitalBio, is the only mycobacterial rapid detection kit approved for marketing in China, capable of simultaneously detecting MTB and NTM. It exhibits good accuracy in diagnosing pulmonary mycobacterial infections, similar to Xpert MTB/RIF, and superior diagnostic accuracy for NTM infections compared to Xpert MTB/RIF [13]. Hence, in this study, using 6M GTC as the liquefaction agent, a POCT method for both MTB and NTM based on CPA technology was established. Mycobacterium nucleic acid detection kit (RT-PCR) from CapitalBio was used as the reference reagent to compare the performance of CPA-POCT, RT-PCR, and AFB smear testing for the detection of pulmonary mycobacterial infections.

This study, conducted on 196 suspected PTB or NTM pulmonary disease patients, found that CPA-POCT showed better clinical sensitivity, specificity, accuracy, and consistency with clinical diagnosis/exclusion results compared to AFB smear but was inferior to RT-PCR. The lower specificity of CPA-POCT may be related to the propensity of isothermal amplification technology to produce non-specific binding [30]. However, the negative coincidence rate of CPA-POCT with RT-PCR was 89.36 %, the positive coincidence rate was 86.27 %, and the total coincidence rate was 84.45 %, with a Kappa coefficients of 0.786, indicating high consistency and good diagnostic accuracy. Therefore, using 6M GTC as the liquefaction agent, the POCT method based on CPA technology, requiring only one-step sampling, can be rapidly conducted on-site in resource-limited primary healthcare settings, providing laboratory evidence for early diagnosis and timely accurate treatment of MTB and NTM infections. This method reduces dependence on nucleic acid amplification laboratories, addresses the shortcomings of current diagnostic methods, and strengthens the control of mycobacterial infections in grassroots or impoverished areas.

Furthermore, the clinical characteristics analysis of the 196 patients included in this study revealed that the proportion of male patients with PTB was significantly higher than that of patients with NTM pulmonary disease and non-mycobacterial disease ($\chi 2 = 24.50$, $P < 0.05$). Additionally, there was a statistical difference between PTB patients and non-mycobacterial disease patients in terms of whether they had diabetes ($\chi 2 = 10.03$, $P < 0.05$). However, there were no statistical differences among the three groups of patients in terms of age, PTB history, and hypertension. This indicates that diabetic patients are at a higher risk for tuberculosis, consistent with the results of Hu et al [31]. Therefore, strengthening the detection of tuberculosis patients among diabetic individuals during PTB screening and implementing standardized treatment management can effectively reduce the spread of tuberculosis and prevent its occurrence.

This study has some limitations. Firstly, the CPA-POCT cannot differentiate between mixed infections of MTB and NTM, nor can it distinguish between different NTM species or MTB drug resistance. The inability to recognize mixed MTB and NTM infections is a common challenge that exists in the rapid diagnosis of MTB and NTM. Currently, the only rapid detection kit for mycobacteria in China [13]; the LAMP method established by Kim et al. [32]; and the multiplex fluorescence PCR method established by Ustinova et al. [33] all suffer from the same limitation. Currently, the rate of mixed infections of MTB and NTM in China is 0–3% [34,35], necessitating further optimization of the POCT system and exploration of other biomarkers or targets, to overcome the challenge of molecular rapid diagnostic methods failing to recognize mixed MTB and NTM infections. Secondly, this study is a single-center study with a small sample size, and the study was only assessed clinically by comparison with RT-PCR technology and not with the gold standard culture method, thus requiring multicenter and larger sample studies.

5. Conclusions

In summary, the POCT method for MTB and NTM established in this study based on CPA technology, using 6M GTC as a pre-processing solution for sputum samples, can be performed under rudimentary laboratory conditions, requiring only 90 min to complete without complex manual operation steps, and is easy to operate. The development of this POCT system provides a new option for rapid and accurate diagnosis of MTB and NTM infections in primary healthcare settings. Its rapid, simple, and convenient characteristics contribute to improving diagnostic efficiency and patient management in clinical practice.

Ethics statement

This research was approved by the Ethics Committee of the Second Hospital of Longyan City, Fujian Province (Approved No. of ethic committee: LYEY2023LSK-009). All samples are residual specimens from routine clinical examinations.

Data availability statement

Data included in article/supp. material/referenced in article.

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CRediT authorship contribution statement

Wenbin Huang: Writing – original draft, Visualization, Validation, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Liping Chen:** Writing – review & editing, Validation, Investigation, Data curation, Conceptualization. **Yuankui Lin:** Writing – review & editing, Validation, Investigation, Formal analysis, Conceptualization. **Ting Xiao:** Validation, Investigation, Formal analysis, Data curation. **Man'e Zhang:** Validation, Investigation, Formal analysis, Data curation. **Dingchang Wu:** Writing – review & editing, Visualization, Resources, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Wenbin Huang reports financial support was provided by Fujian Society of Laboratory Medicine and the Joint Innovation Project of the National (Fujian) Gene Testing Technology Application Demonstration Center. Yuankui Lin is employed by company "Ustar Biotechnologies (Hangzhou) Ltd." If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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