Heliyon 11 (2025) e41384

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Development and evaluation of multiplex real-time PCR for rapid identifying major pathogenic mycobacteria from pulmonary and extrapulmonary clinical samples in eastern China

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ARTICLE INFO

Keywords:
Mycobacterium tuberculosis complex
Non-tuberculous mycobacteria
Multiplex real-time fluorescence PCR
Rapid identification

ABSTRACT

Background: Diseases caused by *M. tuberculosis* (MTB) and non-tuberculous mycobacteria (NTM) have similar clinical symptoms but require different treatments. Rapid and accurate identification of MTB and NTM is essential for proper patient management and treatment.

Methods: To develop and assess a multiplex real-time fluorescence PCR (Multiplex PCR) method for rapid identification of MTB, *M. avium* complex (MAC), M. chelonae-M. abscessus group (MCAG), and *M. kansasii* in clinical samples. The specificity and limit of detection (LOD) were tested using standard strains and clinical isolates. The accuracy of the Multiplex PCR was validated with DNA from 228 known clinical samples confirmed by Targeted next-generation sequencing (tNGS). Additionally, 901 consecutive clinical samples were assessed to evaluate the Multiplex PCR's diagnostic performance in detecting mycobacteria in pulmonary and extrapulmonary samples.

Results: LOD of the four mycobacteria ranged from 11.7 to 360.0 CFU/mL in water, 43.8–922.0 CFU/mL in sputum, and 53.2–859.3 CFU/mL in sputum mixed infection, with 98.7 % sample detection accuracy and 100 % strains identification accuracy. Based on the composite reference standard, the sensitivity of the Multiplex PCR for detecting tuberculosis in pulmonary and extrapulmonary samples was comparable to Xpert ($p > 0.05$) and higher than Culture, especially in extrapulmonary samples ($P < 0.0001$). For NTM detection in pulmonary samples, the sensitivity was slightly lower than Culture ($P > 0.05$) but higher than CapitalBio RT-PCR ($P < 0.05$). Overall, the Multiplex PCR showed significantly higher sensitivity for mycobacterial diseases compared to the other three methods ($P < 0.01$), with a specificity of 96 %.

Conclusions: The Multiplex PCR demonstrated excellent diagnostic performance in both pulmonary and extrapulmonary samples, offering a low-cost, rapid identifying tool for major pathogenic mycobacteria in eastern China.

1. Introduction

Diseases caused by mycobacteria are a major global health issue, leading to infections in the lungs, skin, bones, joints, lymph nodes,

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https://doi.org/10.1016/j.heliyon.2024.e41384
Received 28 July 2024; Received in revised form 7 December 2024; Accepted 19 December 2024
Available online 27 December 2024

and other extrapulmonary tissues [1]. The Mycobacterium genus includes the *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium leprae*, and non-tuberculous mycobacteria (NTM) [2]. *M. tuberculosis* (MTB) is the primary member of the MTBC species, and diseases caused by MTB are known as tuberculosis (TB), one of the world's deadliest infectious diseases. It was reported that in 2022, approximately 1.3 million people worldwide died from TB, with 7.5 million new infections, of which 83 % were pulmonary tuberculosis (PTB) and 17 % were extrapulmonary tuberculosis (EPTB). China, being one of the high-burden TB countries, ranks third globally in incidence [3]. The incidence of NTM is increasing globally, posing a serious threat to human health [4]. NTMs can cause widespread systemic infections, with pulmonary infections being the most common (65–90 %) [5]. The distribution of NTM varies by country and region, with the most common pathogenic NTMs worldwide being *M. avium* complex (MAC), M.chelonae-M. abscessus group (MCAG), *M. kansasii*, *M. xenopi*, and *M. fortuitum* [4,6].In the eastern Zhejiang region of China, the main pathogenic NTMs are MAC (*M.avium*, *M. intracellulare*, and its subspecies *M. paraintracellulare* and *M. chimaera*), MCAG (*M. chelonae*, *M. abscessus* subsp. *abscessus*, and *M. abscessus* subsp. *massiliense*), and *M. kansasii*, accounting for 93 % of all clinical NTM isolates [7].

The clinical manifestations of NTM diseases are similar to those of TB, posing diagnostic challenges even for experienced clinicians [4]. However, their treatment regimens differ, and the treatment strategies for different types of NTM also vary [8]. In China, 4.2 %–6.4 % of suspected PTB cases are actually NTM infections, and misdiagnosis and inappropriate treatment significantly increase the mortality rate associated with NTM infections [9]. Therefore, rapid identification methods for mycobacteria are needed to guide clinicians in making accurate and timely diagnoses.

Traditional culture methods ("gold standard") and acid-fast bacilli (AFB) smears remain important for laboratory diagnosis of mycobacteria. However, due to their time-consuming nature and low sensitivity, they have gradually been replaced by molecular diagnostic techniques, significantly shortening the diagnostic time. The WHO-recommended Xpert MTB/RIF (Xpert, Cepheid, USA) is widely used in China and can provide results for MTB and rifampicin resistance within 2 h, with high sensitivity (59%–75 %) and high specificity (96%–100 %). However, it can only detect MTB and not NTM [10–12]. The CapitalBio Mycobacterium RT-PCR Detection Kit (CapitalBio RT-PCR, CapitalBio Technology, China), developed in China, uses dual real-time fluorescence PCR technology to differentiate between MTB and NTM infections but cannot distinguish NTM species [13]. Complex techniques based on PCR products, such as linear probe detection [14], PCR restriction fragment length polymorphism (RFLP) analysis [15], and microarray technology [16], are cumbersome and susceptible to contamination from amplification products. In many large laboratories in China, gene sequencing has become the primary method for mycobacteria diagnosis. For example, Targeted next-generation sequencing (tNGS) combines ultra-multiplex PCR amplification with high-throughput sequencing to directly detect dozens to hundreds of known pathogenic microorganisms and their virulence and resistance genes from clinical samples. It offers clear pathogen spectra and high detection precision but requires 2–3 days for results and is expensive, making it difficult to promote in routine laboratories [17]. To enhance the diagnostic capabilities of clinical laboratories, there is an urgent need for a rapid, low-cost mycobacteria identification method suitable for routine laboratories in primary hospitals.

This study developed a rapid detection method for the four major pathogenic mycobacteria prevalent in eastern China using multiplex real-time fluorescence PCR technology based on TaqMan probes and evaluated its diagnostic performance in pulmonary and extrapulmonary clinical samples.

2. Materials and methods

2.1. DNA preparation and colony-forming unit (CFU) calculation of bacterial strains

All standard strains and clinical isolates used in this study were obtained from the Tuberculosis Laboratory of Hangzhou Red Cross Hospital (Supplementary Table 1). Colonies in the logarithmic growth phase were picked from solid media, and a bacterial ultrasonic dispersion counter (TB Healthcare, China) was used to adjust each strain to a concentration of 1 McFarland. Each strain was diluted in a 10-fold gradient and inoculated separately in solid culture medium, with CFU counts conducted during the logarithmic growth phase of each strain.

DNA was extracted according to the instructions of the CapitalBio RT-PCR kit. DNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher, USA) and stored at −80 °C for future use.

2.2. Development of multiplex PCR detection method

2.2.1. Selection of target genes

Main *Mycobacterium* Target Gene Selection based on the bioinformatics analysis results, the IS*6110* sequence is a commonly used target for MTBC detection [18]. Specific targets for MAC and MCAG were designed within the 16S-23S rRNA intergenic spacer (ITS) [19]. *M. kansasii* was designed based on the most conservative sequence of mkan_rs*12360* [20]. Specific primers and probes were designed for these four targets, and the sequences are listed in Supplementary Table 2.

2.2.2. Multiplex PCR amplification and interpretation criteria

Multiplex PCR amplification was conducted in a total volume of 10 μL, including 5 μL of 2 × Animal Detection U + Probe qPCR Super PreMix (Nanjing VAzyme Biotech Co., Ltd, China), 3.2 μL of primer/probe mix, and 1.8 μL of DNA template. PCR and fluorescence detection were performed using the Bio-Rad CFX96 deep well real-time system (Bio-Rad Laboratories, USA). The PCR program was as follows: pretreatment at 37 °C for 2 min; denaturation at 95 °C for 30 s; 45 cycles of denaturation at 95 °C for 10 s, annealing, and extension at 60 °C for 30 s. A Ct value < 40 with a significant amplification curve was considered positive, while a Ct

value ≥ 40 (or no value) was considered negative. The interpretation criteria are referenced in Supplementary Table 3.

2.2.3. Specificity evaluation

To evaluate the specificity and diagnostic performance of this method in clinical isolates, we tested 6 standard strains, 64 mycobacterial clinical isolates, and 6 non-mycobacterial clinical isolates, each using 1 ng/μL of DNA.

2.2.4. Limit of detection (LOD)

Standard bacterial suspensions were prepared using six reference strains to generate both single bacterial solutions and mixed bacterial solutions in a 1:1 ratio. Dilutions were performed using RNase-free water and liquefied negative sputum samples as matrices (negative sputum refers to sputum from patients clinically diagnosed with non-mycobacterial infections). Each strain was adjusted from 1 McFarland Standard (MCF) to an initial concentration of 20,480 CFU/mL in both matrices, and then diluted to 2048 CFU/mL at a 1:9 ratio. Gradient dilutions were prepared from 2048 CFU/mL to 4 CFU/mL in a 1:1 ratio. The mixed bacterial suspension was prepared using liquefied negative sputum samples as the matrix, and dilutions were made in the same manner. The final gradient concentrations for each strain ranged from 20,480 CFU/mL to 16 CFU/mL, with corresponding negative controls established for each matrix. Nucleic acids from all gradient dilution samples were extracted using the CapitalBio RT-PCR kit (according to the instructions, negative sputum samples were treated with 4 % NaOH for 30 min). Each concentration was tested ten times. The limit of detection was defined as the lowest concentration that achieved a 95 % detection rate.

2.2.5. Accuracy evaluation

To validate the accuracy of multiplex PCR, retrospectively collected 228 respiratory samples from the tuberculosis laboratory sample bank of Hangzhou Red Cross Hospital. These samples, clinically diagnosed with mycobacterial infections between March 2021 and March 2023, were confirmed by tNGS as MTB, MAC, MCAG, and *M. kansasii* (DNA extraction method same as CapitalBio RT-PCR).

2.3. Clinical diagnostic performance evaluation

2.3.1. Clinical sample study design

A clinical trial was conducted at Hangzhou Red Cross Hospital, which is the tuberculosis diagnosis and treatment center of Zhejiang Province. Between August and November 2023, cases suspected of mycobacterial infection and simultaneously tested by Xpert, CapitalBio RT-PCR, and mycobacterial culture were consecutively and non-selectively included. Exclusion criteria were duplicate patients and cases with unclear diagnoses. All included cases underwent multiplex PCR testing, with DNA sourced from the remaining DNA extracts from CapitalBio RT-PCR. The diagnostic performance of multiplex PCR in clinical samples was evaluated by comparing it with three other detection methods. Cases of mixed infection and false-positive results by multiplex PCR were confirmed by tNGS.

2.3.2. Patient classification

Clinical diagnosis followed composite reference standard (CRS) diagnostic criteria. (I) TB: Following the "Chinese Clinical Guidelines for Tuberculosis Treatment" and tuberculosis treatment guidelines [21], all PTB patients were diagnosed based on chest imaging features and confirmed by etiological (including bacteriological and molecular biological) and pathological results. Patients without etiological evidence were clinically diagnosed with active tuberculosis infection based on clinical symptoms, positive immunological test results, and subsequent treatment response after a one-month follow-up (evidenced by symptom improvement and relief). EPTB diagnosis referred to PTB clinical diagnostic criteria and was primarily confirmed by etiological and pathological results [22].

(II) NTM: Following the Chinese "Non-tuberculous Mycobacteria Disease Diagnosis and Treatment Guidelines" [23], the final diagnosis of NTM lung disease required meeting one of the following criteria: (1) Two separate sputum specimens positive for NTM culture and identified as the same pathogen, and/or NTM molecular biological detection consistent with the same pathogen; (2) One positive NTM culture and/or molecular biological detection from bronchial washings or bronchoalveolar lavage fluid; (3) Histopathological features of mycobacteriosis (granulomatous inflammation or acid-fast staining positive) found in lung biopsy samples obtained via bronchoscopy or other methods, with positive NTM culture and/or molecular biological detection; (4) Histopathological features of mycobacteriosis found in lung biopsy samples, with one or more positive sputum specimens or bronchial washings. The final diagnosis of extrapulmonary NTM disease required local and/or systemic symptoms, exclusion of other diseases, and ensuring no exogenous contamination of the specimen, with positive NTM culture and/or molecular biological detection from puncture or biopsy tissue of the lesion site.

(III) TB and NTM co-infection: Patients meeting the diagnostic criteria for both TB and NTM diseases.

(IV) Non-mycobacterial disease: Patients with a clear diagnosis of diseases other than mycobacterial infection.

2.4. Xpert, CapitalBio RT-PCR, and mycobacterial culture

Xpert, CapitalBio RT-PCR, and mycobacterial culture tests were performed as previously described [24]. Briefly, Xpert was performed according to the manufacturer's instructions, providing automatic MTB detection results. CapitalBio RT-PCR followed the protocol for DNA extraction and real-time fluorescence quantitative PCR detection of MTB-specific multi-copy sequence IS*6110* and the common mycobacterial gene HSP*65*. Mycobacterial culture involved decontaminating clinical samples with

N-acetyl-L-cysteine-2% sodium hydroxide (NALC-NaOH). Notably, specimens from different body sites were processed according to the CDC's Public Health Mycobacteriology guidelines before inoculation into MGIT 960 liquid culture tubes (Becton, Dickinson and Company, USA). All positive cultures were distinguished between MTB and NTM using the CapitalBio RT-PCR detection kit.

2.5. tNGS analysis

The tNGS procedure was developed as follows: Multiplex PCR amplicons were prepared using a Ligation Sequencing Kit (SQK-LSK109; ONT, Oxford, UK) and a Native Barcoding Kit (EXP-NBD196; ONT). End-prep and native barcode ligation to amplicons were performed according to the Native Barcoding Kit protocol using a 300 ng sample diluted in 70 μL of nuclease-free water. Subsequently, adapter ligation and cleaning steps were carried out using a NEB ligation kit and AM Pure XP magnetic beads (Beckman Coulter, USA), respectively, resulting in a final adapter-ligated DNA library containing 100 ng of DNA. The pooled library was loaded into a flow cell (FLO-MIN106 R9.4, ONT), and DNA sequencing was performed using a Min ION Mk1C sequencing device (ONT). Real-time sequencing data were collected using the Min Know v3.6.5 software (ONT). After completing the sequencing run, the Nanopore raw data (fast5) were base - called using Guppy v4.5.2 software (ONT). Trimmed amplicon sequences were compared with mycobacterial reference sequences to obtain results for the MTB and NTM gene analysis. This analysis was conducted using an nBLAST-based bioinformatics pipeline created by Dian Diagnostics Co., Ltd. (Hangzhou, China).

2.6. Data processing and statistical analysis

SPSS was used to calculate the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and statistical differences of different diagnostic methods. The chi-square test was used to compare the differences between multiplex PCR and various detection methods. All statistical tests were two-sided, with a significance threshold set at $p < 0.05$. Data visualization was performed using GraphPad Prism and Origin. The expected value for accuracy validation was 90 %, and the expected values for sensitivity and specificity in clinical performance evaluation were 60 % and 95 %, respectively. According to the sample size estimation formula: $n = [Z_{1-\alpha/2}]^2 P(1-P)/\Delta^2$(n is the sample size, $Z_{1-\alpha/2}$ is the quantile of the confidence standard normal distribution, P is the expected value of the evaluation index, and Δ is the allowable error size of P), the sample size included in this study meets the standards.

Table 1
Specificity validation results of multiplex PCR in strains.

Species	No	Multiplex PCR				
		IS*6110*	ITS -MAC	ITS -MCAG	mkan-rs*12360*	Results interpretation
Standard strain						
H37Rv	1	+	–	–	–	MTB
M.avium	1	–	+	–	–	MAC
M.intracellulare	1	–	+	–	–	MAC
M.chelonae	1	–	–	+	–	MCAG
M.abscessus subsp. abscessus	1	–	–	+	–	MCAG
M.kansasii	1	–	–	–	+	*M. kansasii*
Clinical isolates						
M. tuberculosis	20	+(20)	–	–	–	MTB
M. avium complex						
M.avium	5	–	+(5)	–	–	MAC
M.intracellulare	5	–	+(5)	–	–	MAC
M.paraintracellulare	5	–	+(5)	–	–	MAC
M.chimaera	5	–	+(5)	–	–	MAC
M. chelonae-M. abscessus group						
M. chelonae	5	–	–	+(5)	–	MCAG
M.abscessus subsp. abscessus	5	–	–	+(5)	–	MCAG
M.abscessus subsp. massiliense	5	–	–	+(5)	–	MCAG
M.kansasii	5	–	–	–	+(5)	*M. kansasii*
M. simiae	1	–	–	–	–	Negative
M.parascrofulaceum	1	–	–	–	–	Negative
M. triplex	1	–	–	–	–	Negative
M. lentiflavum	1	–	–	–	–	Negative
Klebsiella pneumoniae	1	–	–	–	–	Negative
Staphylococcus aureus	1	–	–	–	–	Negative
Pseudomonas aeruginosa	1	–	–	–	–	Negative
Escherichia coli	1	–	–	–	–	Negative
Cryptococcus	1	–	–	–	–	Negative
Aspergillus	1	–	–	–	–	Negative
Total	76					
Coincidence rate (%)	100 %					

MAC,*M.avium* complex; MCAG,*M.chelonae-M.abscessus* group; +, detected; -, not detected.

3. Results

3.1. Evaluation of diagnostic performance of multiplex PCR

3.1.1. Specificity evaluation

Cross-detection results of the four pairs of primers/probes designed based on specific target genes with standard strains of six target species (*H37Rv*, *M. avium*, *M. intracellulare*, *M. chelonae*, *M. abscessus* subsp. *abscessus,* and *M. kansasii*) showed high specificity. Each pair of primers and probes could only detect the corresponding target species with no cross-reactivity with other species. Multiplex PCR demonstrated excellent species specificity among 70 clinical isolates of 19 common species, detecting only the corresponding target genes in 9 target species and their subspecies (MTB, *M. avium*, *M. intracellulare*, *M. paraintracellulare*, *M. chimaera*, *M. chelonae*, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, *M. kansasii*). No detection was observed in 4 non-target mycobacteria species (*M. simiae*, *M. parascrofulaceum*, *M. triplex*, and *M. lentiflavum*) and 6 non-mycobacterial species (*Cryptococcus*, *Aspergillus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*). The detection results were 100 % consistent with expectations for all strains (Table 1).

3.1.2. Limit of detection (LOD)

The final LOD in water of the 6 standard strains were as follows: 11.7 CFU/mL for *H37Rv*, 40.5 CFU/mL for *M. avium*, 46.8 CFU/mL for *M. intracellulare*, 360.0 CFU/mL for *M. chelonae*, 201.6 CFU/mL for *M. abscessus*, and 242.1 CFU/mL for *M. kansasii* (Supplementary Fig. 1). The LOD in sputum were 43.8 CFU/mL for *H37Rv*, 73.9 CFU/mL for *M. avium*, 74.5 CFU/mL for *M. intracellulare*, 906.4 CFU/mL for *M. chelonae*, 922.0 CFU/mL for *M. abscessus*, and 791.4 CFU/mL for *M. kansasii* (Supplementary Fig. 1), the fluorescence curve is shown in Supplementary Fig. 2. Multiplex PCR cannot distinguish between *M. avium* and *M.intracellulare*, so when validating the LOD of respiratory mixed infections, the 6 standard strains were mixed in a 1:1 ratio, effectively doubling the actual concentration of MAC. Similarly, since multiplex PCR cannot differentiate between *M. chelonae* and *M. abscessus*, the actual concentration of MCAG also doubled. Therefore, the accurate LOD of mixed infections in sputum were 53.2 CFU/mL for *H37Rv*, 75.6 CFU/mL for MAC, 859.3 CFU/mL for MCAG, and 823.1 CFU/mL for *M. kansasii* (Supplementary Fig. 3).

3.1.3. Accuracy evaluation

To evaluate the accuracy of multiplex PCR in clinical samples, DNA from 228 clinical positive samples confirmed by tNGS as MTB, MAC, MCAG, and *M. kansasii* was retrospectively collected. Among these, 210 samples were single infections (54 TB and 156 NTM), and 18 were mixed infections. In single infection samples, 1 MAC sample was not detected; 2 mixed infection samples with MAC and MCAG only detected MAC. A total of 225 samples (98.7 %) were accurately detected, with a 100 % accuracy rate in strain typing (Table 2).

3.2. Diagnostic performance of multiplex PCR in clinical samples

3.2.1. Clinical sample study participants

To evaluate the diagnostic performance of multiplex PCR in clinical samples, 994 clinical samples were consecutively included, excluding 63 duplicate cases and 30 cases with unclear diagnoses. Ultimately, 901 clinical samples were included in the study (Fig. 1). Among them, 724 pulmonary samples included 529 bronchoalveolar lavage fluid (73.1 %) and 195 sputum samples (26.9 %); 177 extrapulmonary samples included 116 tissue samples (65.5 %), 30 puncture fluid samples (16.9 %), 14 pleural fluid samples (7.9 %), 13 urine samples (7.3 %), 2 ascites samples (1.2 %), 1 cerebrospinal fluid sample (0.6 %), and 1 pericardial effusion sample (0.6 %). The proportion of males with PTB and EPTB was higher than females, with approximately 50 % of patients being over 55 years old. The NTM disease group had more female patients, mostly over 55 years old, accounting for 78.9 % (Supplementary Table 4).

Table 2
Accuracy Validation Results of Multiplex PCR in Clinical Samples Based on tNGS.

tNGS	Multiplex PCR			Sample detection accuracy	Identification accuracy of detected strains
Strain type(n)	Correct detection (n)	Partial detection (n)	Not detection (n)		
Single Infection					
MTB(54)	54	/	/	100 %	100 %
MAC(120)	119	/	1	99.2 %	100 %
MCAG(33)	33	/	/	100 %	100 %
M.kansasii(3)	3	/	/	100 %	100 %
Multiple infections					
MTB&MAC(3)	3	/	/	100 %	100 %
MTB&MCAG(1)	1	/	/	100 %	100 %
MAC&MCAG(11)	9	2(MAC)	/	81.8 %	100 %
MAC&*M.kansasii*(3)	3	/	/	100 %	100 %
Total(228)	225	2	1	98.7 %	100 %

MTB,*Mycobacterium tuberculosis*;MAC,*M.avium* complex; MCAG,*M.chelonae-M.abscessus* group.

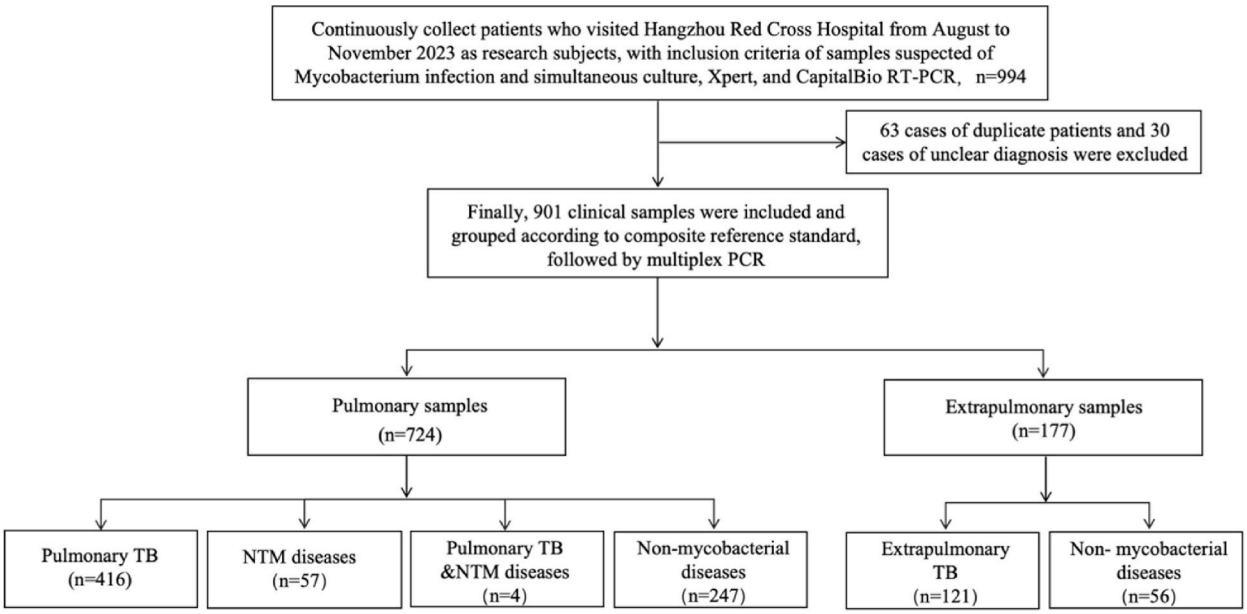

Fig. 1. Sample inclusion flowchart for clinical test.

3.2.2. Comparison of diagnostic performance of different methods in clinical samples

In pulmonary samples, the sensitivity, specificity, PPV, and NPV of multiplex PCR for PTB detection were 67.4 %, 97.4 %, 97.3 %, and 68.4 %, respectively. The sensitivity of multiplex PCR was comparable to Xpert (67.6 %) ($P > 0.05$). The sensitivity of multiplex PCR was significantly higher than CapitalBio RT-PCR and Culture ($P < 0.05$). The specificity and PPV of multiplex PCR were relatively lower compared to the other three methods ($P < 0.05$) (Fig. 2a). For NTM disease detection, the sensitivity, specificity, PPV, and NPV of multiplex PCR were 80.3 %, 99.2 %, 90.7 %, and 98.2 %, respectively. Its sensitivity was higher than CapitalBio RT-PCR (63.9 %) ($P < 0.05$), with no statistical difference in other diagnostic performance indicators ($P > 0.05$) (Fig. 2b). Detailed data are shown in Supplementary Tables 5 and 6

In extrapulmonary samples, the sensitivity, specificity, PPV, and NPV of multiplex PCR for EPTB detection were 66.1 %, 100.0 %, 100.0 %, and 57.7 %, respectively. The sensitivity and NPV of multiplex PCR were significantly higher than Culture ($P < 0.05$). There was no statistical difference in all diagnostic performance indicators of multiplex PCR compared to Xpert and CapitalBio RT-PCR ($P > 0.05$) (Fig. 2c). Detailed data are shown in Supplementary Table 7.

In all samples, the sensitivity, specificity, PPV, and NPV of multiplex PCR for mycobacterial disease detection were 68.9 %, 96.0 %, 97.2 %, and 61.0 %, respectively. The sensitivity was significantly higher than the other three methods, and the NPV was significantly higher than CapitalBio RT-PCR and Culture ($P < 0.05$). The specificity and PPV were relatively lower (Fig. 2d). Detailed data are shown in Supplementary Table 8.

Multiplex PCR detected only NTM and not MTB in four clinically diagnosed mixed infections of MTB and NTM, with Culture results also showing NTM. tNGS confirmed 2 cases of mixed MTB and NTM infections, while the other 2 cases only detected NTM strains (Supplementary Table 9).

Using CRS as the standard, multiplex PCR detected 13 false positives, all from pulmonary samples, including 8 MTB, 3 MAC, and 1 MCAG in 12 cases diagnosed as non-mycobacterial infections, MTB and MAC co-infection in 1 PTB sample. tNGS confirmed 9 of these samples as true positives, with 4 samples showing no detection by tNGS. Among these 4 samples, two had Culture results for NTM, confirmed as MAC by multiplex PCR, consistent with clinical sample detection results (Table 3).

4. Discussion

In recent years, several multiplex real-time fluorescence PCR methods for the direct detection and identification of mycobacteria in clinical samples have been described. For example, in 2020, Shin et al. [25] validated a multiplex real-time fluorescence PCR in respiratory samples that could identify MTB and NTM but could not differentiate NTM species. In 2016, Rocchetti et al. [26] and in 2021, Sarro et al. [27] developed detection methods that could differentiate MTB and MAC in clinical samples, but these methods missed several globally common pathogenic NTMs. In 2015, Kim et al. [28] developed a multiplex real-time fluorescence PCR technique that could identify 20 mycobacterial species, but it required three PCR steps and five sets of tests, making the complexity of this assay a potential hindrance for routine laboratory use.

To effectively address the shortcomings of the aforementioned methods, this study utilized TaqMan probe-based multiplex real-time fluorescence PCR technology to develop a method for rapidly distinguishing MTB, MAC, MCAG, and *M. kansasii*, the major pathogenic mycobacteria prevalent in eastern China, directly from clinical samples. This method covers MTB and 93 % of NTMs in this

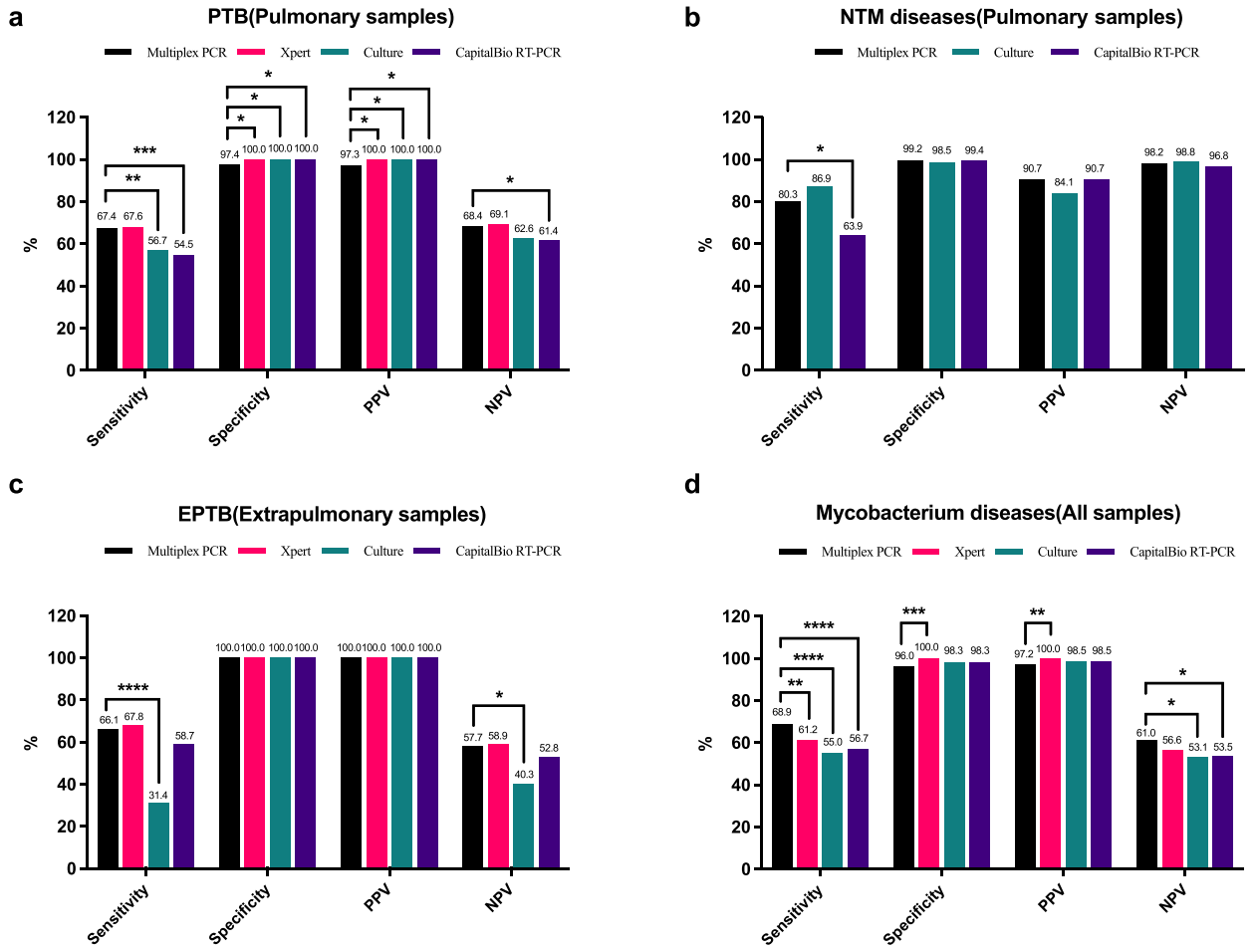

Fig. 2. Comparison of Diagnostic Performance Indicators such as Sensitivity, Specificity, PPV, and NPV of Various Methods.a: Comparison of Diagnostic Performance Indicators of Four Methods in Detecting PTB in Pulmonary Samples; b: Comparison of Diagnostic Performance Indicators of Three Methods in Detecting NTM in Pulmonary Samples; c:Comparison of Diagnostic Performance Indicators of Four Methods in Detecting EPTB in Extrapulmonary Samples of EPTB Cases; d: Comparison of Diagnostic Performance Indicators of Four Methods in Detecting Mycobacteria in All Samples.Only groups with statistically significant differences compared to multiplex PCR are marked in all figures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Groups without statistical differences are not marked.

Table 3
Analysis of false positive results of multiplex PCR.

S/N	Composite Reference Standard Group	Sample type	Xpert	CapitalBio RT-PCR	Culture	Multiplex PCR	tNGS
16	Non- mycobacterial disease	BALF	–	–	NTM	MAC	–
91	Non- mycobacterial disease	BALF	–	–	–	MCAG	MCAG
323	Non- mycobacterial disease	BALF	–	NTM	NTM	MAC	–
326	Non- mycobacterial disease	BALF	–	–	–	MTB	MTB
402	Non- mycobacterial disease	BALF	–	–	–	MTB	MTB
448	Non- mycobacterial disease	Sputum	–	–	–	MAC	MAC
463	Non- mycobacterial disease	BALF	–	–	–	MTB	MTB
466	Non- mycobacterial disease	BALF	–	–	–	MTB	MTB
584	Non- mycobacterial disease	BALF	–	–	–	MTB	MTB
585	Non- mycobacterial disease	BALF	–	–	–	MTB	–
686	Non- mycobacterial disease	BALF	–	–	–	MTB	–
712	Non- mycobacterial disease	BALF	–	–	–	MTB	MTB
281	TB	BALF	MTB	TB	NTM	MTB&MAC	MTB&MAC

S/N,Sample Number; TB,Tuberculosis; NTM,Non-tuberculous mycobacteria; MTB,*Mycobacterium tuberculosis*; BALF,broncho-alveolar lavage fluid;-, not detected.

region, is simple to operate, and cost-effective (only $1.3 per test). Species identification can be completed within 2 h in a single reaction tube, including 50 min for nucleic acid extraction and 70 min for nucleic acid amplification, making it faster and cheaper than other molecular detection methods (Supplementary Table 10) [29–34]. Validation with 6 standard strains and 70 clinical isolates confirmed by Whole Genome Sequencing (WGS) showed highly specific target genes and 100 % species specificity, with a low LOD of 11.7 CFU/mL for MTB and 40.5–360.0 CFU/mL for other NTMs in water. The LOD range in sputum was 43.8–922.0 CFU/mL, which was similar to the LOD of mixed infection in sputum (53.2–859.3 CFU/mL). The main reason for the higher LOD in sputum may be the presence of inhibitory substances in clinical sputum samples that inhibit PCR reactions [35].

Multiplex PCR also demonstrated good diagnostic performance in clinical samples, with a detection accuracy of 98.7 % and 100 % accuracy in strain typing in 228 samples validated against tNGS. Multiplex PCR missed one MAC in single infections and two MCAG in mixed infections, likely due to target gene concentrations below the LOD of multiplex PCR, as tNGS sequencing reads were all below 300 (data not provided). The detection accuracy for mixed infections was lower than that for single infections, as confirmed in the evaluation of 901 clinical samples, where multiplex PCR failed to detect MTB in 4 clinically diagnosed mixed infections of MTB and NTM. tNGS detected MTB in 2 cases, but the reads were around 100 (data not provided). These 4 patients had undergone anti-tuberculosis treatment, and the failure of multiplex PCR to detect MTB may be due to the low concentration of target genes.

Using CRS as the standard, the sensitivity of multiplex PCR for detecting PTB and EPTB showed no significant difference compared to Xpert. Although multiplex PCR targets the IS*6110* multicopy gene for MTBC detection while Xpert uses the rpoB single-copy gene, the two methods employ different DNA extraction techniques. Both methods extract nucleic acid from 1 mL clinical samples, but Xpert has a 100 % utilization rate of extracted DNA, while multiplex PCR only has a 3.6 % utilization rate of extracted DNA (the nucleic acid is finally dissolved in 50 μL of nucleic acid extraction solution and only 1.8 μL is used for PCR amplification). Xpert achieves higher DNA utilization rate, which is crucial for sensitivity. We will explore different DNA extraction methods and attempt to prepare reagents into freeze-dried powder to improve the utilization rate of nucleic acid extraction, thereby enhancing the sensitivity of the method and further reducing LOD values. It is worth mentioning that multiplex PCR shows significantly higher sensitivity in detecting mycobacteria. This is mainly because Xpert cannot identify NTM, although it can detect rifampicin resistance.

The specificity of Xpert, CapitalBio RT-PCR, and Culture for PTB and EPTB was 100 %, while the specificity of multiplex PCR for PTB and mycobacteria was lower than these three methods. This is because clinicians use positive results from these three methods as the etiological diagnosis basis for tuberculosis, thus their diagnostic specificity for tuberculosis is 100 %. Additionally, multiplex PCR detected 13 false-positive cases in 901 clinical samples (Table 3), 9 of which were confirmed by tNGS, 2 were consistent with Culture results, and 2 are pending further follow-up. These 13 false positive cases are the main reason for the low specificity of multiplex PCR, but the 11 cases confirmed by tNGS, and culture may be the cases of clinical missed diagnosis, and the true sensitivity and specificity of multiplex PCR may be higher than the data in this paper. Therefore, multiplex PCR is an effective identification tool for major pathogenic mycobacteria.

The limitations of this study include: first, the inability to differentiate mycobacteria other than MTB, MAC, MCAG, and *M. kansasii* in clinical samples, which may lead to missing less common mycobacterial diseases. Second, the study did not include human internal control genes to monitor the entire testing process, including sample quality and DNA extraction efficiency. Third, as NTM infections are mostly pulmonary diseases, this study lacked the evaluation of extrapulmonary NTM cases, and some types of extrapulmonary samples (e.g., cerebrospinal fluid, pericardial effusion) had too few cases.

5. Conclusions

In conclusion, multiplex PCR can rapidly and accurately identify MTB, MAC, MCAG, and *M. kansasii* directly from pulmonary and extrapulmonary clinical samples at a low cost, making it a useful rapid identification tool for the major pathogenic mycobacteria in eastern China.

CRediT authorship contribution statement

Tingting Fang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Data curation. **Lijun Peng:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Conceptualization. **Tingting Yang:** Software, Formal analysis. **Qingshan Cai:** Resources, Funding acquisition. **Huanyu Li:** Project administration, Investigation. **Hao Li:** Project administration, Investigation. **Long Cai:** Writing – review & editing, Supervision.

Ethical approval statement

This retrospective study received ethical approval from the Ethics Committee of Hangzhou Red Cross Hospital (Approval No. 2021-274), with a granted waiver of informed consent. The research was conducted in strict accordance with the ethical principles of the Helsinki Declaration as delineated by the World Medical Association.

Data availability statement

The WGS data obtained in this study have been deposited under NCBI BioProject PRJNA1036710.

Funding source

This work was supported by the Zhejiang Provincial Natural Science Foundation of China [LTGC23H200003]; Zhejiang Provincial Medical and Health Science and Technology Project[2023KY967,2023KY972]; Hangzhou Medical Health Science and Technology Project [Z20220032]; Hangzhou Municipal Science and Technology Bureau Hangzhou Agricultural and Social Development Research Project[202204B02].

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

We greatly appreciate the provision of strains and clinical samples by the Tuberculosis Laboratory at Hangzhou Red Cross Hospital.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e41384.

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