Heliyon 10 (2024) e37640

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Point-of-care testing of *rpoB* in *Mycobacterium tuberculosis* using multiply-primed-RCA coupled with CRISPR/Cas12a

Yingying Sun [a,b,1], Yaozhou Wu [c,1], Yulin Wang [d], Keke Li [b], Yanbin Chang [b,**], Lianhua Wei [a,b,*]

[a] School of Public Health, Gansu University of Chinese Medicine, Lanzhou, 730000, PR China
[b] Department of Clinical Laboratory, Gansu Provincial Hospital, Lanzhou, 730000, PR China
[c] First School of Clinical Medicine, Lanzhou University, Lanzhou, 730000, PR China
[d] First School of Clinical Medicine, Ningxia Medical University, Yinchuan, 750000, PR China

ARTICLE INFO

Keywords:
Multiply-primed-RCA
CRISPR/Cas12a
Mycobacterium tuberculosis
POCT
dsDNA

ABSTRACT

Purpose: Due to the serious threat of tuberculosis to global health and limitations of existing diagnostic methods, this study combined the CRISPR/Cas12a system with Multiply-primed-RCA (MRCA) technology for *Mycobacterium tuberculosis* Point-of-care Testing (POCT).
Method: We utilized T4 and Taq DNA ligases, compared the effects of specific primers and random $6N^S$ primers on the method, and integrated MRCA and the CRISPR-Cas12a system in one tube. By optimizing conditions such as the concentration of DNA ligase, the concentration of padlock probes, and the number of cycles, we finally established T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods for both stepwise and one-step.
Results: The limits of detection of the one-step T4/Taq-MRCA-Cas12a were 10^4aM and 10^3aM. With no cross-reactivity with DNA from other bacterial strains. The accuracy and specificity were 88 % and 100 % for T4-MRCA-Cas12a, and 96 % and 100 % for Taq-MRCA-Cas12a, respectively.
Conclusion: We developed a POCT method that can directly identify MTB through the naked eye.

1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB) primarily affecting the respiratory system of the host, especially the lungs [1]. The infection and mortality rates of pulmonary tuberculosis are increasing annually worldwide, making it one of the infectious diseases with the highest incidence and mortality rates globally [2]. Traditional methods for identifying MTB, including sputum culture, sputum smear, and molecular biology techniques such as qPCR, GeneXpert, and Next-Generation Sequencing (NGS), each have their limitations. Sputum culture takes a long time and cannot meet the clinical need for rapid testing [3]; Sputum smear has low sensitivity and poor stability [4]; However, methods such as qPCR, GeneXpert, and NGS have high specificity in identifying MTB, but their reliance on complex instrumentation and the high requirements for laboratory conditions and operator skills limit their application in resource-limited areas [5–7]. Given the difficulties current diagnostic methods have in meeting the clinical needs for MTB detection, there is an urgent need for the development of an efficient, economical, and rapid MTB

* Corresponding author. School of Public Health, Gansu University of Chinese Medicine, Lanzhou, 730000, PR China.
** Corresponding author. Department of Clinical Laboratory, Gansu Provincial Hospital, Lanzhou, 730000, PR China.
E-mail addresses: 1109605451@qq.com (Y. Chang), 3752131518@qq.com (L. Wei).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e37640
Received 23 May 2024; Received in revised form 6 August 2024; Accepted 6 September 2024
Available online 7 September 2024

detection technology.

Previously, the CRISPR/Cas system was primarily used for gene editing, but the discovery of its trans-cleavage activity has revealed its tremendous potential in rapid molecular diagnostics [8,9]. CRISPR/Cas12a can specifically recognize the target gene, and by designing a guide RNA(gRNA) complementary to the target gene, its trans-cleavage activity can be activated, indiscriminately cutting single-stranded DNA (ssDNA) reporter probes in the system that carry fluorescent and quenching groups [10]. Rolling circle amplification (RCA) is a simple and efficient isothermal amplification technique that, under the action of Phi29 DNA polymerase, can produce a large amount of repetitive ssDNA. This technique has been widely used in early disease detection and the identification of biomarkers [11,12]. For example, by combining RCA with the CRISPR system, a one-pot isothermal EXTRA-CRISPR method for detecting miRNA and a method for detecting lung cancer-associated miRNA have been developed, as well as enabling the identification of methicillin-resistant *Staphylococcus aureus* (MRSA) strains [13–15]. Multiply-primed-RCA (MRCA) is an exponential amplification of target DNA based on RCA, achieved by simultaneously using multiple primers [16]. However, currently RCA technology is primarily used for detecting RNA and ssDNA, with few reports on its application in detecting double-stranded DNA (dsDNA).

The RNA polymerase β subunit encoded by the *rpoB* gene plays a crucial role in the transcription process of MTB. Mutations in this gene are closely related to the rifampicin resistance of MTB. Compared with other genes, the *rpoB* gene is relatively conserved in MTB, making it an ideal detection target. Therefore, in this study, we established a method for identifying MTB based on the MRCA combined with the CRISPR/Cas12a system to detect the *rpoB* gene.

This method achieves the detection of dsDNA through MRCA, expanding its potential applications in molecular biology research and clinical diagnosis. This method does not rely on complex laboratory equipment; it only requires basic laboratory devices such as a water bath and a UV irradiation unit to achieve rapid detection of MTB. Therefore, this method provides an experimental basis for Point-of-Care Testing (POCT) of MTB, and is expected to significantly improve the efficiency of rapid screening and diagnosis of TB.

2. Materials and methods

2.1. Collection of bacterial strains

All the bacterial strains in the article were obtained from Clinical Laboratory Center of Gansu Provincial Hospital. The strains used for the specificity test were clinically cultivated from the microbiology laboratory of the Clinical Laboratory Center of Gansu Provincial Hospital, and all the strains have undergone mass spectrometry identification. The 38 clinical specimens used for clinical validation were collected from the microbiology laboratory of the Clinical Laboratory Center of Gansu Provincial Hospital. All the sputum and bronchoalveolar lavage fluid specimens have been verified by sequencing.

2.2. Design and synthesis of nucleic acid targets and sgRNAs

The *rpoB* gene sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). The accession number of the *rpoB* gene is NC_000962.3. After the sequence alignment, corresponding specific gRNAs were designed based on the conserved sequences of the *rpoB* gene that match the Cas12a protospacer adjacent motif (5′-TTTN). Subsequently, the gRNA was synthesized by Takara Bio (Beijing, China). The specificity of the target sequence for the *rpoB* gene and the sequence alignment was examined using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Primer and padlock probe design

Primers and Padlock (PL) were designed using Primer6.0, with MRCA primers being $6N^{S}$. The design of PL should follow the following principles: (1) The suitable length of PL is 70-125bp; (2) The detection arm length at both ends of PL is approximately 15-30bp; (3) Eliminate secondary structures such as dimers and hairpin structures at both ends of PL, with a GC content of less than 60 %; (4) Due to the special properties of PL, the Tm value of the probe's 5′ end sequence should be higher than the 3′ end sequence. Through nucleic acid secondary structure prediction websites, test and adjust the secondary structure of PL after looping to ensure that PL has the best secondary structure.

2.4. DNA preparation

DNA was extracted from the sputum and bronchoalveolar lavage (BAL) of TB patients using a nucleic acid extraction kit according to the manufacturer's instructions (Dilan Biotechnology, Hangzhou, China). Briefly, 0.5–1 mL sputum or purulent secretion samples were digested with 2–2.5 vol of the digestion buffer, and agitated in a vortex mixer to ensure complete homogenization. BAL samples were similarly processed with an equal volume of the digestion buffer and vortexed for up to 15 min (depending on the viscosity of the sample) at 37 °C. The homogenized samples were centrifuged and washed once, and the pellets were resuspended in 300 μL lysis buffer and transferred into a centrifuge tube containing DNA-binding substances. The samples were homogenized in a high-speed vortex mixer and then transferred to a 96-well deep plate. The plate was subsequently inserted into an automated extraction device where samples were processed according to preset programs. The extracted solutions were transferred to clean tubes and stored at −20 °C ± 10 °C.

2.5. Construction of psgRNAc-rpoB plasmid

A fragment of the *rpoB* gene was amplified from the genome of MTB, and the PCR product was purified using a PCR product purification kit (TIANGEN, Beijing, China). The purified fragment was cloned into the *psgRNAc* plasmid (Addgene, Massachusetts, USA) using the Golden Gate Assembly Cloning Kit (NEB, Ipswich, MA, USA). *PsgRNAc* was a gift from David Bikard (Addgene plasmid # 114006; http://n2t.net/addgene:114006; RRID:Addgene_114006). The primers are listed in Table S1. The reaction mix consisted of 1 μL *psgRNAc* plasmid vector, 1 μL purified PCR product (100 fmol), 1 μL 10x T4 DNA ligase buffer, 0.5 μL T4 DNA ligase, 0.5 μL *Bsa*I-HF, and nuclease-free water to make up the total volume to 10 μL. The thermal cycling conditions were as follows: 37 °C for 2 min and 16 °C for 5 min for a total of 25 cycles, 50 °C for 5 min, and 80 °C for 15 min. The amplified product was transformed into DH5α competent Escherichia coli cells, and the antibiotic-resistant colonies were screened on chloramphenicol-containing M − H agar plates. Selected single colonies were incubated overnight in the chloramphenicol-containing medium, and the recombinant *psgRNAc-rpoB* plasmid with the cloned *rpoB* gene was extracted using a mini-prep plasmid extraction kit (TIANGEN, Beijing, China).

2.6. Application and operating methods of UV lamp

In the experiment, we used a UV lamp with a wavelength of 302 nm for the results interpretation. The operating method is as follows: after the completion of the reaction, the samples were placed under the UV lamp for illumination. If the sample shows visible fluorescence to the naked eye, it indicates a positive (MTB) result; if there is no fluorescence, it indicates a negative (Others) result. During the operation, it is important to ensure that the samples are evenly exposed to the UV light and to avoid direct eye contact with the UV light to prevent eye damage.

2.7. Padlock ligation

PL 3 μL (10 μM), template DNA 5 μL, ddH_2O 8 μL, mix well by shaking and briefly centrifuge. 95 °C for 5 min; 46 °C for 1 min; then add 2 μL T4 DNA ligase and 2 μL of T4 DNA ligase Buffer. Incubate at 37 °C for 1 h 0.2 μL Taq DNA ligase (40U/μL), 2 μL 10 × Taq DNA ligase Buffer, 3 μL PL (1 μM), 1 μL template DNA, and top up with ddH_2O to 20 μL, mix well by shaking, and briefly centrifuge. Reaction conditions: 94 °C for 4 min; perform 10 ligation cycles: 94 °C for 30 s; 46 °C for 5 min 95 °C for 10 min.

2.8. MRCA reaction

T4 DNA ligation product 10 μL, phi29 DNA polymerase 1 μL (10U/μL), phi29 DNA polymerase Buffer 2 μL, dNTP Mixture 4 μL, $6N^S$ 1 μL (10 μM), add ddH_2O to make up to 20 μL. Incubate at 37 °C for 2 h. Taq DNA ligation product 10 μL, use primers Bca-R (5 μM) and Bca-F (5 μM) each 1 μL, the rest of the reaction system and quantities as mentioned above.

2.9. Establishment of CRISPR/Cas12a detection method

Cas12a trans-cleavage activity was assayed using EnGen® LbaCas12a (NEB, Ipswich, MA, USA). Mix 2 μL Cas12a (1 μM) with 2 μL gRNA (1 μM) and incubate at room temperature for 10 min; add 2 μL 10 × NEB Buffer2.1 (NEB, Ipswich, MA, USA), 0.25 μL RNase inhibitor, 2 μL reporter probe (2 μM), and 4 μL MRCA product. Adjust to 20 μL with ddH_2O, incubate at 37 °C, and measure every 60 s for a total of 120 cycles. (ssDNA FQ reporter gene = λex: 492 nm; λem: 522 nm).

2.10. Establishment of T4-MRCA-Cas12a and Taq-MRCA-Cas12a one-step methods

Mix 2 μL Cas12a (1 μM), 2 μL gRNA (1 μM), 2 μL reporter probe (2 μM), 0.25 μL RNase inhibitor, 1 μL phi29 DNA polymerase (10U/μL), 2 μL phi29 DNA polymerase Buffer (10X), 4 μL dNTP Mixture, 1 μL BSA protein (2 mg/mL), 1 μL ATP (10 mM), 10 μL circular product, and 1 μL $6N^S$ (10 μM). For the Taq DNA ligase primer, use 1 μL each of Bca-F (5 μM) and Bca-R (5 μM).

2.11. Optimization of T4-MRCA-Cas12a and Taq-MRCA-Cas12a systems

Optimize the concentration of T4 DNA ligase, the concentration of PL, and the amplification time for MRCA. Stepwise method: Set a gradient of T4 DNA ligase concentrations at 35U/μL, 25U/μL, 15U/μL, 10U/μL, 5U/μL, 1U/μL for the reaction; One-step method: Set a gradient of T4 DNA ligase concentrations at 350U/μL, 200U/μL, 100U/μL, 50U/μL, 10U/μL, 1U/μL. Under the optimal T4 DNA ligase concentration, optimize the concentration of PL using a stepwise method, with gradients of 10 μM, 8 μM, 6 μM, 4 μM, 2 μM, 1 μM; and for the one-step method, optimize the PL concentration with gradients of 50 μM, 10 μM, 5 μM, 1 μM. Under the optimal ligase concentration and PL concentration, optimize the MRCA amplification time with gradients of 0.5 h, 1 h, 1.5 h, 2 h, and perform fluorescent detection after amplification is complete. Optimize the concentration of Taq DNA ligase and the number of ligation cycles. The gradient for Taq DNA ligase concentration is set at 40 U/μL, 100U/μL, 200U/μL, and perform fluorescent detection according to the stepwise reaction system. Under the optimal ligase concentration and PL concentration, set the number of cycles gradient at 4, 6, 8, 10 for optimizing the number of ligation cycles.

2.12. Evaluation of sensitivity and specificity

To accurately assess the lower limit of detection (LOD) of T4-MRCA-Cas12a and Taq-MRCA-Cas12a using one-step method, we performed gradient dilutions of plasmid DNA at concentrations of 10^0,10^1,10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 aM to clarify the detection capabilities of the methods at different concentration levels. To further evaluate the specificity of T4-MRCA-Cas12a and Taq-MRCA-Cas12a using one-step method, we selected the following bacterial DNA strains for experiments: *Stenotrophomonas maltophilia, Acinetobacter baumannii, Corynebacterium striatum*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Staphylococcus aureus*, *and Kelbsiella aerogenes*. To ensure the accuracy and repeatability of the experimental results, each sample will be tested in triplicate.

2.13. Clinical sample validation

To verify the applicability of T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods in a clinical setting, we utilized actual clinical samples for testing. The collection of these samples strictly followed standard operating procedures to ensure their high quality and representativeness. By comparing the experimental results with the "gold standard" Sanger sequencing results, we assessed the performance and suitability of these techniques in a real clinical setting.

2.14. Statistical analysis

The Cas12a detection assay was conducted in triplicates across three independent experiments. Data was presented as the mean ± standard error of the mean (SEM). All statistical analyses were conducted using GraphPad Prism v.8.1.2 software. (GraphPad Software, San Diego, USA).

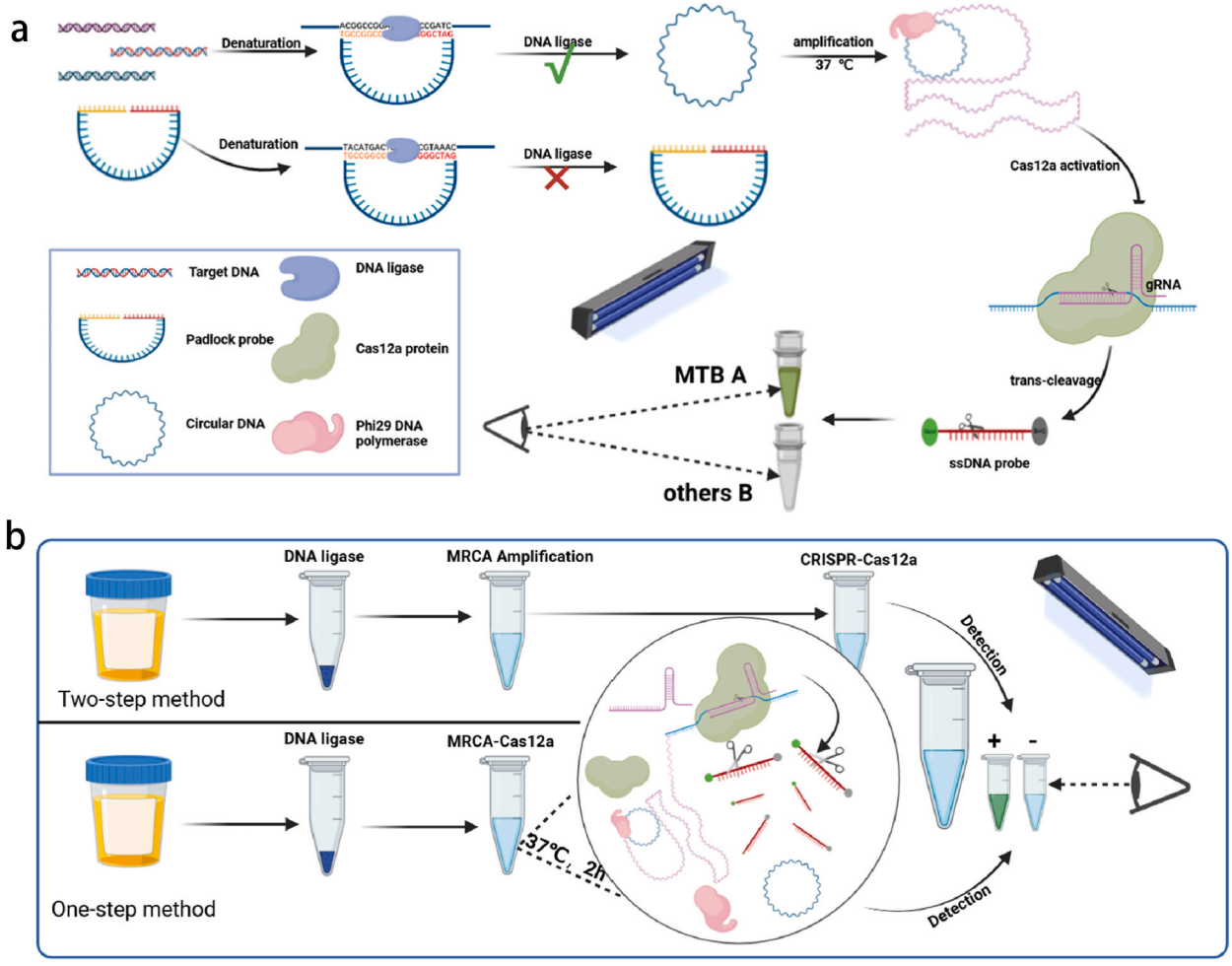

Fig. 1. Principle and flowchart of the MRCA-Cas12a MTB detection experiment. (a)Schematic diagram of the experimental principle. (b)Flowchart of the experimental procedure.

2.15. Ethical statement

Bacterial strain collection was in accordance with the relevant clinical guidelines. Ethical approval was waived for this study, as all individual patient identifiers were removed during the experiments.

3. Results

3.1. Experimental principles and operational procedures

In this study, we have established the T4/Taq-MRCA-Cas12a methods (utilizing T4 DNA ligase and Taq DNA ligase) for detecting MTB. The experimental principle, as illustrated in Fig. 1a, demonstrates that in the presence of the target gene, the PL hybridizes with the target gene and, under the action of DNA ligase, forms a circle; the generated circular ssDNA serves as a template for exponential amplification under the action of phi29 DNA polymerase, using $6N^S$ primers. The amplification product can activate the non-specific ssDNA cleavage activity of Cas12a protein, separating the fluorophore and quencher of the reporter probe, thus releasing fluorescence. Finally, by simple illumination with a UV lamp, a positive (MTB) result is indicated by visible fluorescence to the naked eye, while no fluorescence indicates a negative (Others) result. The operational procedure, as shown in Fig. 1b, comprises three steps: 1) DNA template denaturation and PL ligation; 2) MRCA amplification; 3) CRISPR/Cas12a fluorescence detection. The one-step method integrates MRCA amplification and CRISPR/Cas12a detection into a single tube.

3.2. Padlock ligation and $6N^S$ in enhancing validation of RCA efficiency

In this study, we conducted a strict verification of the PL loop formation process. We used the *psgRNAc* empty plasmid as a negative template. By comparing it with the positive template, it was concluded that PL could be connected into a loop only in the presence of the target for subsequent RCA, as shown in Fig. 2a. When PL was utilized as the template, no fluorescence could be detected. That is to say, in the situation where the target was absent, PL was not connected to form a loop, as depicted in Fig. 2b. In the absence of PL, no fluorescence appeared either (Fig. 2b). In conclusion, the above results indicate that only when the positive target exists can PL be connected into circular DNA for the subsequent MRCA process.

On the premise of using T4 DNA ligase, we compared the amplification efficiency of $6N^S$ primers and specific primers and observed significant differences. When amplification was performed using $6N^S$ primers, the fluorescence value was approximately 3000 at 60 min, while the fluorescence value of the specific primers at the same time was only 1200 (Fig. 2c). Moreover, the amplification curve using $6N^S$ primers showed a relatively steep upward trend; while the amplification curve using specific primers was relatively gentle.

In summary, the results of the loop formation verification indicate that only in the presence of a positive target can PL be connected into circular DNA for the subsequent MRCA process; and there are obvious differences in the amplification efficiency between $6N^S$ and specific primers, which has important guiding significance for subsequent related research and applications.

3.3. Establishment of T4-MRCA-Cas12a and Taq-MRCA-Cas12a stepwise and one-step methods

In the T4-MRCA-Cas12a stepwise method, the fluorescence value for WT exceeds 5000 at about 50 min (Fig. 3a), and in the one-step method, the fluorescence value reaches over 4000 at 100 min (Fig. 3b). In the Taq-MRCA-Cas12a stepwise method, the fluorescence value for WT is close to 6000 at about 100 min (Fig. 3c), and in the one-step method, the fluorescence value exceeds 4000 (Fig. 3d). Both methods can accurately distinguish WT from the negative control, indicating that we have preliminarily established the T4-MRCA-Cas12a and Taq-MRCA-Cas12a stepwise and one-step methods for the identification of MTB.

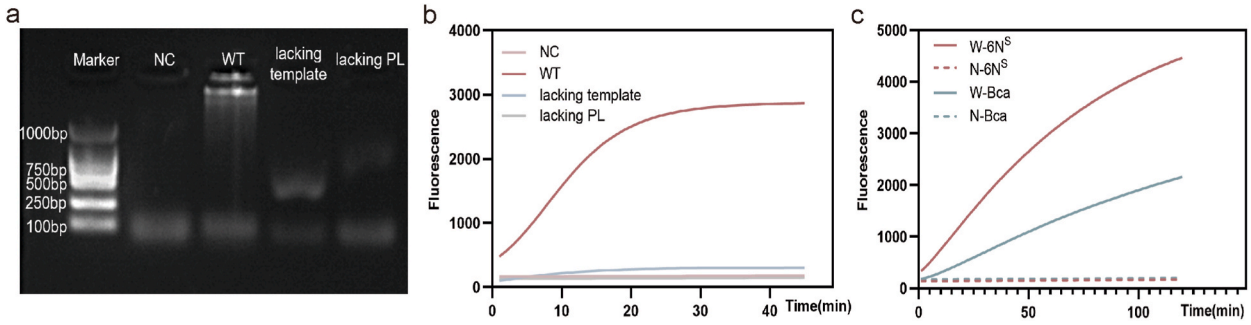

Fig. 2. Padlock ligation and $6N^S$ in Enhancing Validation of RCA Efficiency. (a) Gel electrophoresis image for padlock ligation validation. (b) Real-time fluorescence image for padlock ligation validation. (c) Real-time fluorescence image for validating that $6N^S$ primers can improve the amplification efficiency of MRCA. NC: Negative Control; WT: Plasmid containing MTB *rpoB* gene fragment. W-Bca refers to the RCA amplification reaction of the WT template using specific primers; N-Bca refers to the RCA amplification reaction of the NC template using specific primers; W-$6N^S$ refers to the RCA amplification reaction of the WT template using $6N^S$ primers; N-$6N^S$ refers to the RCA amplification reaction of the NC template using $6N^S$ primers.

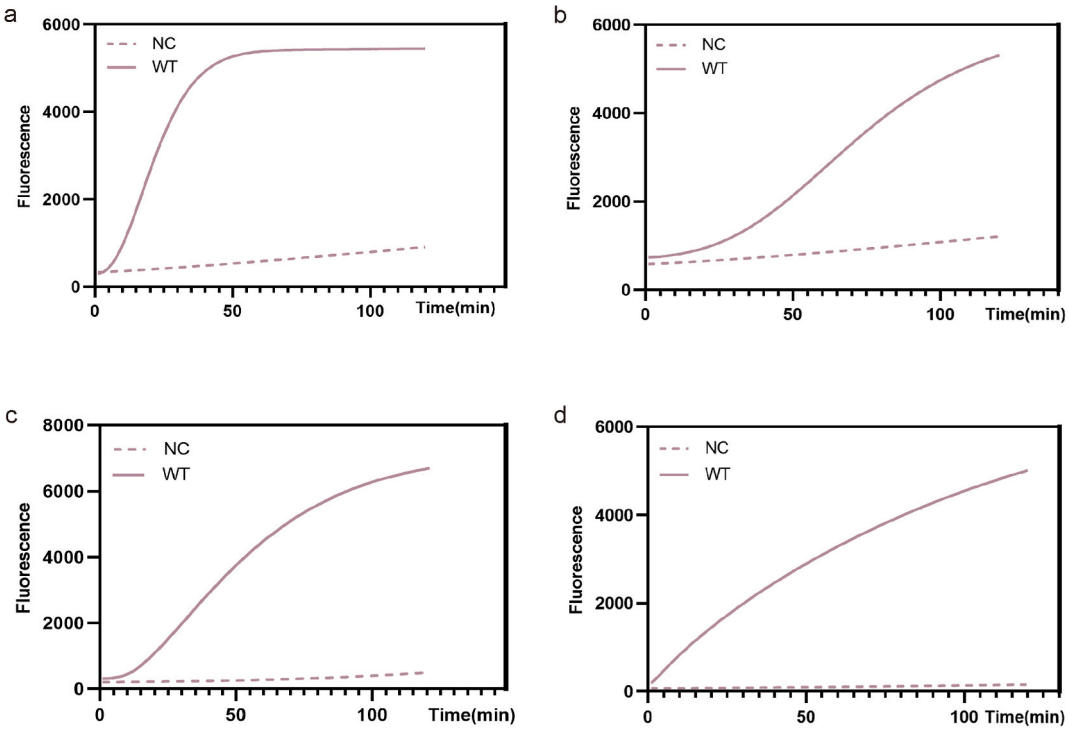

Fig. 3. Establishment of T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods using stepwise and one-step approaches. (a)Establishment of T4-MRCA-Cas12a stepwise method. (b) Establishment of T4-MRCA-Cas12a one-step method. (c) Establishment of Taq-MRCA-Cas12a stepwise method. (d) Establishment of Taq-MRCA-Cas12a one-step method. NC: Negative Control; WT: Plasmid containing MTB *rpoB* gene fragment.

3.4. Optimization of T4-MRCA-Cas12a stepwise and one-step methods

When using a concentration of 350 U/μL of T4 DNA ligase, PL can still form a loop even in the absence of target DNA (Fig. S1a). This can subsequently facilitate amplification and activate the CRISPR/Cas12a system, generating a fluorescence signal that exceeds 2000 at around 100 min. Based on this, we optimized the concentration of T4 DNA ligase in both the stepwise and one-step methods. The optimal concentrations for T4 DNA ligase in the stepwise and one-step methods are 1 U/μL and 50 U/μL, respectively (Fig. 4a and b). Under these optimal conditions for T4 DNA ligase, the concentrations of PL were further optimized. The optimal concentrations of PL in the stepwise and one-step methods are 4 μM and 1 μM, respectively (Fig. 4c and d). Additionally, as shown in Fig. S1b, the fluorescence value of the MRCA amplification is highest after 2 h, exceeding 6000 at around 100 min. To sum up, for the stepwise method of T4-MRCA-Cas12a, the optimal concentrations of T4 DNA ligase and the PL are 1 U/μL and 4 μM, and the optimal amplification time of MRCA is 2 h. For the one-Step method, they are 50 U/μL and 1 μM respectively.

3.5. Optimization of the Taq-MRCA-Cas12a method

When the concentration of Taq DNA ligase is reduced, the fluorescence value of the negative control remains relatively high when using $6N^S$ (Fig. S2a). After reducing the number of ligation cycles, the issue of false positives still remains unresolved (Fig. S2b). Therefore, when using different DNA ligases, the influence of different primer pairs on the method is crucial. Questioning whether $6N^S$ are the cause, we replaced them with specific primers. To confirm our conjecture, we set up a control experiment. The results are shown in Fig. 5. In the presence of T4 DNA ligase, the results showed that both had good specificity in this system (Fig. 5a). In the presence of Taq DNA ligase, only the positive and negative samples using the specific primer can be significantly distinguished; the fluorescence values of the negative and positive samples using the $6N^S$ primer are almost the same and cannot be distinguished; In addition, we also added both the specific primer and the $6N^S$ primer to a negative sample, and its fluorescence value reached 1267 (Fig. 5b). The above results suggest that in the presence of Taq DNA ligase, only the specific primer can significantly differentiate between the positive and negative. In other words, after introducing the specific primer into the Taq-MRCA-Cas12a system, the specificity of the method can be improved.

When using specific primers, we optimized the concentration and the number of cycles for Taq DNA ligase. The results indicate that the best performance of the Taq-MRCA-Cas12a method for detecting MTB occurs when the Taq DNA ligase concentration is set at 100 U/μL and the number of ligation cycles is 6 (Fig. 5c and d). Consequently, within the Taq-MRCA-Cas12a methodology, employing specific primers significantly enhances specificity compared to the use of $6N^S$.

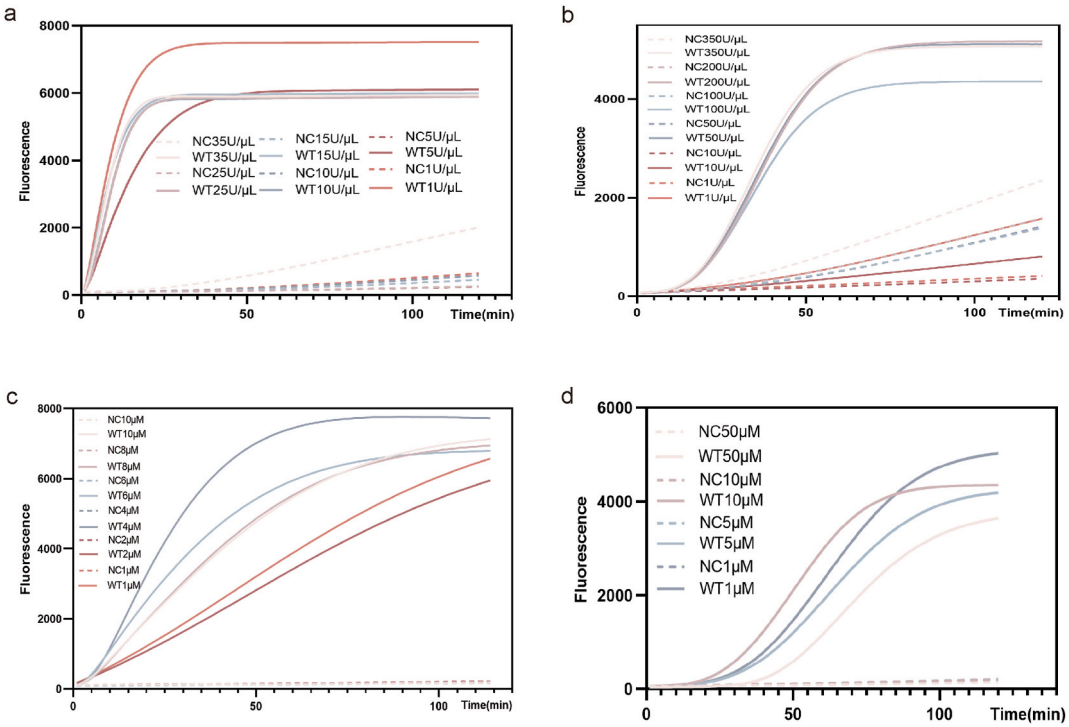

Fig. 4. Optimization of T4-MRCA-Cas12a stepwise and one-step methods. (a)Optimization of DNA ligase concentration for T4-MRCA-Cas12a stepwise method (PL concentration, 10 μM). (b)Optimization of DNA ligase concentration for T4-MRCA-Cas12a one-step method (PL concentration, 10 μM). (c)Optimization of PL concentration for T4-MRCA-Cas12a stepwise method (T4 DNA ligase concentration, 1U/μL). (d)Optimization of PL concentration for T4-MRCA-Cas12a one-step method (T4 DNA ligase concentration, 50U/μL). NC: Negative Control; WT: Plasmid containing MTB *rpoB* gene fragment; PL: Padlock probe.

3.6. Optimization of the CRISPR/Cas12a reaction system

The fluorescence reporting system is crucial for T4/Taq-MRCA-Cas12a methods. Optimizing the CRISPR/Cas12a reaction system can not only improve the sensitivity of the method but also make the distinction between positive and negative more significant. Components that affect the efficiency of the CRISPR/Cas12a system include the Cas12a protein, gRNA, reporter, and the amount of added products. We optimized the ratio of Cas12a protein to gRNA (1:0.8, 1:1, 1:1.2, 1:1.4, 1:1.6, 1:1.8, 1:2) and found that the fluorescence value was the highest when the ratio of gRNA to Cas12a protein was 1:1.2(Fig. 6a). In addition, we also optimized the influence of the reporter probe concentration on the experiment. The concentrations of the reporter (2 μM, 4 μM, 6 μM, 8 μM, 10 μM). The results showed that when the reporter probe with a concentration of 10 μM was used, the fluorescence could best distinguish between positive and negative (Fig. 6b).

3.7. Sensitivity assessment of T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods

We evaluated the limit of detection of the T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods. When using T4 DNA ligase, the sensitivity of the one-step T4-MRCA-Cas12a method with $6N^S$ primers was 10^4 aM, which was 100 times higher than that of the one-step T4-MRCA-Cas12a method with specific primers (Fig. 7a–d); while the sensitivity of the one-step Taq-MRCA-Cas12a method with specific primers was 10^3aM (Fig. 7e and f). Therefore, $6N^S$ primers can significantly improve the sensitivity of the method compared to specific primers. Although the Taq-MRCA-Cas12a method does not use $6N^S$ primers, it demonstrates superior sensitivity. This advantage may stem from the characteristics of Taq DNA ligase or the influence of the thermal cycling ligation method. The relative advantage of the Taq-MRCA-Cas12a method indicates that it can achieve or even surpass the detection sensitivity of traditional methods without using special primers, providing new insights and possibilities for future detection technology development.

3.8. Specificity assessment of T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods

As shown in Fig. 8a and b, one-step approaches of T4-MRCA-Cas12a and Taq-MRCA-Cas12a can accurately identify the *rpoB* gene of MTB and have no cross-reaction with DNA from other common clinical bacterial strains.

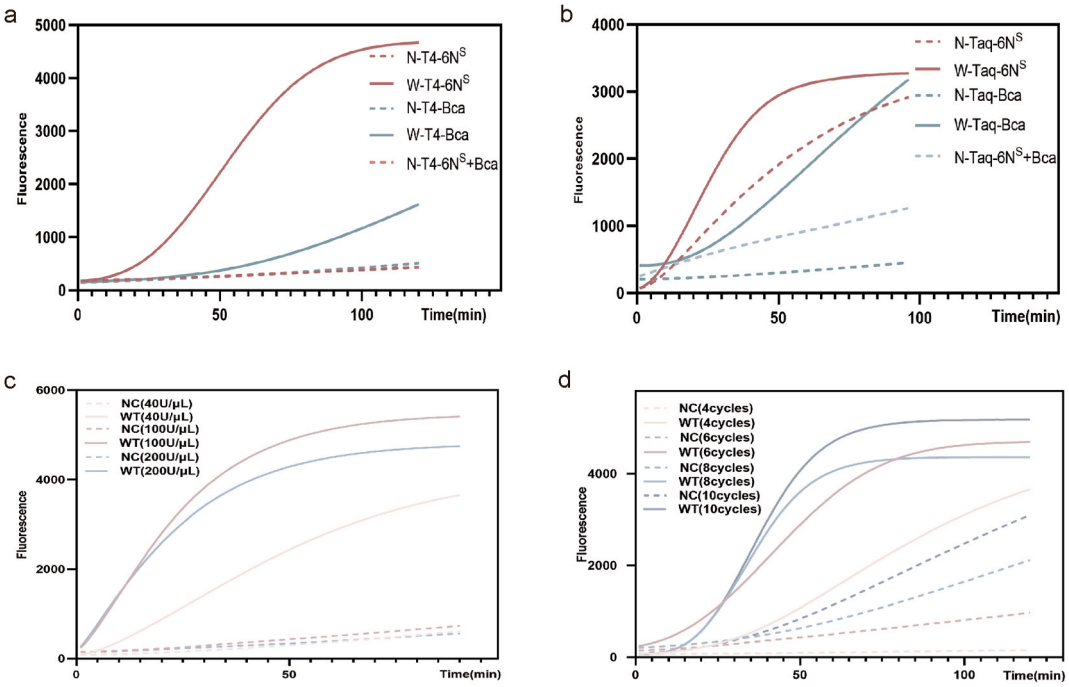

Fig. 5. Optimization of Taq DNA ligase reaction system. (a) Verification that 6N^S primers can not cause false positives, utilizing T4 DNA ligase. (b) Verification that 6N^S primers can cause false positives, utilizing Taq DNA ligase. (c) Optimization of DNA ligase concentration for Taq-MRCA-Cas12a method (Bca-F/R, 10 cycles) (d) Optimization of ligation cycle number for Taq-MRCA-Cas12a method (Bca-F/R, 100U/μL). NC: Negative Control; WT: Plasmid containing MTB *rpoB* gene fragment; PL: Padlock probe. W-T4/Taq-Bca refers to the RCA amplification reaction of the WT template using specific primers and T4/Taq DNA ligase; N-T4/Taq-Bca refers to the RCA amplification reaction of the NC template using specific primers and T4/Taq DNA ligase; W- T4/Taq -6N^S refers to the RCA amplification reaction of the WT template using 6N^S primers and T4/Taq DNA ligase; N- T4/Taq -6N^S refers to the RCA amplification reaction of the NC template using 6N^S primers and T4/Taq DNA ligase.

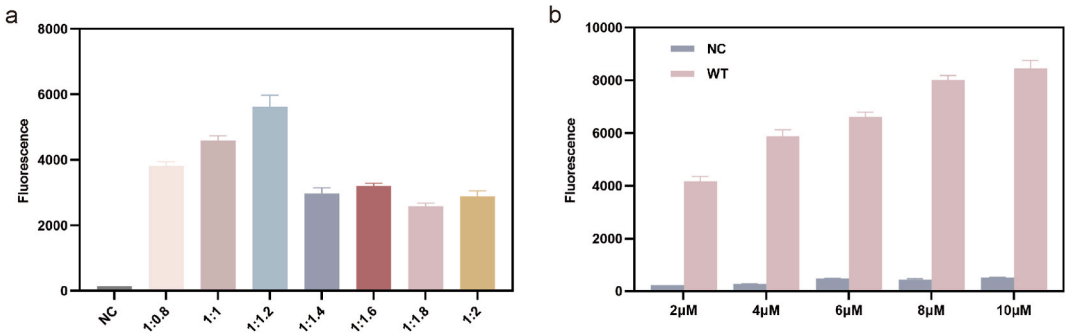

Fig. 6. Optimization of the CRISPR/Cas12a system. (a) Optimization of the ratio between gRNA and Cas12a protein. (b) Optimization of the reporter probe concentration. NC: Negative Control; WT: Plasmid containing MTB *rpoB* gene fragment.

3.9. Clinical sample validation

To further validate the applicability of the established methods in clinical samples, we tested 38 clinical sputum and bronchoalveolar lavage fluid samples, and the results are shown in Fig. 9. When comparing the results obtained using the T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods with those from "gold standard" Sanger sequencing, the former showed three differing results, and the latter showed one differing result. The accuracy, specificity, positive predictive value, and negative predictive value for the one-step method of T4-MRCA-Cas12a were 88 %, 100 %, 100 %, and 81.25 %, respectively; for the one-step method of Taq-MRCA-Cas12a, they were 96 %, 100 %, 100 %, and 92.86 %, respectively (Table 1). These results suggest that while our methods exhibit high sensitivity and specificity, there are still some discrepancies with the gold standard sequencing method in individual cases.

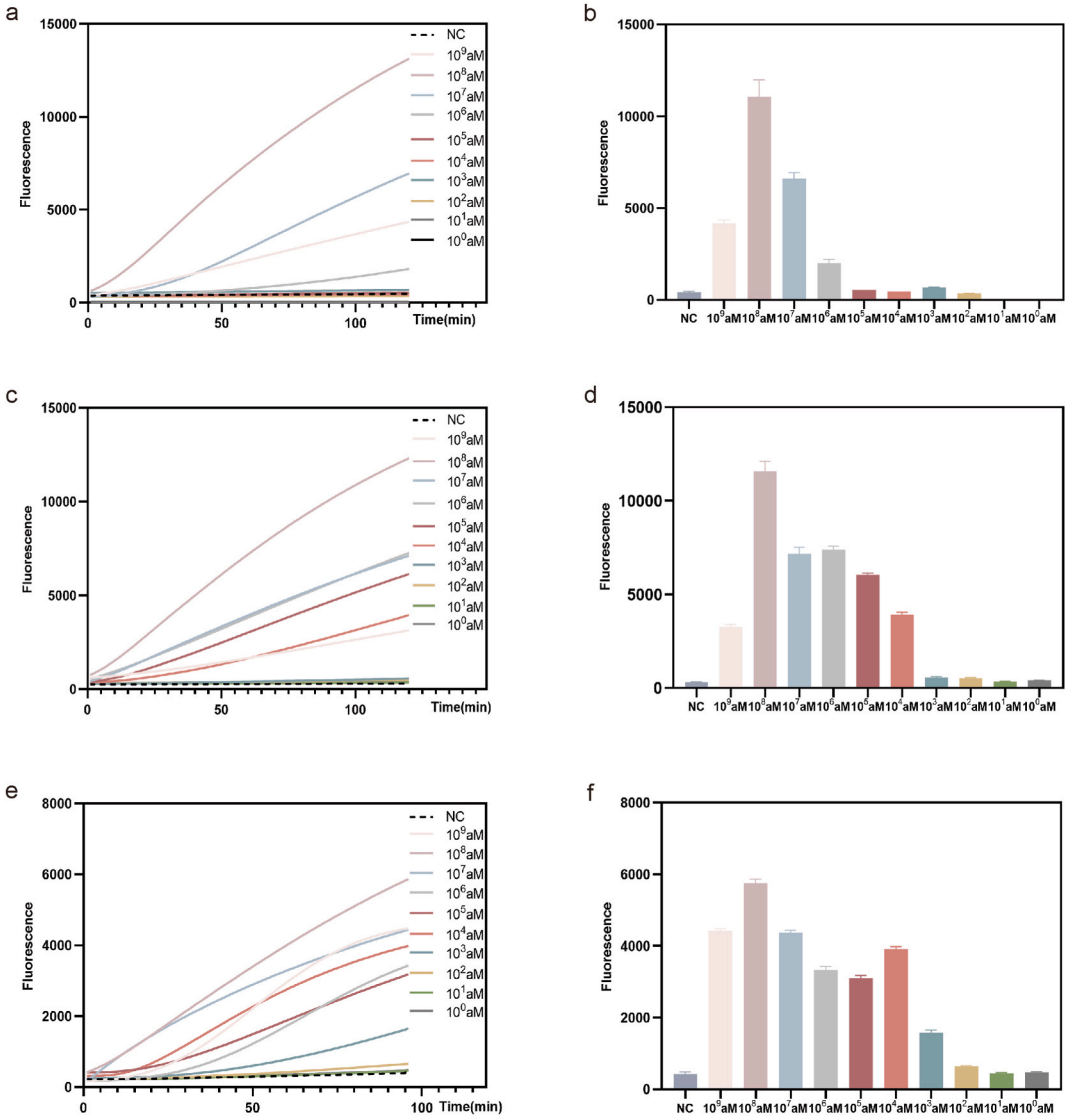

Fig. 7. Sensitivity evaluation of T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods. (a,b)Sensitivity of T4-MRCA-Cas12a one-step method, utilizing special primers. (c,d) Sensitivity of T4-MRCA-Cas12a one-step method, utilizing $6N^S$ primers. (e,f)Sensitivity of Taq-MRCA-Cas12a one-step method, utilizing special primers. NC: Negative Control.

4. Discussion

In this study, we optimized the concentrations of DNA ligase and PL in both stepwise and one-step methods to reduce the incidence of false-positive results. Our investigation yielded two findings. First, in the Taq-MRCA-Cas12a method, the use of $6N^S$ continued to produce false-positive results, even with reduced ligation cycles and DNA ligase concentration. Second, we observed a substantial difference in detection sensitivity between the T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods, which may be attributed to the distinct characteristics of the two DNA ligases employed.

When the concentration of DNA ligase is too high, it leads to false-positive results. We speculate that in a high-concentration environment of T4/Taq DNA ligase, the increased number of ligase molecules greatly enhances their opportunities to contact and bind with DNA ends, thus accelerating the self-ligation process of DNA ends [17]. Our research also revealed that the concentration of PL significantly impacts the efficiency of circularization. When the PL concentration is too low, the number of circularization events is relatively limited. However, as the PL concentration increases, the fluorescence value does not increase as expected but instead shows a decreasing trend. This phenomenon occurs because when the PL concentration exceeds a certain threshold, competition among probes ensues, leading to reduced circularization efficiency and, consequently, a decrease in the production of amplification products [18]. It is noteworthy that we discovered significant differences in the performance of $6N^S$ in reactions mediated by T4 DNA ligase and Taq DNA ligase. In the T4-MRCA-Cas12a reaction system, the incorporation of $6N^S$ significantly enhanced reaction sensitivity. However, in

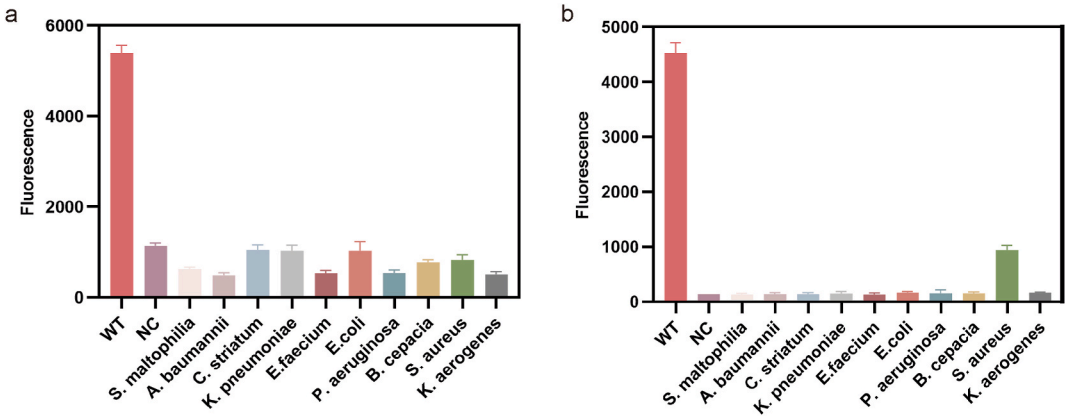

Fig. 8. Specificity evaluation of T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods. (a)Specificity of T4-MRCA-Cas12a one-step method. (b)Specificity of Taq-MRCA-Cas12a one-step method. WT: Plasmid containing MTB *rpoB* gene fragment; NC: Negative Control.

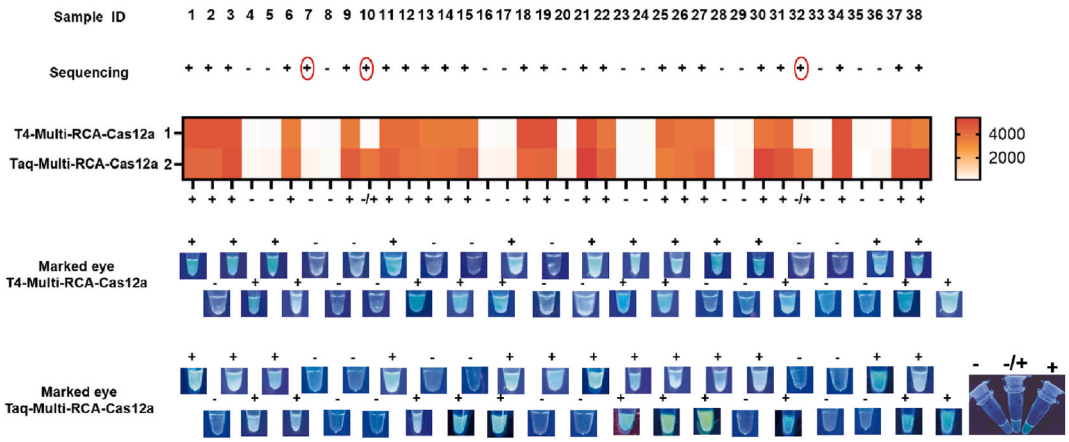

Fig. 9. Clinical sample verification.

Table 1
Comparison of T4/Taq-Multi-RCA-Cas12a Method with Sequencing Results. PPV: positive predictive value; NPV: Negative predictive value.

		Sequencing		Accuracy(%)	Specificity(%)	PPV(%)	NPV(%)
		MTB(B)	Other(n)				
T4-Multi-RCA-Cas12a	MTB	22	0				
	Others	3	13	88	100	100	81.25
	Total	25	13				
Taq-Multi-RCA-Cas12a	MTB	24	0				
	Others	1	13	96	100	100	92.86
	Total	25	13				

reactions mediated by Taq-MRCA-Cas12a, no significant difference in fluorescence values between positive and negative samples was observed, regardless of whether stepwise or one-step methods were employed. We speculate that this may be related to the excessive use of Taq DNA ligase or too many ligation cycles. Yet, even with reduced amounts of ligase and ligation cycles, the false-positive problem persists. Given the short length and highly universal nature of $6N^S$, they exhibit the capacity to bind at multiple positions on RNA/DNA molecules, rather than exclusively at specific target sequences. This characteristic potentially contributes to the occurrence of false-positive results [19]. Therefore, even in the absence of target DNA, it is more likely for ssDNA strands to undergo self-ligation [20]. To test this hypothesis, we replaced $6N^S$ with specific primers in our experiments, and the results showed that the specific primers effectively eliminated false-positive results.

When considering the characteristics of $6N^S$ and Taq DNA ligase in combination, their simultaneous presence in a reaction system substantially increases the probability of false-positive results. Consequently, in experimental settings that demand high accuracy and specificity, it is advisable to substitute $6N^S$ with specific primers, particularly in experimental systems employing Taq DNA ligase. This

helps to reduce non-specific ligation and ensure the reliability of experimental results. Additionally, in the Taq-MRCA-Cas12a reaction system, the use of specific primers and an increase in cycle number can also lead to a decrease in specificity. This may be due to the fact that as the temperature increases, the activity of Taq DNA ligase also increases, which makes it more effective in promoting the formation of a phosphodiester bond between the 5′-phosphate end and the 3′-hydroxyl end of two adjacent DNA strands. Under elevated temperature conditions, molecular thermal motion is accelerated, resulting in increased collision frequency between terminal bases and enhanced ligation efficiency. These findings from our experimental groundwork provide a valuable reference for the development of platforms incorporating Taq DNA ligase and random primers.

We further substantiated the T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods using sputum and BAL samples from TB patients. Compared to the traditional smear microscopy method, the T4/Taq-MRCA-Cas12a methods demonstrated higher sensitivity and stability [21]; Compared to sputum culture, our method can complete the identification of MTB within 3 h, meeting the needs for rapid clinical testing [22]; Additionally, compared to GeneXpert, our established method is more convenient, economical, and efficient, enabling POCT for MTB detection [23]. A comparison of various molecular biological detection techniques is provided in Table S2. However, we found differences in detection sensitivity between the T4-MRCA-Cas12a and Taq-MRCA-Cas12a methods, with the latter demonstrating higher sensitivity. This disparity mainly stems from the mechanism of the Taq DNA ligase in the ligation cycling reaction, where the circular product of a previous cycle can serve as a template for the next cycle, greatly enhancing the efficiency of circularization and increasing the amplification template for subsequent RCA. In contrast, T4 DNA ligase performs a single circularization, which is relatively less efficient [24,25]. While the Taq-MRCA-Cas12a method offers advantages in terms of detection sensitivity, it necessitates precise thermocycling equipment for operation, which somewhat constrains its application scenarios, particularly in resource-limited settings. In contrast, the T4-MRCA-Cas12a method can be executed using simple constant temperature devices such as water baths, thus fulfilling the requirements for POCT. Additionally, compared to the stepwise approach, the one-step method integrates MRCA and CRISPR detection into a single tube, simplifying the experimental operations and workflow. Choosing which method to use requires a comprehensive consideration of the specific application requirements, available equipment conditions, and the desired detection sensitivity.

The development of methods based on RCA for dsDNA detection holds significant potential and application value in the field of molecular diagnostics. For instance, the combination of CRISPR/Cas12a with Hyperbranched Rolling Circle Amplification (HRCA) by Juneseok You and others for the identification of various cancer ctDNAs contributes to early screening and diagnosis of cancer [26]. However, this technology is complex in both design and operation, imposing high demands on laboratory technicians. Moreover, it necessitates sophisticated electrochemical detection instruments, which not only increases costs but also limits its application in resource-constrained areas. Additionally, the difficulty in primer design within the HRCA technology adds to the complexity of the experiments and the potential failure rate [27]. In comparison, the technology we developed, combining CRISPR-Cas12a with MRCA for dsDNA detection, requires only simple equipment such as a water bath and a UV lamp for results to be read with the naked eye. This avoids reliance on complex instruments, greatly broadening its range of applications, and features simple, economical, and rapid experimental performance, achieving POCT for dsDNA.

5. Conclusion

In this study, we developed a novel MTB detection method that combines MRCA with CRISPR/Cas12a. This technique extends the application range of MRCA in detecting dsDNA. Notably, this method requires only simple laboratory equipment such as UV lamps and water baths to achieve POCT for MTB. Additionally, the technique has shown great potential in detecting other clinical pathogen DNA, viral DNA, circulating tumor DNA and other dsDNA types. Notwithstanding these advantages, there remains considerable scope for enhancing the sensitivity of this methodological approach. Future enhancements may involve the integration of dual fluorescent probes in the CRISPR detection system to increase the fluorescent signal, further boosting the detection sensitivity to meet broader clinical and public health needs.

CRediT authorship contribution statement

Yingying Sun: Writing – review & editing, Writing – original draft, Software, Project administration, Methodology, Data curation. **Yaozhou Wu:** Methodology. **Yulin Wang:** Methodology. **Keke Li:** Software. **Yanbin Chang:** Writing – review & editing, Supervision, Methodology. **Lianhua Wei:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The author reports no conflicts of interest in this work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Acknowledgements

This work was supported by the Hospital Fund of Gansu Provincial Clinical Research Center for Laboratory Medicine (No. 21GSSYC-41) and Doctoral supervisor cultivation project at Gansu Provincial Hospital (No. 22GSSYA-2). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37640.

References

[1] K.C. Rahlwes, et al., Pathogenicity and virulence of Mycobacterium tuberculosis, Virulence 14 (1) (2023) 2150449.
[2] F. Guo, et al., Immunological effects of the PE/PPE family proteins of Mycobacterium tuberculosis and related vaccines, Front. Immunol. 14 (2023) 1255920.
[3] L.S. Li, et al., From immunology to artificial intelligence: revolutionizing latent tuberculosis infection diagnosis with machine learning, Mil Med Res 10 (1) (2023) 58.
[4] F. Zhang, et al., New insights into biomarkers for evaluating therapy efficacy in pulmonary tuberculosis: a narrative review, Infect. Dis. Ther. 12 (12) (2023) 2665–2689.
[5] U.B. Singh, et al., Expedited diagnosis of pediatric tuberculosis using Truenat MTB-Rif Dx and GeneXpert MTB/RIF, Sci. Rep. 13 (1) (2023) 6976.
[6] S.H. Wu, et al., Development and assessment of a novel whole-gene-based targeted next-generation sequencing assay for detecting the susceptibility of Mycobacterium tuberculosis to 14 drugs, Microbiol. Spectr. 10 (6) (2022) e0260522.
[7] M. Beviere, et al., The role of next-generation sequencing (NGS) in the management of tuberculosis: practical review for implementation in routine, Pathogens 12 (8) (2023).
[8] H. Yang, et al., CRISPR-based nucleic acid diagnostics for pathogens, Trends Analyt Chem 160 (2023) 116980.
[9] N. Bhokisham, et al., CRISPR-cas system: the current and emerging translational landscape, Cells 12 (8) (2023).
[10] X. Lei, et al., Non-canonical CRISPR/Cas12a-based technology: a novel horizon for biosensing in nucleic acid detection, Talanta 271 (2024) 125663.
[11] L. Xu, et al., Recent advances in rolling circle amplification-based biosensing strategies-A review, Anal. Chim. Acta 1148 (2021) 238187.
[12] C. Li, et al., Construction of rolling circle amplification products-based pure nucleic acid nanostructures for biomedical applications, Acta Biomater. 160 (2023) 1–13.
[13] H. Yan, et al., Extraction-free, one-pot CRISPR/Cas12a detection of microRNAs directly from extracellular vesicles, Chem. Commun. 59 (67) (2023) 10165–10168.
[14] X. Liu, et al., Accurate detection of lung cancer-related microRNA through CRISPR/Cas9-assisted garland rolling circle amplification, J. Thorac. Dis. 14 (11) (2022) 4427–4434.
[15] L. Xu, et al., Accurate MRSA identification through dual-functional aptamer and CRISPR-Cas12a assisted rolling circle amplification, J. Microbiol. Methods 173 (2020) 105917.
[16] Z. Chu, et al., Cyclic multiple primer generation rolling circle amplification assisted capillary electrophoresis for simultaneous and ultrasensitive detection of multiple pathogenic bacteria, Anal. Chem. 96 (4) (2024) 1781–1788.
[17] H. Kuhn, M.D. Frank-Kamenetskii, Template-independent ligation of single-stranded DNA by T4 DNA ligase, FEBS J. 272 (23) (2005) 5991–6000.
[18] Y. Li, et al., Rolling circle amplification based colorimetric determination of Staphylococcus aureus, Mikrochim. Acta 187 (2) (2020) 119.
[19] V. Oliveira, et al., Characterization of putative circular plasmids in sponge-associated bacterial communities using a selective multiply-primed rolling circle amplification, Mol Ecol Resour 21 (1) (2021) 110–121.
[20] J. Hetmańczyk, et al., Investigation of hydrogen bonds in proton transfer complexes derived from the reaction of 2- and 4-(N,N-dimethylamino)pyridines with chloranilic acid, Spectrochim. Acta Mol. Biomol. Spectrosc. 315 (2024) 124241.
[21] E. Kotei, R. Thirunavukarasu, Computational techniques for the automated detection of mycobacterium tuberculosis from digitized sputum smear microscopic images: a systematic review, Prog. Biophys. Mol. Biol. 171 (2022) 4–16.
[22] K. Naidoo, et al., Rapid diagnosis of drug-resistant tuberculosis-opportunities and challenges, Pathogens 13 (1) (2023).
[23] K. Naidoo, N. Dookie, Can the GeneXpert MTB/XDR deliver on the promise of expanded, near-patient tuberculosis drug-susceptibility testing? Lancet Infect. Dis. 22 (4) (2022) e121–e127.
[24] Y. Cui, et al., Cyclization of secondarily structured oligonucleotides to single-stranded rings by using Taq DNA ligase at high temperatures, RSC Adv. 8 (34) (2018) 18972–18979.
[25] J. Kim, M. Mrksich, Profiling the selectivity of DNA ligases in an array format with mass spectrometry, Nucleic Acids Res. 38 (1) (2010) e2.
[26] J. You, et al., Sensitive and selective DNA detecting electrochemical sensor via double cleaving CRISPR Cas12a and dual polymerization on hyperbranched rolling circle amplification, Biosens. Bioelectron. 224 (2023) 115078.
[27] C. Zhang, et al., Comparison of loop-mediated isothermal amplification with hyperbranched rolling circle amplification as a simple detection method for Heterosigma akashiwo, Harmful Algae 73 (2018) 1–11.