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ORIGINAL ARTICLE

A Novel Terahertz-Based Metamaterial System for the Detection of Multidrug-Resistant *Mycobacterium tuberculosis*

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SUMMARY

Background: **Terahertz metamaterial is a label-free affinity sensor that is particularly sensitive to changes in the surface dielectric environment, so it can be used to detect protein molecules, human tissues, and drug reaction processes. This study aimed to construct a detection system for multidrug-resistant *Mycobacterium tuberculosis* (MTB) based on terahertz metamaterials.**
Methods: **Linear padlock probes (PLP) were designed to contain sequences complementary to the DNA sequences of drug-resistant MTB (target sequences), and linear PLP hybridizes to the target sequence to form a circular PLP. The primers fixed on the magnetic beads hybridize with the circular PLP to initiate rolling circle amplification (RCA). We placed RCA nucleic acid products on terahertz metamaterials for terahertz measurements.**
Results: **We determined the detection system primer concentration to be 15 μmol/L and the RCA reaction time to be 2 hours. Under the abovementioned optimal conditions, the minimum detection limits of the terahertz metamaterial detection system for the target sequences of katG315, rpoB531, and rpsL43 were 7.7×10^{-5} pmol/L, 1.03×10^{-5} pmol/L, and 1.3×10^{-4} pmol/L, respectively.**
Conclusions: **This terahertz metamaterial-based detection system can achieve high-sensitivity and rapid detection of multiple drug-resistant MTB.**
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KEYWORDS

terahertz, *Mycobacterium tuberculosis*, rolling circle amplification, multidrug-resistant

INTRODUCTION

According to the "Global Tuberculosis Report 2024" released by the World Health Organization, the total number of new tuberculosis (TB) patients in the world in 2023 was 10.8 million, the incidence rate reached 134/100,000, caused 1.25 million deaths worldwide, and TB had once again become the leading cause of death from a single infectious disease in the world [1]. Importantly, the incidence of tuberculosis in the Chinese population accounts for 6.8% of the world, making China a country

with a high burden of TB. Unfortunately, the incidence of multidrug-resistant TB is increasing due to delayed tuberculosis diagnosis and misuse of anti-tuberculosis drugs, which was also one of the main causes of death among TB patients [2,3]. Currently, the confirmation of multidrug-resistant TB happens mainly through drug sensitivity testing after culture of *Mycobacterium tuberculosis* and direct sequencing, but both methods have obvious shortcomings. The disadvantages of drug sensitivity testing are long cycle and low positive results, and the high cost is an obstacle to large-scale clinical application of direct sequencing [4,5]. Therefore, it is of great practical significance to establish a new method for rapid and simple detection of multidrug-resistant *Mycobacterium tuberculosis*.

Rolling circle amplification (RCA) is a constant temperature amplification technology developed based on the rolling circle replication of DNA/RNA molecules of circular pathogenic microorganisms in nature [6,7]. RCA avoids the technical defects of traditional PCR, such as non-specific amplification and incompatibility caused by complex thermal cycles, and achieves simple operation (no need for special instruments), high detection sensitivity, and multiple analysis. At the same time, RCA allows the immobilization of primers or capture probes on a solid support, and after hybridization with the target sequence, a rolling circle amplification system can be established on the surface of a biosensor chip [8, 9]. However, the traditional method of detecting RCA products by agarose gel electrophoresis not only has a long detection cycle but also has poor result stability [10]. Therefore, a fast, accurate, and stable RCA product detection method is necessary.

Terahertz radiation is an electromagnetic wave with a frequency of 0.1 - 10 THz, and its wavelength is between the millimeter wave in wireless waves and infrared rays. Terahertz metamaterial is a label-free affinity sensor that is particularly sensitive to changes in the surface dielectric environment, so it can be used to detect protein molecules, human tissues, and drug reaction processes [11,12]. In this study, we developed a terahertz metamaterial detection system based on RCA technology to detect multidrug-resistant Mycobacterium tuberculosis.

MATERIALS AND METHODS

Chemicals and reagents

Streptavidin magnetic beads with a diameter of 1 μm were purchased from Thermo Fisher Scientific (MA, USA). DNA polymerase, dNTP Mix, DNA ligase, exonuclease I, and exonuclease III were purchased from TAKARA (BeiJing, China). All nucleic acid sequences used in this study were synthesized by Sangon Biotechnology (Shanghai) Co., Ltd., including linear PLP, primers for labeling organisms, and target sequences. The design of linear PLP is shown in Figure 1A, and the sequences of linear PLP are shown in Figure 1B. At the same time, the sequences of primers were as follows:
katG315:
Biotin-$(CH_2)_6$-AGGTCGATGCTGAGGTCGCA-3′,
rpoB531:
Biotin-$(CH_2)_6$-CGATAGGTGGTCTACCGCTG-3′,
rpsL43:
Biotin-$(CH_2)_6$-TACCAGCTCGCATACCGGTC-3′.
And the target sequences are shown in Table 1.

Circular ligation reaction of linear PLP

Hybridization reaction was performed in 20 μL system with linear PLP and target sequence at a ratio of 10:1 (95°C for 5 minutes, 60°C for 30 minutes). Next, 5 U DNA ligase were added into the abovementioned reaction system to incubate at 37°C for 45 minutes to prepare the circular PLP. Subsequently, 10 U exonuclease I and exonuclease III were added to the abovementioned reaction system for 30 minutes at 37°C to remove uncircularized linear PLP and target sequences. At last, the reaction system was heated to 95°C for 5 minutes to terminate the reaction.

Preparation of functionalized magnetic beads

First, 10 μL of streptavidin magnetic beads were washed 3 times with magnetic bead buffer solution and then resuspended in 30 μL of magnetic bead buffer solution. Next, 1 μL of biotinylated primers was added to the magnetic beads for 30 minutes at room temperature to prepare functionalized magnetic beads. Finally, functionalized magnetic beads were washed with sterile enzyme-free DEPC water and were then used.

Solid phase RCA on magnetic beads

At first, the hybridization system between circular PLP and primer was constructed according to the system with a volume ratio of 10:1 between the circular PLP and the functionalized magnetic beads, and the hybridization reaction was carried out for 30 minutes at 37°C. Subsequently, the beads were washed 2 times with ultrapure water to remove unbound oligonucleotides. Finally, the RCA reaction was initiated with 10 U of DNA polymerase, and the amplified beads were washed (3 times) and resuspended with ultrapure water for terahertz detection.

Preparation of THz metamaterials

As previously studied [13], we use conventional photolithography to target the THz metamaterial with a double-layer cross-shaped plate-hole structure. Briefly, a cross-shaped pattern layer was made on the silicon wafer using inductively coupled plasma etching technology, and then a 200 nm thick gold layer (a chromium layer was added to increase the adhesion of the gold) was deposited on the pattern layer using an electron beam evaporator system (Figure 2).

Measurement of zeta potential of magnetic beads

Ultrapure water was used as the Zeta potential measurement medium and blank control. The Zeta potential of

Table 1. Wild-type and mutant MTB target sequences.

Name (WT→MUT)	Target sequences (5′-3′)
katG315 (G→C)	TGGCACCGGAACCGGTAAGGACGCGATCACCA***C***CGGCATCGAGGTCGTATGGACGAA
rpoB531 (C→T)	TCGGGGTTGACCCACAAGCGCCGACTGT***T***GGCGCTGGGGCCCGGCGGTCT
rpsL43 (A→G)	GCACCCGCGTGTACACCACCACTCCGA***G***GAAGCCGAACTCGGCGCTTCGG

Italic, and underscore are mutation sites.

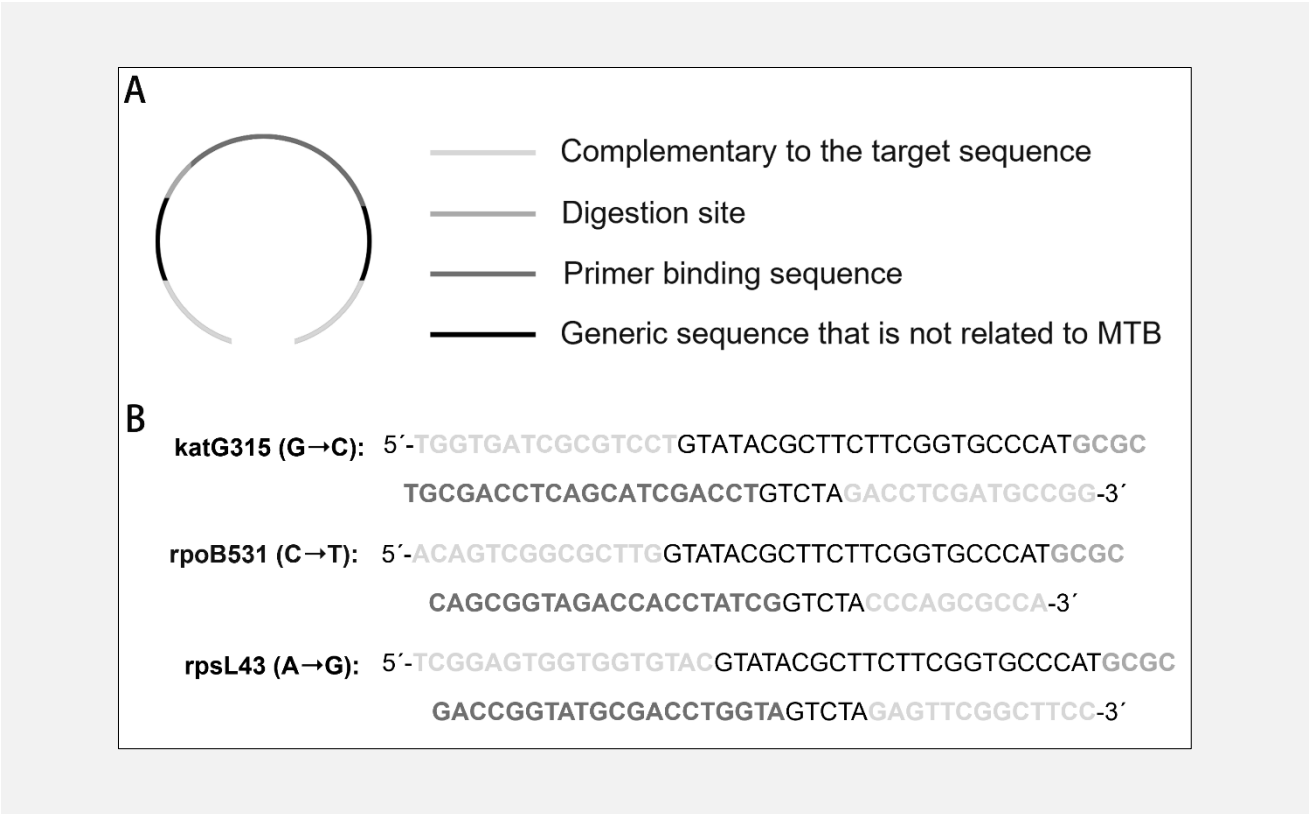

Figure 1. Structure (A) and sequence (B) of PLP.

samples containing different magnetic beads (10 μL beads in 1 mL ultrapure water) was measured using an Zetasizer Nano-ZS analyzer (Malvern, UK) to characterize the surface charge of different magnetic beads. Each measurement was repeated 3 times, and the measurement results were expressed as (mean ± standard deviation).

THz measurement of nucleic acid amplification products

The magnetic beads connected to the RCA product, and the functionalized magnetic beads were evenly dropped onto the terahertz metamaterial. The terahertz measurement was performed using an TAS7500SP THz-TDS analyzer (Advantest, Japan) in reflection mode at room temperature. During the measurement, the terahertz optical path was filled with helium to reduce the absorption of terahertz waves by water vapor, and the humidity was less than 5%. The frequency shift values of the RCA product magnetic beads ($F_{MB\text{-}RCA}$) and the functionalized magnetic beads ($F_{MB\text{-}P}$) were obtained respectively.

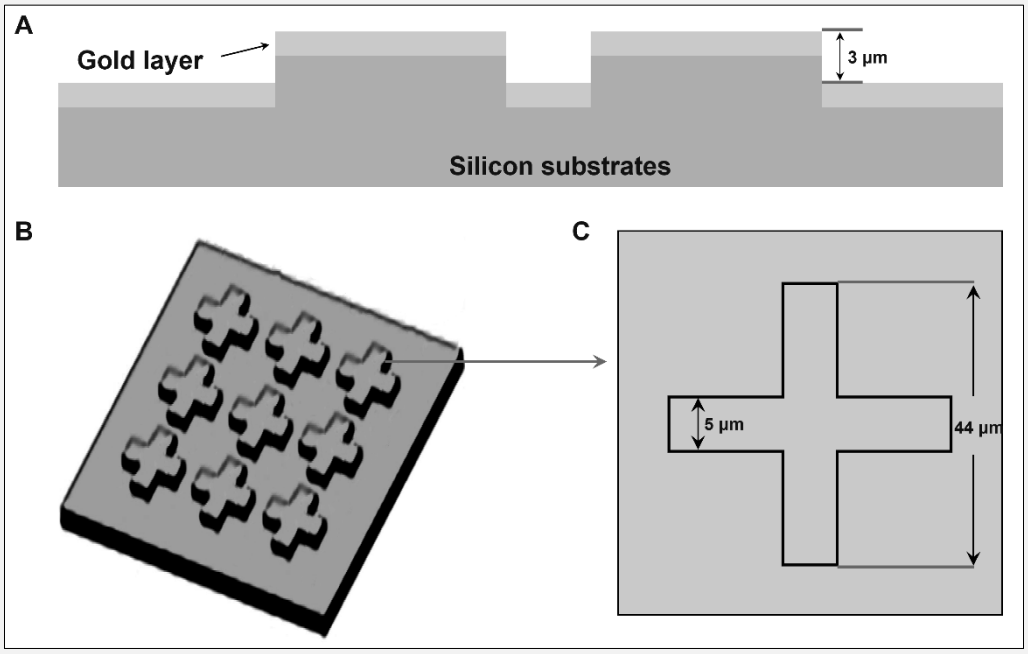

Figure 2. Structure of THz metamaterial chip.

A Schematic diagram of cross-section of THz metamaterial chip.
B Schematic diagram of top view of THz metamaterial chip.
C Unit cell of the metasurface consisting of cross-shaped modules on a dielectric slab.

RESULTS

Solid phase RCA on THz metamaterials

As shown in Figure 3, this detection system based on terahertz metamaterials mainly includes four steps. First, the complementary sequence of the target sequence on the linear PLP hybridizes with the target sequence to form a circular PLP. At the same time, linear PLP that cannot hybridize to the target sequence is processed by nuclease (Figure 3A). Second, magnetic beads carrying streptavidin bind to biotin-labeled primers to form functionalized magnetic beads (Figure 3B). Third, the primers on the functionalized magnetic beads hybridize to the primer complementary sequences on the circular PLP and then initiate RCA to form RCA products of the target sequence (Figure 3C). Fourth, the RCA product was magnetically separated and then tested for terahertz signals (Figure 3D).

Zeta potential of magnetic beads

To evaluate the performance of RCA, we measured the zeta potential of blank magnetic beads (MB), functionalized magnetic beads (MB-P), and RCA product magnetic beads (MB-RCA). We found that the zeta potential of MB, MB-P (katG315), MB-RCA (katG315), MB-P (rpoB531), MB-RCA (rpoB531), MB-P (rpsL43), and MB-RCA (rpsL43) was (23.81 ± 0.23), mV (24.71 ± 0.09) mV, (31.86 ± 0.50) mV, (24.85 ± 0.24) mV, (33.13 ± 0.26) mV, (24.77 ± 0.19) mV, and (32.02 ± 0.29) mV, respectively, which indicated that RCA on the magnetic beads surface occurred successfully (Figure 4).

Optimization of THz metamaterial detection conditions

To maximize the detection performance, we optimized two conditions that may affect the terahertz metamaterial detection system, including primer concentration and RCA reaction time. As shown, when the primer concentration is lower than 15 μmol/L, the mobile frequency of terahertz detection (ΔF) increases with the increase of primer concentration. However, when the primer concentration exceeds 15 μmol/L, ΔF did not change strongly with the change of primer concentration (Figure 5A - 5C). For the time of RCA reaction, we found that when the RCA reaction time is 2 hours, ΔF reached its peak (Figure 5D - 5F). Therefore, we set the primer concentration and RCA reaction time of the detection

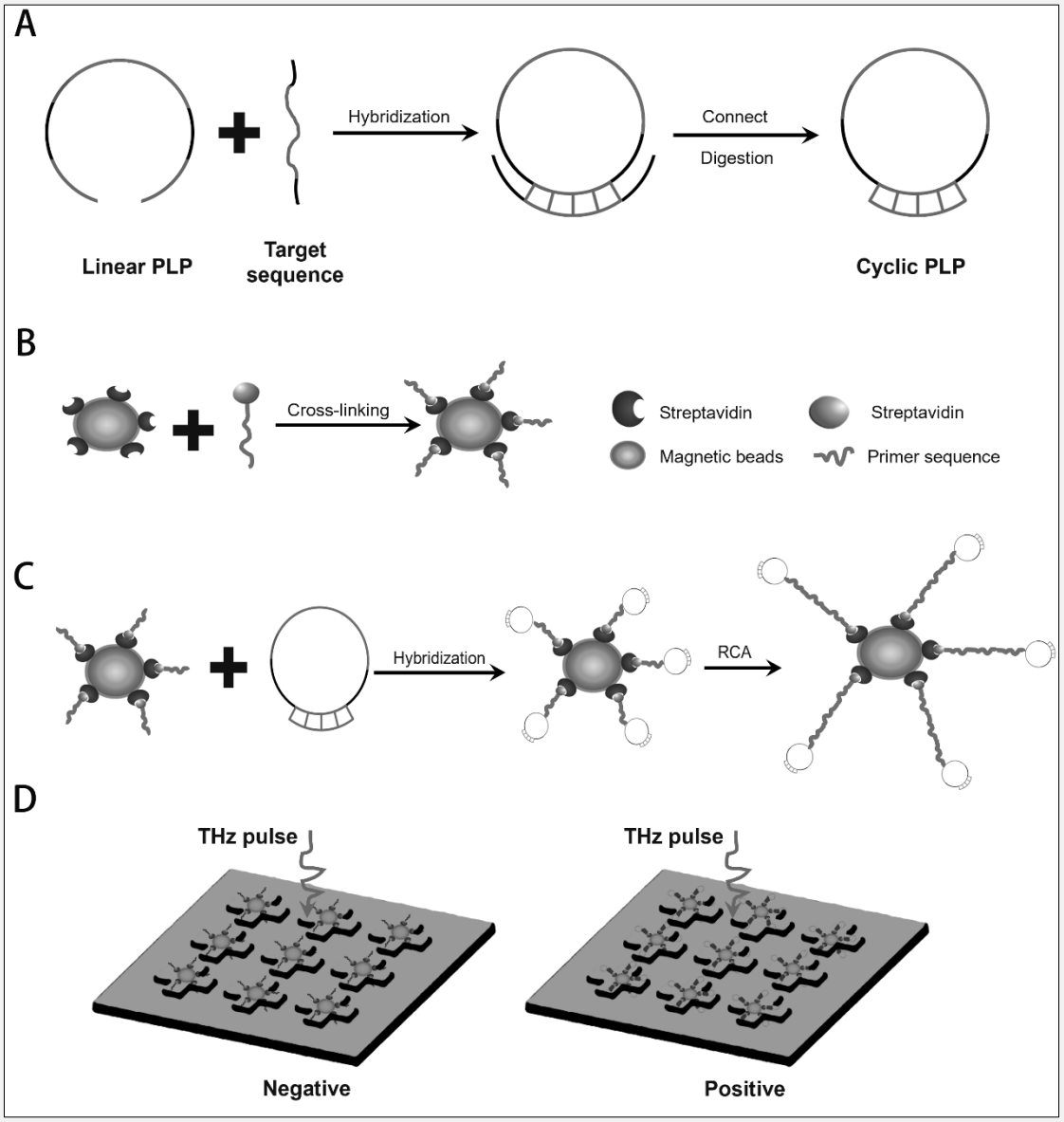

Figure 3. Schematic description of THz spectroscopy for MTB DNA detection by MB-based RCA.

A Schematic diagram of the circumferential connection reaction of linear PLP.
B Schematic diagram of functional MB preparation.
C Schematic diagram of solid phase RCA on magnetic beads.
D Measurement of zeta potential of magnetic beads using THz pulse.

system to 15 μmol/L and 2 hours, respectively.

Sensitivity of THz detection system

After determining the optimal detection conditions of the detection system, we used different concentrations of target sequences to evaluate the detection performance of the terahertz metamaterial. As shown in Figure 6, the terahertz detection frequency increased gradually as the concentration of the target sequence increased, and ΔF was linearly correlated with the logarithm of the target sequence concentration (Log (C)), which was ΔF = 3.6312 Log (katG315) + 18.573 (R^2 = 0.9945) (Figure 6A), ΔF = 2.9615 Log (rpoB531) + 17.729 (R^2 = 0.9719) (Figure 6B), and ΔF = 3.2826 Log

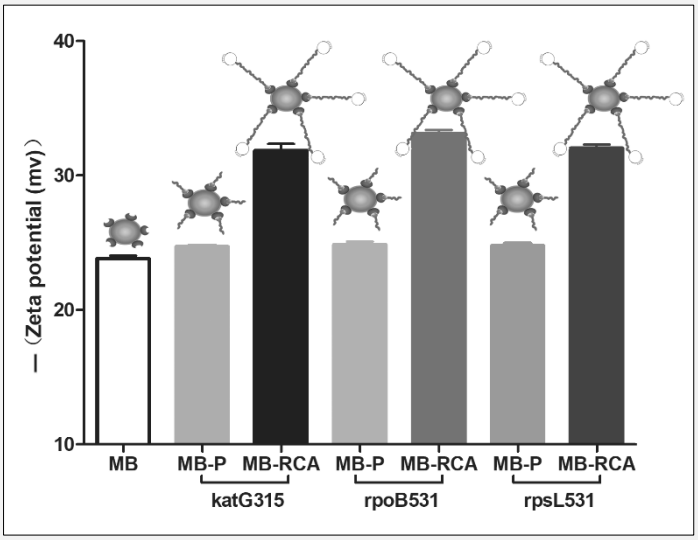

Figure 4. Zeta potential of different magnetic beads.

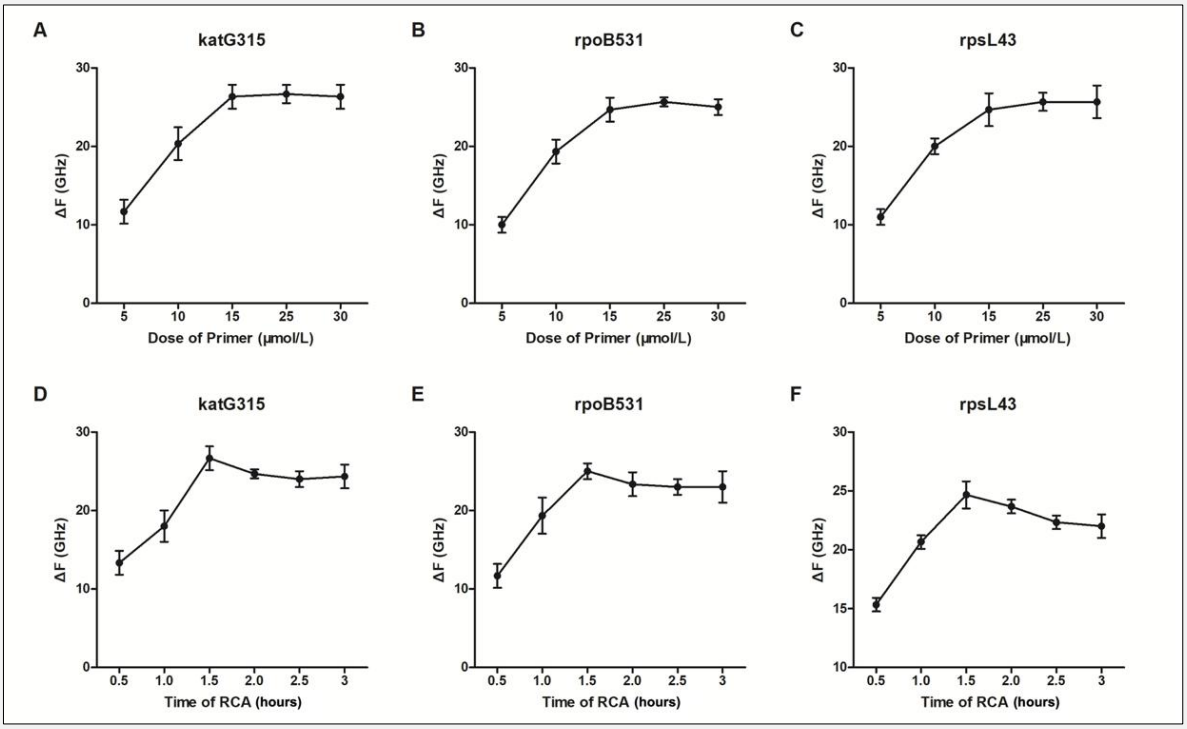

Figure 5. Effects of different primer concentrations and RCA reaction time on the detection results of THz metamaterials.

A - C Effect of different primer concentrations on the detection results of katG315 (A), rpoB531 (B), and rpsL43 (C) resistant MTBs.
D - F Effect of different RCA reaction times on the detection results of katG315 (D), rpoB531 (E), and rpsL43 (F) resistant MTBs.

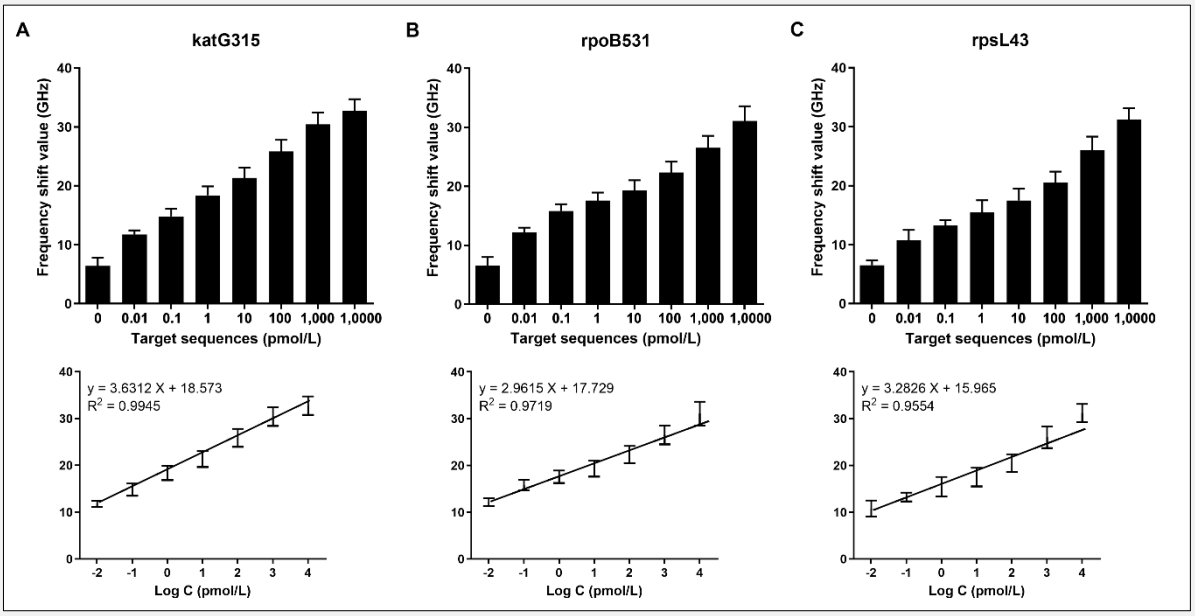

Figure 6. Sensitivity assessment of target sequence detection.

The graph below shows the relationship between the analytical signal and the logarithm of the target sequence concentration.

(rpsL43) + 15.965 (R^2 = 0.9554) (Figure 6C), respecttively. Meanwhile, the detection limit of this method was estimated to be 7.7 × 10^{-5} pmol/L(katG315), 1.03 × 10^{-5} pmol/L (rpoB531), and 1.3 × 10^{-4} pmol/L (rpsL43).

DISCUSSION

Although the rate of increase in tuberculosis incidence has slowed, it remains the single deadliest infectious disease in the world [1]. The emergence of multidrug-resistant *Mycobacterium tuberculosis* is the main cause of death in tuberculosis patients. This is mainly because ordinary tuberculosis can be cured in about half a year by taking first-line anti-tuberculosis drugs (such as isoniazid and rifampicin) in the principle of early, combined, appropriate, regular, and full-course treatment [14,15]. However, the tuberculosis bacteria in multidrug-resistant tuberculosis patients have been proven to be resistant to at least two first-line drugs (isoniazid, rifampicin, etc.) *in vitro*, resulting in failure to take medication in a timely and accurate manner. In this study, we used RCA signal amplification technology and terahertz metamaterial detection methods to build a system for rapid detection of multidrug-resistant *Mycobacterium tuberculosis*.

Firstly, nucleic acid amplification technology has been shown by many studies to be able to be combined with a variety of technologies to improve the sensitivity of detection of *Mycobacterium tuberculosis* (including drug-resistant *Mycobacterium tuberculosis*).

Researchers such as Bai et al. [16] combined the PCR method with the electrochemical biosensing technology differential pulse voltammetry to detect 3 fmol/L of *Mycobacterium tuberculosis* insertion sequences, but PCR requires a complex temperature control system and special instruments. Meanwhile, Aryan et al. used loop-mediated isothermal amplification to detect *Mycobacterium tuberculosis*, and the sensitivity was as high as 5 fg and did not require complex temperature control or special instruments, but this method required two external primers and two internal primers [17]. In contrast, the advantages of RCA technology are very obvious; isothermal amplification and only one primer is required. RCA is a process of cyclically amplifying circular DNA templates at a constant temperature to obtain long-chain nucleic acids [18,19]. The amplification template of RCA is PLP, and single gene mutations can be sensitively identified under the action of DNA ligase. In addition, RCA can amplify a large number of tandem repeat nucleic acid long chains complementary to the circular membrane plate in a short period of time under constant temperature conditions using a single primer. In this study, we found that the RCA reaction could amplify nucleic acid products for terahertz detection in just half an hour.

There are many methods that can be used to detect RCA amplified nucleic acid products, including nucleic acid electrophoresis and fluorescence detection, but their disadvantages are very obvious, such as the long detection cycle of nucleic acid electrophoresis and the low sensitivity of fluorescence detection [20,21]. In this study, we used terahertz metamaterials to detect RCA amplified nucleic acid products. Terahertz waves have low single-light molecular energy and will not ionize biological tissues, making them an ideal nondestructive testing method. In addition, the vibration and rotation energy levels of biological macromolecules are just in the terahertz band, and optical information can be extracted through the electric field vibration and phase of different biological samples to achieve differential analysis of different samples [11,12]. Terahertz metamaterial is a periodic unit structure material composed of a subwavelength microstructure array. It is widely used in the detection of bacteria [22,23], proteins [24,25], and cells [26] because of its ability to regulate electromagnetic waves and its sensitivity to changes in the refractive index of surface deposited substances. Herein, we found that the sensitivity of terahertz metamaterials to detect katG135, rpoB531, and rpsL43 drug-resistant Mycobacterium tuberculosis target sequences is 7.7×10^{-5} pmol/L, 1.03×10^{-5} pmol/L, and 1.3×10^{-4} pmol/L, respectively.

CONCLUSION

All in all, our study provides a detection solution for multidrug-resistant Mycobacterium tuberculosis based on RCA technology and terahertz metamaterials.

Declaration of Interest:

The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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