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Original Article

Enhanced detection of rifampicin and isoniazid resistance in mycobacterium tuberculosis using AuNP-qPCR: a rapid and accurate method

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Abstract: Objectives: To evaluate the resistance of Mycobacterium tuberculosis to Rifampicin (RIF) and Isoniazid (INH) using enhanced qPCR methodologies. Methods: This study compared the detection of drug-resistant mutations in the rpoB and katG genes using AuNP-qPCR and No-AuNP-qPCR. Calibration curves were constructed to correlate the amount of template with the Ct values for resistant strains. Results: The AuNP-qPCR method demonstrated high efficacy in detecting RIF resistance with an area under the curve (AUC) of 0.951, sensitivity of 97.92%, specificity of 87.5%, and overall accuracy of 95.31%. Similarly, INH resistance detection by AuNP-qPCR showed an AUC of 0.981, sensitivity of 98.08%, specificity of 94.44%, and accuracy of 97.14%. Comparatively, No-AuNP-qPCR yielded lower performance metrics for RIF resistance (AUC: 0.867, sensitivity: 91.67%, specificity: 75%, accuracy: 87.5%) and INH resistance (AUC: 0.882, sensitivity: 88.46%, specificity: 83.33%, accuracy: 87.14%). Conclusions: AuNP-qPCR exhibits over traditional qPCR methods, making it a promising tool for rapid and precise detection of drug resistance in Mycobacterium tuberculosis. This method's robust performance underscores its potential to improve diagnostic protocols and contribute to more effective management of tuberculosis treatment.

Keywords: Mycobacterium tuberculosis, rifampicin and isoniazid, gold nanoparticles, PCR, drug resistance

Introduction

Tuberculosis (TB) is a major public health threat caused by Mycobacterium tuberculosis (MTB). The World Health Organization estimates that about one-fourth of the world's population is infected with MTB, positioning it as a leading global health hazard. Treatment for TB can be lengthy, and the risk of developing drug resistance is a significant concern, reducing the effectiveness of therapeutic interventions against pathogenic bacteria [1]. Antibiotics, crucial in combating life-threatening bacterial infections, face challenges due to their excessive and inappropriate use, which has contributed to the emergence of drug-resistant strains. This resistance complicates treatments, leading to harder-to-treat infections and elevating the risks of disease spread and mortality, particularly with drug-resistant tuberculosis, now a pressing global issue [2].

Both rifampicin (RIF) and isoniazid (INH) are vital for their bactericidal properties against all stages of M. tuberculosis infection. Multidrug-resistant TB (MDR-TB), characterized by resistance to both RIF and INH, poses a severe global challenge. The lower treatment success rates of MDR-TB result in significant loss of life and escalate healthcare costs, particularly burdening developing countries [3, 4].

Bacteria can acquire antibiotic resistance through various mechanisms, including primary and secondary resistance. Primary resistance occurs when individuals are infected with strains already resistant to drugs. Conversely, secondary resistance results from inadequate drug absorption or incorrect treatment regimens in tuberculosis therapy. In MTB, resistance is often linked to genetic mutations at multiple sites within the pathogen's genome. Resistance to RIF typically involves point mutations in the rpoB gene, while INH resistance

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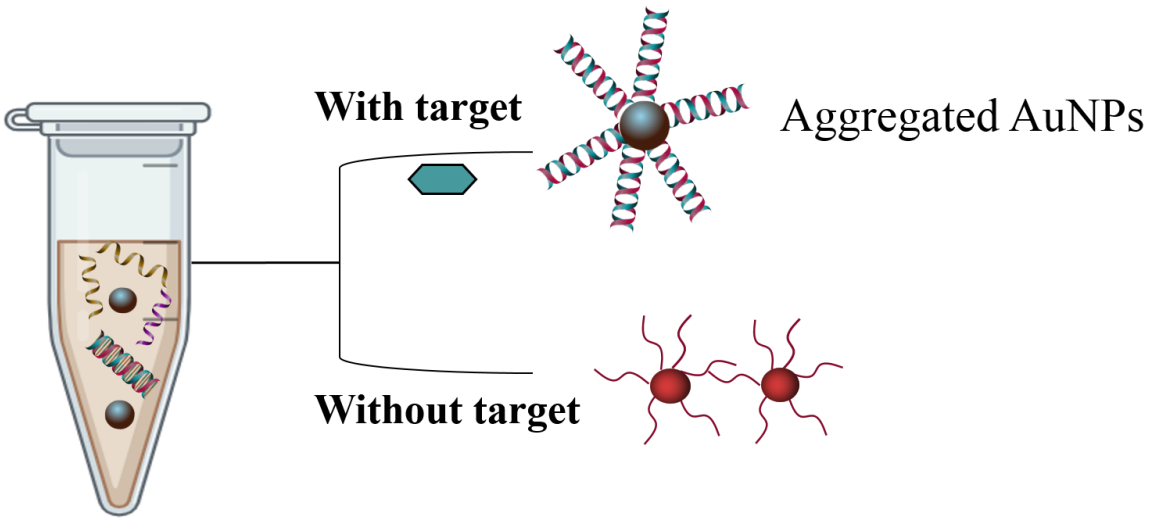

Figure 1. Schematic diagram of detection of antibiotics based on gold nanoparticles PCR.

commonly arises from mutations in the inhA promoter region and katG gene [5].

Rapid detection of drug resistance is crucial for optimizing anti-tuberculosis treatment and preventing the spread of resistant strains [6]. Molecular techniques, offering greater speed, sensitivity, and specificity, have superseded traditional culture-based methods in identifying tuberculosis and its drug-resistant forms. Real-time fluorescence quantitative PCR (qRT-PCR), widely adopted for diagnosing drug-resistant tuberculosis, integrates amplification and mutation detection via fluorescence labeling [7]. However, this method has limitations, such as potential instrument error and increased background noise beyond 35 cycles, which can lead to false positives.

Over the past two decades, advancements in nano-diagnostic technologies have significantly enhanced infectious disease diagnostics. These methods utilize labeled nanoparticles or probes that interact with target biomolecules, producing a quantifiable electrical signal characteristic of the biomolecules. This approach holds substantial promise for future diagnostic applications [8].

Gold nanoparticles (AuNPs) are distinguished by their exceptional properties, including intense light absorption, robust light scattering, unique chemical, biological, and electronic features, low or negligible toxicity, and fluorescence quenching capabilities. These characteristics render AuNPs highly suitable for clinical applications, especially in pathogen detection [9]. AuNPs can be functionalized with a variety of molecular attachments, such as proteins, drugs, antibodies, enzymes, nucleic acids (DNA or RNA), and fluorescent dyes, making them versatile optical probes and imaging agents [10].

Although the precise mechanisms are not fully understood, AuNPs have been shown to enhance the efficacy of qRT-PCR. It has been observed that AuNPs exhibit strong electrostatic interactions with single-stranded DNA (ssDNA) and weaker affinity for double-stranded DNA. At high concentrations, AuNPs bind to ssDNA, creating steric hindrance that can inhibit the reaction. Additionally, the concentration of AuNPs influences the fluorescence signal change by absorbing polymerase and modulating the amount of active polymerase to reduce inhibition [11].

AuNPs have proven effective in diagnosing drug-resistant tuberculosis. Utilizing a combination of AuNP-enhanced probe detection and loop-mediated isothermal amplification (LAMP), this approach can rapidly and accurately detect mutations in MTB associated with resistance to RIF and INH [12, 13]. In this study, we employed qRT-PCR augmented with gold nanoparticles to detect resistance to RIF and INH in MTB, investigating the impact of AuNPs on the molecular diagnosis of tuberculosis infection. A schematic diagram of the antibiotic testing process is presented in **Figure 1**.

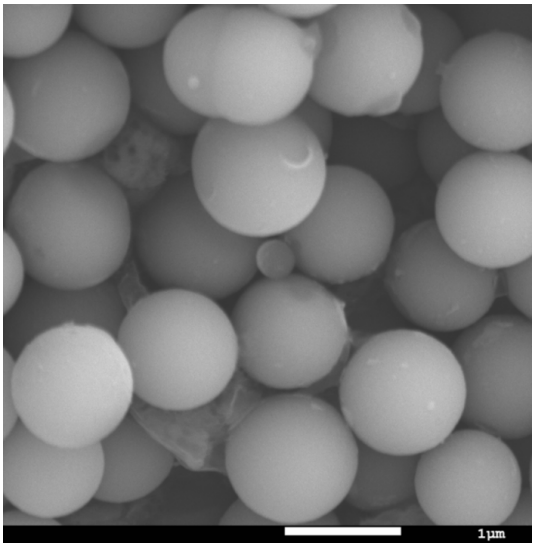

Figure 2. The electron microscope diagram of gold nanoparticles.

Table 1. Primer sequence

Gene	Primer name	Primer sequence (5'-3')
rpoB	rpoB-F	TCACACCGCAGACGTTGATC
	rpoB-R	CGTAGTGCGACGGGTGC
katG	katG-F	GGGCTTGGGCTGGAAGA
	katG-R	GGAAACTGTTGTCCCATTTCG

Materials and methods

Bacterial isolates

In this study, we collected strains from TB patients with documented drug sensitivity and sequenced rpoB and katG genes. The isolates were preserved and cultured at the National Key Laboratory for Prevention and Control of Infectious Diseases. We identified 134 MTB strains. Drug resistance to RIF and INH was assessed using the Lowenstein-Jensen (LJ) proportion method. Resistant strains were further analyzed for mutations in the rpoB and katG genes via DNA sequencing. The study received approval from the Hengyang Central Hospital Ethics Committee.

DNA extraction

Colonies of MTB grown on LJ medium (Gibco) were suspended in 100 μL of lysis buffer (50 mmol/L Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0; 100 mmol/L NaCl). The suspension was heated at 100°C for 15 minutes, centrifuged at 5000× g for 1 minute, and then the pellet was discarded. The extracted DNA was stored at -20°C for subsequent qRT-PCR analysis.

Synthesis and characterization of gold nanoparticles

AuNPs were synthesized as described in the literature [13]. All glassware was pre-rinsed with distilled water. Initially, 1 mM tetrachloroauric acid trihydrate (Sinopharm Chemical Reagent Co., Ltd.) was dissolved in 250 mL of distilled water and brought to a vigorous boil. Subsequently, 38.8 mM trisodium citrate dihydrate was added. Upon cooling to room temperature, the solution transitioned from yellow to transparent, then to black, turning purple, and finally settling into a crimson hue. The resultant AuNPs were filtered through a 0.22 μm membrane and stored at 4°C. An electron microscope diagram of the gold nanoparticles is shown in **Figure 2**.

qRT-PCR methodology

We used SYBR Green DNA dye (Beijing Baiaolaibo Technology Co., Ltd.) and a LightCycler480 qRT-PCR (Roche) to detect known mutations in the RIF resistance-determining gene rpoB and INH resistance-related gene katG in drug-resistant strains. The PCR reaction mixture, totaling 20 μL, included 2 mM of DNA template, 10 μL of 2× SYBR Green qPCR mix, 0.5 mM of each primer (upstream and downstream, details in **Table 1**), and nuclease-free water. The study was divided into two groups: the observation group, where AuNPs were added prior to the qPCR reaction, and the control group, which did not include AuNPs. The amplification protocol involved a pre-denaturation at 95°C for 1 minute, denaturation at 94°C for 20 seconds across 40 cycles, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 75°C for 2 minutes.

Statistics and analysis

Data analysis was performed using GraphPad Prism 8.2. Measurement data were presented as mean ± standard deviation, and the t-test was used for comparisons between groups. Categorical data were expressed as percentages, and the chi-square test was used for their

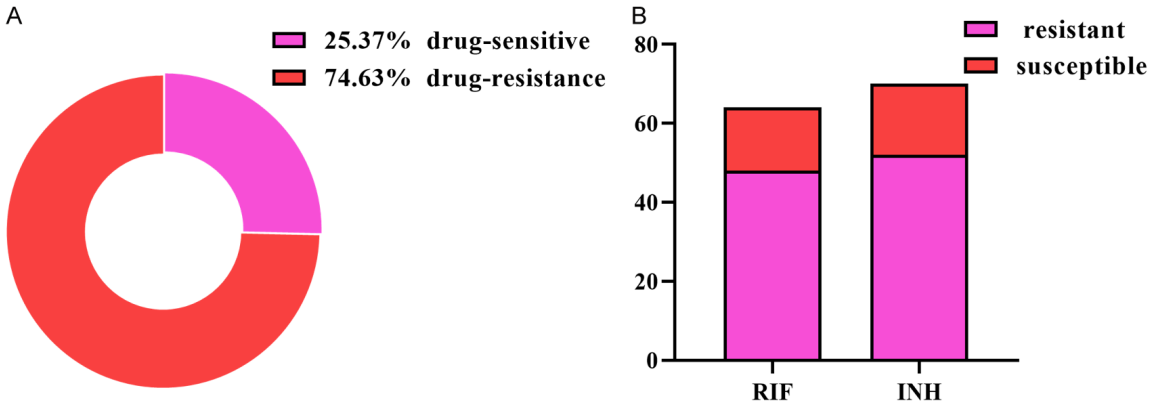

Figure 3. Characterization of drug resistance of isolated strains (A, B). Note: RIF: rifampicin; INH: isoniazid.

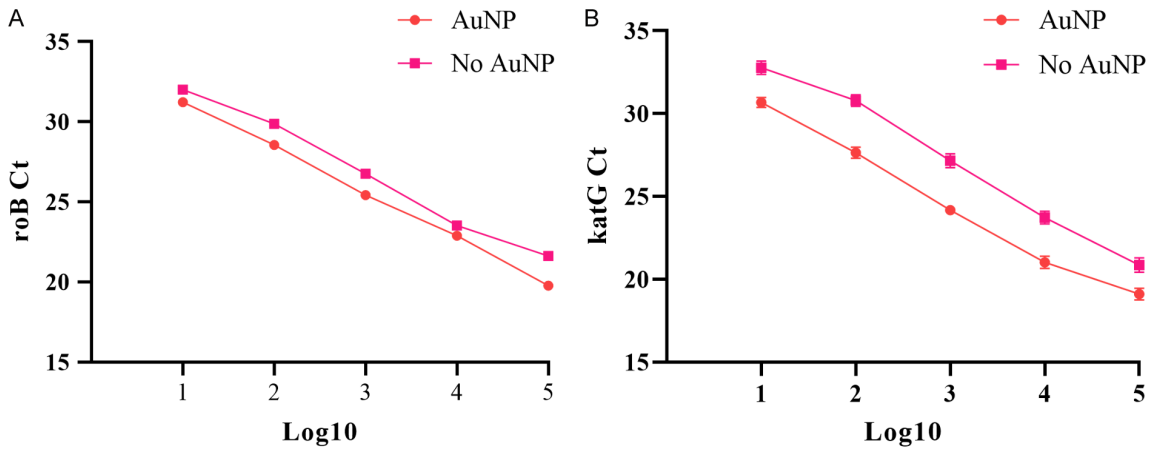

Figure 4. Comparison of Ct values of roB (A) and katG (B) genes in drug-resistant strains. Note: AuNP: gold nanoparticles.

analysis. The SPSS software was employed to generate Receiver Operating Characteristic (ROC) curves for drug resistance to various drugs, calculating the area under the curve. A *p*-value of <0.05 was considered statistically significant.

Results

Characterization of drug resistance of isolated strains

Out of 134 analyzed strains based on drug sensitivity tests, 100 (74.63%) were drug-resistant, while 34 (25.37%) were sensitive (**Figure 3A**). Specifically, of these, 48 strains (48%) were resistant to RIF, and 52 strains (52%) were resistant to INH. Conversely, 16 strains (47.06%) were sensitive to RIF and 18 strains (52.94%) were sensitive to INH (**Figure 3B**).

Detection of resistance genes roB and katG in MTB

The expression of drug-resistant mutant genes rpoB and katG was quantified using AuNP-qPCR and No-AuNP-qPCR, and a calibration curve was constructed. There was a linear relationship between the Ct value and the copy number of drug-resistant strains across a 10-fold dilution gradient (10 to 10^5 copies). The linear regression coefficients for the rpoB gene in the AuNP and No-AuNP groups were R2B0.999 and R2N0.998, respectively (**Figure 4A**), while for the katG gene, they were R2H0.996 and R200.997, respectively (**Figure 4B**). The Ct values detected by AuNP-qPCR were consistently lower than those by No-AuNP. However, t-test analysis revealed no significant difference between the data sets of the two groups (P<0.05).

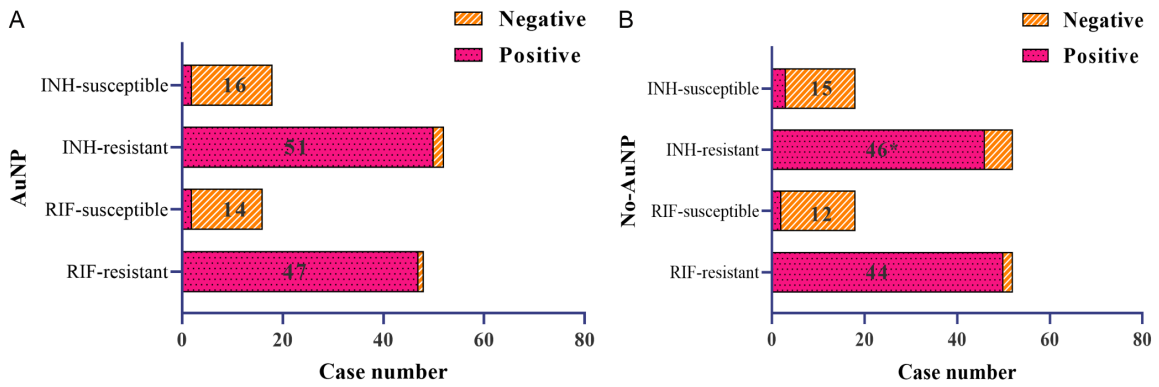

Figure 5. Effect of AuNP-qPCR and No-AuNP-qPCR on detecting Mycobacterium tuberculosis isolates. A. AuNP-PCR method; B. No-AuNP-qPCR method. Note: RIF: rifampicin; INH: isoniazid; AuNP: gold nanoparticles.

Detection of RIF/INH-resistant strains by AuNP-qPCR and No-AuNP-qPCR

According to the LJ proportion method, both AuNP-qPCR and No-AuNP-qPCR accurately identified cases of RIF and INH resistance, with one false negative each. AuNP-qPCR detected 14 cases of rpoB gene mutation (with 2 false positives) and 16 cases of katG gene mutation (with 2 false positives) in sensitive strains. The positive detection rate determined by AuNP-qPCR was not significantly different from the gold standard (**Figure 5A**). No-AuNP-qPCR identified 44 RIF-resistant strains (with 4 false negatives), 46 INH-resistant strains (with 6 false negatives), 12 strains without rpoB gene mutation (with 4 false positives), and 15 strains without katG gene mutation (with 3 false positives). The positive rate for INH resistance detected by No-AuNP-qPCR was significantly different from the gold standard ($P<0.05$) (**Figure 5B**).

Sensitivity and specificity of AuNP-qPCR and No-AuNP-qPCR for detecting drug resistance in RIF/INH

The ROC curve analysis was employed to evaluate the effectiveness of AuNP-qPCR and No-AuNP-qPCR in detecting RIF/INH drug resistance. For RIF resistance, AuNP-qPCR demonstrated an AUC of 0.951, sensitivity of 97.92%, specificity of 87.5%, and accuracy of 95.31%. In contrast, No-AuNP-qPCR showed an AUC of 0.867, sensitivity of 91.67%, specificity of 75%, and accuracy of 87.5% (**Figure 6A**). For INH resistance, AuNP-qPCR yielded an AUC of 0.981, with a sensitivity of 98.08%, specificity of 94.44%, and accuracy of 97.14%. The values for No-AuNP-qPCR were AUC of 0.882, sensitivity of 88.46%, specificity of 83.33%, and accuracy of 87.14% (**Figure 6B**). The comparative analysis revealed higher sensitivity, specificity, and accuracy in AuNP-qPCR.

Discussion

MTB, the causative agent of tuberculosis, has been treated with antibiotics for over 50 years. Despite this, various factors such as inadequate medical infrastructure, inappropriate treatment prescriptions, and poor patient compliance have led to the evolution of drug-resistant strains. These strains have progressively developed resistance to existing anti-tuberculosis drugs, complicating clinical management and the treatment process, and thereby imposing a serious burden [14]. Consequently, rapid, accurate, and sensitive detection methods for drug resistance in MTB are essential to improve treatment success rates and curb the disease's spread.

It is well-established that drug resistance in MTB typically results from mutations at multiple genomic sites [15]. Clinical studies have demonstrated that detecting mutations in specific genes associated with anti-tuberculosis drug resistance is more efficient and effective. For instance, RIF resistance is often due to point mutations in a specific region of the rpoB, whereas INH resistance is linked to mutations in the katG and inhA genes. Notably, the katG gene encodes a catalase-peroxidase that activates the prodrug INH into its reactive form [16].

qRT-PCR is a rapid and straightforward method used to identify gene mutations associated

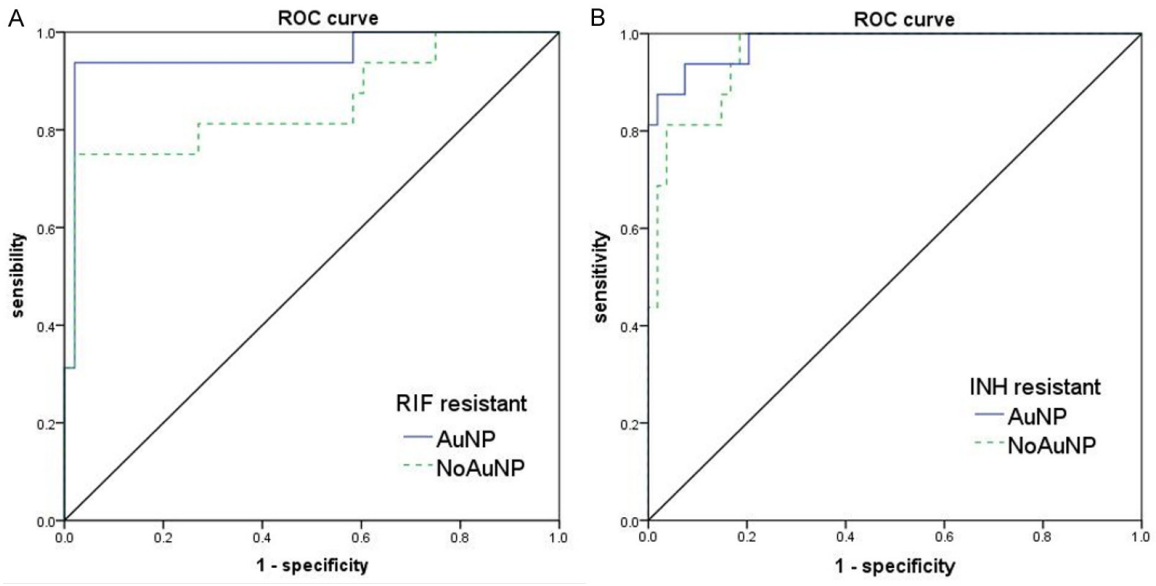

Figure 6. AuNP-qPCR and No-AuNP-qPCR in detecting the sensitivity and specificity of RIF/INH resistance. A. ROC curve of RIF resistance; B. ROC curve of INH resistance. Note: RIF: rifampicin; INH: isoniazid; AuNP: gold nanoparticles.

with antibiotic resistance in MTB. It offers a high degree of automation and the capability to simultaneously detect multiple drug resistance gene mutations. Nonetheless, further enhancements are needed to improve its sensitivity and selectivity. In recent years, significant advancements have been made in DNA detection technologies, particularly those involving nanoparticles. AuNPs, due to their unique optical and magnetic properties, have become prominent in the field of DNA detection. AuNPs are known for their high sensitivity and accuracy, water solubility, and straightforward synthesis. Depending on the proximity to other fluorescent substances, AuNPs can cause two distinct effects: fluorescence enhancement and fluorescence quenching [17, 18].

In this study, AuNPs were incorporated into the qPCR reaction system to optimize the detection of the most prevalent mutations associated with RIF and INH resistance in MTB. Our results demonstrated that as few as five copies of MTB genes (rpoB and katG) could be detected using qRT-PCR. There was a clear linear relationship between the copy number and Ct value, with higher expression levels observed in the AuNP group, likely due to the enhanced qPCR amplification efficiency provided by AuNPs. Previous research has consistently shown that AuNPs are frequently used as biosensors and have been optimized for PCR applications [19]. They improve the binding to single-stranded DNA and increase the thermal conductivity of conventional PCR. By including AuNPs in the PCR mix, non-specific amplification at lower annealing temperatures can be minimized, thereby increasing the assay's sensitivity and extending the PCR elongation phase [20].

The application of AuNPs has emerged as a crucial analytical tool in pathogen diagnosis. Integrating PCR with AuNPs enhances the detection of pathogen drug resistance with high sensitivity. Due to its low cost and ease of use, this combination not only quickly identifies mutations but also meets the requirements for high sensitivity and early detection in a shorter timeframe, significantly improving the specificity of PCR assays [21]. A novel, rapid, sensitive, and simple colorimetric nano-diagnostic platform using ribonuclease-gold nanoparticles has been developed [22], capable of identifying pathogenic organisms and related antibiotic resistance genes within 2 hours, greatly enhancing sensitivity compared to traditional qRT-PCR.

Previous studies have developed AuNP biosensors for analyzing drug resistance in MTB [23]. These biosensors, combining AuNP probes with LAMP, effectively detect multidrug-resistant

tuberculosis strains and characterize mutations associated with isoniazid resistance [12, 13]. Another study utilized allele-specific PCR and AuNP biosensors to achieve 100% sensitivity and specificity in detecting mutations related to INH resistance [24-26]. Compared to conventional qRT-PCR, our findings demonstrate that AuNP-qPCR-based detection of RIF and INH resistance-related mutations offers superior sensitivity, specificity, and accuracy, thus providing a reliable prediction of whether the tested strain is MDR-TB.

One limitation of this study is the small sample size, focusing primarily on the most significant mutations in genes related to RIF and INH resistance. Further research is needed to optimize the verification method and determine the best approach for direct application to clinical samples, which should include larger sample sizes and a broader gene pool.

In conclusion, the development of cost-effective and reliable methods for diagnosing drug-resistant tuberculosis remains a major goal. qRT-PCR is an ideal technique for detecting slow-growing drug-resistant bacteria and holds significant potential for clinical implementation. The incorporation of gold nanoparticles enhances the performance of qPCR, leading to improved sensitivity, specificity, and accuracy, making the method more dependable for diagnosing drug resistance in TB.

Disclosure of conflict of interest

None.

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