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Original Research Article

Development of a novel detection technology for drug resistance mutation sites of *Mycobacterium tuberculosis* using Luminex liquid chip technology

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Abstract

Purpose: *To develop a novel detection technology for drug-resistance mutation sites of Mycobacterium tuberculosis (MTB) using a Luminex liquid chip.*
Methods: *Using polymerase chain reaction (PCR) amplification and hybridization analysis, MTB infection and drug-resistant mutation sites of the first-line and second-line anti-MTB drugs were simultaneously identified. A novel detection method was applied to analyze the wild-type standard strains of MTB and 33 clinical samples, and the results were compared with Sanger sequencing results for PCR products.*
Results: *It was revealed that the sensitivity (100 %) and specificity (100 %) of the novel detection method for 31 samples were satisfactory, and all mutation sites were correctly detected. Compared with traditional PCR and culture-based drug sensitivity test, the novel detection method increased the speed of identification of drug-resistant TB, reduced clinicians' workload, and decreased treatment cost. Among 31 samples, 12.90 % were resistant to isoniazid (4/31), 35.48 % to rifampicin (11/31), and 12.90 % to ofloxacin ($p < 0.05$). Furthermore, 2 (6.45 %) samples were resistant to both isoniazid and rifampicin, 2 (6.45 %) samples to both rifampicin and ofloxacin, and 1 (3.22 %) sample to both isoniazid and ofloxacin, and 1 (3.22%) sample to all the three drugs ($p < 0.05$).*
Conclusion: *Development and wide application of this novel detection method will facilitate the treatment of MTB, thus reducing the spread of drug-resistant MTB, and improving the prevention and treatment of MTB.*

Keywords: *Luminex liquid chip technology, Mycobacterium tuberculosis, Drug-resistant mutation, Polymerase chain reaction*

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INTRODUCTION

Tuberculosis is one of the most common infectious diseases seriously threatening human health, and the emergence of drug resistance is a major challenge in its treatment. About 2 billion people worldwide are infected with *Mycobacterium tuberculosis* (MTB), and up to 1.3

million people die of the disease each year [1]. Rifampicin, isoniazid, streptomycin, and other anti-tuberculosis drugs have been widely prescribed since the 1940s, and the incidence and mortality rates of MTB dropped substantially. However, over the past decade, the widespread and inappropriate use of antibiotics led to the emergence of multidrug-resistant tuberculosis (MDR-TB). Moreover, extensively drug-resistant tuberculosis (XDR-TB) strains have induced the resurgence of tuberculosis, posing a serious challenge to the prevention and treatment of tuberculosis [2,3]. No new effective anti-tuberculosis drugs have been developed for XDR-TB. In China, the emergence and spread of drug-resistant MTB strains have always been a major challenge in the treatment of tuberculosis, which in turn has also put a heavy burden on public health care and poses a great threat to people's health [4].

Rapid culture and drug sensitivity determination of MTB using BACTEC 460TB and other systems have been developed in recent years. Liquid chip technology is promising and practical, and the core of this technology involves the application of tiny latex particles to fluorescent color development, and nucleic acid for detection is coupled. After the hybridization of microspheres with target molecules in suspension, fluorescence is stimulated by laser. The results can be interpreted directly after reading the fluorescence values with an appropriate detection equipment (Luminex). Liquid chip technology has been widely used in clinical practice, health examination and scientific research and development.

The present study seeks to develop a novel detection technology for drug-resistance mutation sites of MTB using a Luminex liquid chip.

METHODS

Ethical approval and collection of tuberculosis samples

The study was approved by the Ethics Committee of Hainan Medical University, Haikou, China (approval no. LW2020265). From January 2020 to May 2022, 33 sputum samples from patients with smear-positive tuberculosis who were admitted to the Department of Tuberculosis of the Second Affiliated Hospital of Hainan Medical University were collected. Reference strain RV37 (ATCC 27294) of MTB without drug-resistant mutations was used as the wild-type control in this study, and two non-MTB strains, *Mycobacterium abscessus* and *Mycobacterium coli*, were selected as negative controls for the identification of MTB. MTB identification, amplification and sequencing of drug-resistant genes were carried out at the Second Affiliated Hospital of Hainan Medical University and Hainan Medical University-The University of Hong Kong Joint Laboratory of Tropical Infectious Diseases. Informed consent forms were signed by all patients before enrollment.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of 16SrDNA and drug-resistant genes of MTB

MTB DNA was extracted using Roche Cobas Amplicor extraction kit (Roche Diagnostics, Grenzach-Wyhlen, Germany) according to the manufacturer's instructions. The PCR amplification and sequencing methods of 16SrDNA of MTB and genes related to isoniazid, rifampicin, and ofloxacin resistance were the same as those described previously [8].

Multiple amplification and instrumental analysis

The Luminex 200 platform (Luminex Inc., Austin, TX, USA) was used in this study. To identify MTB and to detect isoniazid-, rifampicin-, and ofloxacin-resistant gene mutations, a series of 16SrRNA and rpoB, katG, inhA, and gyrA gene fragments of MTB were designed and synthesized. Biotin was labeled at the 5 'end of each pair of primers. The PCR amplification of 16SrRNA, rpoB, katG, inhA, and gyrA gene fragments of each sample was performed in a total reaction volume of 50 µL, including 2 µL template DNA, forward and reverse primers of each gene (0.2 µM), 25 µL PCR master mix (TIANGEN Biotech Co. Ltd, Beijing, China), and the remaining volume was filled with double distilled water. The reaction conditions of PCR were summarized as follows: denaturation at 95 °C for 5 min, 30 cycles of amplification, denaturation for 30 s at 95 °C, renaturation for 30 s at 55 °C, extension for 30 s at 72 °C; and at 72 °C for 5 min, followed by cooling to 4 °C. To facilitate the detection of the PCR-amplified product, one end of the forward and reverse primers was conjugated with biotin.

Hybridization analysis

The hybridization analysis was performed according to a previously reported method. 33 µL 1.5× tetramethylammonium chloride (TMAC) hybrid buffer was prepared, which contained 5,000 microspheres of each type, and 12 µL TRIS-EDTA buffer (pH 8.0), and 5 µL PCR products were added to the buffer.

Table 1: Demographic profiles of patients from whom clinical samples were obtained

Variable	MTB group (N = 31)	Non-MTB group (N = 2)
Age (years)	42 ± 7	43 ± 7
Gender		
Male	17	1
Female	14	1
Newly treated tuberculosis	12	1
Re-treated tuberculosis	19	1

The negative control products of the PCR reaction were added to the blank control. The mixed solution was denaturated at 95 °C for 5 min, and then, hybridized at 54 °C for 10 min. An aliquot (10 μL) streptavidin-alginin (50 μg/mL) was added and incubated at 54 °C for 10 min. Luminex 200 was then used to determine the results of the hybridization analysis. A mean fluorescence value > 1.5 times that of a negative result was considered positive.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (IBM, Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation, and categorical variables were presented as the frequency. Chi-square test (univariate analysis) or Fisher's exact test was used to compare the differences between the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic profiles of clinical samples

In this study, 33 sputum samples from sputum smear-positive tuberculosis patients who were admitted to the Department of Tuberculosis of the Second Affiliated Hospital of Hainan Medical University from January 2020 to May 2022 were collected. Demographic profiles of clinical samples are listed in Table 1.

Identified MTB

One MTB strain (as reference), 33 clinical MTB sputum smear-positive samples, and 2 non-MTB strains were identified. These 32 samples (32/32, 100 %) were all positive, and non-MTB samples (2/2, 100 %) were negative.

Identified MTB and drug-resistant mutations

The novel liquid chip detection method identified the 16SrRNA gene of MTB and detected 17 drug-resistant mutations at 8 sites, including mutation of rifampicin-resistant rpoB gene at 516GAC→516GGC or 516GTC, 526CAC→526TAC or 526GAC, 531TCG→531TTG and 533CTG→533CCG, mutations of isoniazid-resistant KatG gene at 315AGC→315ACC, isoniazid-resistant inhA gene at 15C→15T and 8T→8A, and mutations of ofloxacin-resistant GyrA gene at 90GCG→90GTG, 91TCG→91CCG and 94GAC→94GGC, 94GCC, 94CAC, 94TAC or 94AAC. The mean fluorescence value of all positive samples was 1.5 times greater than that of the negative control. Among these samples, the largest fluorescence value was found in the mutation of drug-resistant rpoB gene at 526CAC→526TAC (7.8), and the smallest fluorescence value in mutation of drug-resistant rpoB gene at 516GAC→516GGC (rpoB) (2.4). The results showed that the novel detection method accurately detected rpoB, katG, inhA, and gyrA gene mutation sites. The sequences of primers used for PCR are listed in Table 2. The test results of reference strain RV37 and one strain resistant to isoniazid and rifampicin are shown in Figure 1 A and B.

Table 2: Sequences of primers used for PCR

Gene	Sequence
16SrRNA-F	TTAGATACCCTGGTAGTCCAC
16SrRNA-R	ACGACACGAGCTGACGACAG
rpoB-F	GGCGAGCTGATCCAAAACCAGA
rpoB-R	CGACAGCGAGCCGATCAGAC
katG-F	GTCGGCGGTCACACTTTCGGTA
katG-R	AACGGGTCCGGGATGGTGCC
inhA-F	AAGGGATCCGTCATGGTCGAAGT
inhA-R	GTTGGACACCAGCACCTCGAC
gyrA-F	AGGAGATGCAGCGCAGCTACA
gyrA-R	CATTGCCTGGCGAGCCGAAGT

Microbial resistance

In this study, 31 clinical MTB samples were detected, of which 12 (38.71 %) samples were identified with drug-resistant gene mutations by the novel detection method. Out of 31 samples, 12.90 % were resistant to isoniazid (4/31), 35.48 % to rifampicin (11/31), and 12.90 % to ofloxacin ($p < 0.05$). Furthermore, 2 (6.45 %) samples were resistant to both isoniazid and rifampicin, 2 (6.45 %) samples to both rifampicin and ofloxacin, 1 (3.22 %) sample to both isoniazid and ofloxacin, and 1 (3.22%) sample to all the three drugs ($P < 0.05$). All mutation detection results were fully consistent with PCR sequencing results, indicating that the accuracy of drug-resistant mutation sites achieved by the novel detection method was 100 %.

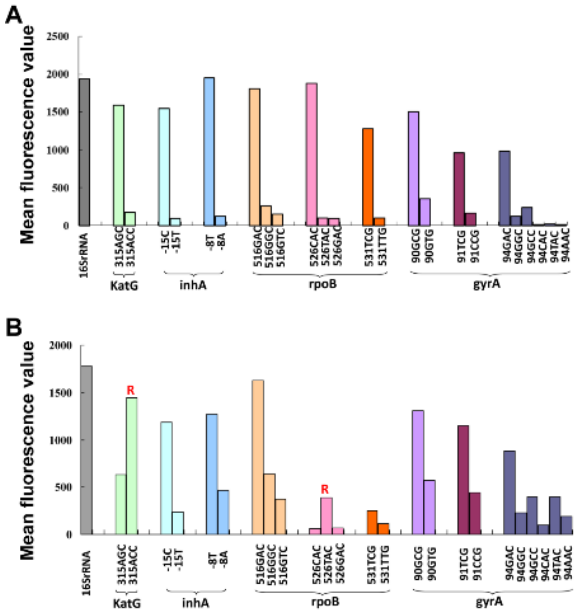

Figure 1: The test results of reference strain RV37 and one strain resistant to isoniazid and rifampicin. (A) Detection results of tuberculosis reference strain (H37Rv sensitive strain); (B) Detection results of isoniazid and rifampicin-resistant samples (R: drug resistance)

DISCUSSION

In this study, a novel detection method was proposed based on the Luminex xMAP liquid chip for the simultaneous identification of MTB and the determination of resistant mutation sites for the first-line drugs (isoniazid and rifampicin) and second-line drugs (ofloxacin). Luminex xMAP liquid chip is a liquid detection method based on multiple fluorescent microsphere technology. Up to 500 types of target genes can be simultaneously identified by the novel detection method, making it highly appropriate for the development of clinical assays that require multiple and high-throughput examinations simultaneously, including SNP genotyping, screening for genetic diseases, and human leukocyte antigen typing.

In recent years, several techniques have been broadly used in clinical microbiology laboratories for the detection of bacterial infections, including multiple detections for respiratory pathogens [9], and typing of human papillomavirus [10]. In addition to the detection of pathogens, the novel detection method has also been used to identify antibodies and infection-related cytokine responses in serum [11].

In recent years, there have been several reports on the detection of MTB and its drug-resistant mutation sites based on liquid chip technology. Compared with these reports, the detection method proposed in this study is unique in the following points: most of the reported tests can only detect first-line drugs, including isoniazid, rifampicin/or ethambutol, and some cannot simultaneously identify MTB [12-14]. Only one reported test simultaneously identified MTB and first-line and second-line drug-resistant mutation sites [14], while it identified fewer mutation sites than the novel detection method. More importantly, none of these methods has been used to evaluate MTB strains in China, which is one of the countries with the most severe drug resistance. The novel detection method simultaneously identified MTB and the resistance mutation sites of the first-line and second-line anti-tuberculosis drugs, and its widespread utilization for detecting MTB strains in China is highly suggested.

This study modified the previous liquid-based detection method of MTB and its drug-resistant mutation sites [7]. Based on the original method, two rounds of PCR amplification were reduced to one round of PCR amplification, which effectively increased the efficiency, shortened analysis time, and avoided PCR-related pollution. On the basis of the original method, the identification of rifampicin-resistant rpoB gene mutation (533CTG→533CCG) was introduced in this study, and the detection of mutation sites was more comprehensive.

The Xpert MTB/RIF assay is recommended by the World Health Organization (WHO) for identification of MTB and drug-resistant mutation sites. Compared with Xpert MTB/RIF, 4 h is required for detection using the novel detection method, which is longer than 2 h, and the number of detected drug-resistant mutation sites was significantly higher than that of Xpert MTB/RIF. Xpert MTB/RIF only identifies MTB and rifampicin resistance, while the novel method simultaneously identifies MTB and resistance of rifampicin, isoniazid, and ofloxacin. The detection results are available within one day. In addition, after determination of 31 clinical sputum smear-positive samples, including 1 reference strain and 2 non-MTB strains, the accuracy of the novel detection method was 100% for identification of MTB and detection of drug-resistant mutation sites in samples.

Compared with the traditional culture-based methods that identify MTB and its drug resistance, the limitation of the method based on liquid chip technology is that it only identifies the drug-resistant mutation sites covered by the method. However, uncovered drug-resistant mutation sites cannot be detected. Although the most common drug-resistant mutation sites have

been covered in this study, the novel detection method still does not completely cover all drug-resistant mutation sites, which is a typical shortcoming of Xpert MTB/RIF technology.

CONCLUSION

An accurate and sensitive method for identification of MTB and detection of gene mutations related to the resistance of isoniazid, rifampicin, and ofloxacin using Luminex® liquid chip. Widespread application of this method should optimize clinical treatment regimens for tuberculosis management, and prevent and control drug-resistant tuberculosis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All the authors actively participated in this study, and they read and approved the final manuscript.
Changjiang Liu, Mingliang Tang and Liyuan Zhang contributed equally to this work.

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