ORIGINAL RESEARCH

Using Microfluidic Chip and Allele-Specific PCR to Rapidly Identify Drug Resistance-Associated Mutations of *Mycobacterium tuberculosis*

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Background: The currently used conventional susceptibility testing for drug-resistant *Mycobacterium tuberculosis (M.TB)* is limited due to being time-consuming and having low efficiency. Herein, we propose the use of a microfluidic-based method to rapidly detect drug-resistant gene mutations using Kompetitive Allele-Specific PCR (KASP).

Methods: A total of 300 clinical samples were collected, and DNA extraction was performed using the "isoChip®" Mycobacterium detection kit. Phenotypic susceptibility testing and Sanger sequencing were performed to sequence the PCR products. Allele-specific primers targeting 37 gene mutation sites were designed, and a microfluidic chip (KASP) was constructed using 112 reaction chambers to simultaneously detect multiple mutations. Chip validation was performed using clinical samples.

Results: Phenotypic susceptibility of clinical isolates revealed 38 rifampicin (RIF)-resistant, 64 isoniazid (INH)-resistant, 48 streptomycin (SM)-resistant and 23 ethambutol (EMB)-resistant strains, as well as 33 multi-drug-resistant TB (MDR-TB) strains and 20 strains fully resistant to all four drugs. Optimization of the chip-based detection system for drug resistance detection showed satisfactory specificity and maximum fluorescence at a DNA concentration of 1×10^1 copies/μL. Further analysis revealed that 76.32% of the RIF-resistant strains harbored *rpoB* gene mutations (sensitivity, 76.32%; specificity 100%), 60.93% of the INH-resistant strains had *katG* gene mutations (sensitivity, 60.93%; specificity, 100%), 66.66% of the SM-resistant strains carried drug resistance gene mutations (sensitivity, 66.66%; specificity, 99.2%), and 69.56% of the EMB-resistant strains had *embB* gene mutations (sensitivity, 69.56%; specificity, 100%). Further, the overall agreement between the microfluidic chip and Sanger sequencing was satisfactory, with a turnaround time of the microfluidic chip was approximately 2 hours, much shorter than the conventional DST method.

Conclusion: The proposed microfluidic-based KASP assay provides a cost-effective and convenient method for detecting mutations associated with drug resistance in *M. tuberculosis*. It represents a promising alternative to the traditional DST method, with satisfactory sensitivity and specificity and a much shorter turnaround time.

Keywords: *Mycobacterium tuberculosis*, multidrug-resistance, drug resistance, resistance-conferring gene mutations, polymerase chain reaction, microfluidic analytical techniques, Sanger sequencing

Introduction

Tuberculosis (TB) is one of the most common causes of death and the leading cause of a single infectious agent.[1] According to the 2022 Global TB Report of the World Health Organization (WHO), 10.6 million people were infected with TB in 2021, and although the annual number of deaths from TB decreased between 2005 and 2019 worldwide, this trend was reversed in 2020 and 2021.[2] The burden of TB varies significantly among countries, with low-income countries experiencing the highest prevalence.[3] In some countries, including India, China, the Philippines and South Africa, the incidence of TB is even higher, reaching up to 500/100,000.[4–7] Although TB is a treatable and curable disease, the emergence of drug resistance has become a major challenge

Graphical Abstract
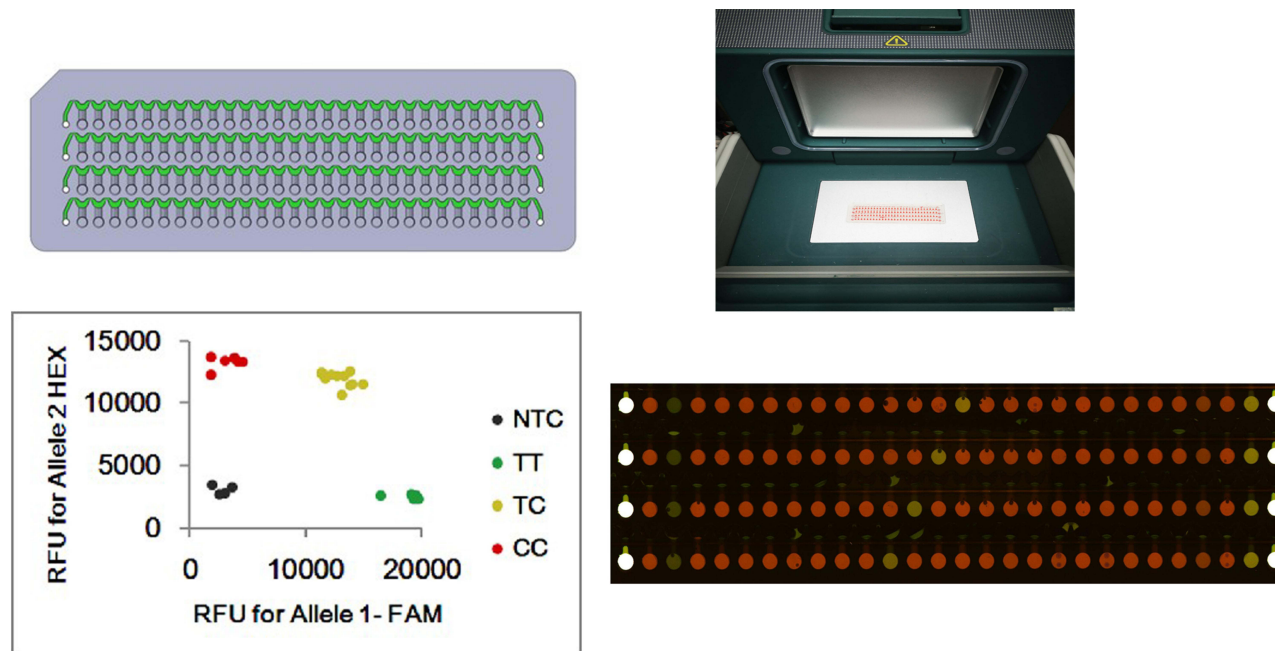
in its management. Additionally, mismanagement of patients often leads to multidrug-resistant TB (MDR-TB), posing a considerable financial burden on patients and increasing mortality rates. For instance, in 2017 alone, there were 560,000 new cases of TB resistant to rifampicin (RR)-TB, among whom 82% were classified as MDR-TB and among these MDR-TB patients, approximately 8.5% were diagnosed with extensively drug-resistant TB (XDR-TB). Overall, the presence of MDR-TB is particularly concerning as it signifies resistance to the most effective drugs for treating TB. As a result, the remaining treatment options are less effective, have more side effects, are costlier, and are generally associated with higher mortality risks. Despite an annual decline in the incidence of TB by 2% since 2005, drug-resistant TB remains a significant obstacle to effectively managing and eradicating the disease.[1,2]

Early and accurate identification of drug-resistant *Mycobacterium TB (M.TB)* is of great significance for the treatment and prevention of the spread of multidrug-resistant disease.[8,9] However, current diagnostic methods based on bacterial culture are time-consuming and have low efficiency,[10,11] thereby hindering the proper management of TB. Traditional culture-based tests for TB diagnosis typically require a lengthy period of 3 to 6 weeks to deliver results for sensitive cases and even longer for MDR and XDR-TB cases. During this waiting period, clinicians have to rely solely on the patient's clinical symptoms, imaging assessments, and other available information to develop a treatment plan, which carries the risk of uncertain outcomes. Furthermore, ineffective therapy is considered the driving force for the emergence and spread of MDR-TB and XDR-TB, with the lack of highly accurate and affordable diagnostic techniques remaining one of the causes of the high incidence and prevalence of drug-resistant TB in economically underdeveloped regions.

It has been demonstrated that polymerase chain reaction (PCR)-based methods, similar to traditional culture-based tests, can accurately detect drug resistance with high specificity.[12,13] However, the risk of carry-over contamination of samples and the ability to detect multiple mutations has not been fully addressed in the present literature. One of the primary concerns in TB diagnosis is the high infectiousness of the disease and the risk of cross-contamination of PCR test amplicons. To address this issue, the GeneXpert MTB/RIF system from Cepheid has been proposed as it can detect TB in a microfluidic cassette, following a sample-in and answer-out approach.[14,15] It significantly reduces manual handling, and the PCR product is isolated within the reaction chamber of the cassette. Consequently, the GeneXpert system has gained wide usage in TB diagnosis due to its ability to minimize contamination risks.

Various approaches have been explored to simultaneously detect common mutations in *M.TB*, such as microarray,[16] qualitative DNA strips[17] and microfluidic chips.[18] While these assays offer rapid detection and the ability to identify a wide range of mutation sites, they are prone to false diagnoses due to issues with multiplex PCR and the open testing environment. Thus, sealing PCR products within an enclosed microfluidic chip has been proposed as a feasible solution to mitigate the risk of contamination.

In this study, we aimed to develop a microfluidic method to rapidly detect gene mutations associated with drug resistance in *M.TB* with high sensitivity and high specificity while also prioritizing cost-effectiveness and convenience for practical implementation.

Methods

Samples Collection and Preparation

A total of 300 clinical samples comprising 140 sputum samples, 125 alveolar lavage fluid samples, two urine samples, 21 pleural fluid samples and 12 samples of other types of specimens, were obtained from TB patients treated at the Shenzhen Third People's Hospital between February 2017 to January 2018. The clinical specimens were either used for DNA extraction and drug susceptibility testing (DST), as previously reported.[19] DNA extraction from the clinical samples was performed using the "isoChip®" Mycobacterium detection kit (CapitalBio Technology Company, Beijing). After extraction, the concentration of genomic DNA was measured using Nanodrop 1000, and the DNA samples were stored at −20°C. All samples were de-identified, and all participants provided written informed consent. This study was approved by the Institutional Review Board of the Shenzhen Third People's Hospital (ID: 20180022).

Phenotypic Susceptibility Test

All clinical samples were decontaminated using the NALC-NaOH method with a final NaOH concentration of 1%. After centrifugation at 10,000 rpm for 20 minutes, the precipitate was re-suspended in 1.5 mL of 0.5 M phosphate buffer (pH 6.8) and inoculated on the BACTEC MGIT 960 mycobacteria culture identification system (Becton Dickinson, USA) following the manufacturer's instructions. The WHO recommended ratio method was used to perform the drug sensitivity test for rifampicin (RIF), isoniazid (INH), streptomycin (SM), and ethambutol (EMB), with a critical concentration of 40 mg/L (RIF), 0.2 mg/L (INH), 4 mg/L (SM), and 2 mg/L (EMB), following which the results were categorized as resistant (R) or sensitive (S).[20]

Sanger Sequencing

The following loci were extended by PCR for 300 TB clinical isolates, namely *rpoB* (RIF), *katG* (INH), *embB* (EMB), and *rpsL* and *rrs* (SM), using the primers listed in Table 1. Sanger sequencing was performed to sequence the PCR products.

Table 1 Primer Sequences for Target Gene Sequencing

Target Gene	Primer Sequence (5′ to 3′)	Product Size (bp)
rpoB-F *rpoB*-R	CAGACGTTGATCAACATCCG GTTGATCGAAACGCCGTA	305
katG-F *katG*-R	ACGTTTCGGCGCATGGCCATG CGCCGCCGCCCATGCGGTCGA	690
rrs-F *rrs*-R	CATGCAAGTCGAACGGAAAGGT CAGCGTCAGTTACTGCCCAGAG	693
rpsL-F *rpsL*-R	GTCAAGACCGCGGCTCTGAA TTCTTGACACCCTGCGTATC	272
embB-F *embB*-R	AACTTCGTCGGGCTCAAG TAACGCAGGTTCTCGGTATA	1097

Abbreviations: bp, base pair; F, forward; R, reverse.

Allele-Specific Primers

Thirty-seven pairs of primers were designed based on the TB resistance-related database and the published literature on the high-frequency resistance mutation sites of *rpoB, katG, embB, rrs* and *rpsL* genes.[18] Each primer pair contained two allele-specific upstream primers and one shared downstream primer. These primers targeted mutation codons of the most common resistance-related genes and are considered to cover approximately 90% of *rpoB* mutations in RIF-resistant strains, 87% of *katG* mutations in INH-resistant strains, 68% of *embB* mutations in EMB-resistant strains, and 70% of *rpsL* and *rrs* mutations in SM-resistant strains. A pair of specific primers targeting the 16S rRNA was designed as the positive control for the PCR reaction. All primers and the master mix of Kompetitive Allele-Specific PCR (KASP) were provided by LGC Corp. (Shanghai, China). The mutation sites are listed in Table 2, and the sequences of primer pairs are shown in Table S1.

Table 2 Resistant Gene Loci and Coverage

Drug	Drug-Resistant Gene	Coverage Rate (%)	Mutation Site	Codon Change	Amino Acid Change
Rifampicin	*rpoB*	84	T511C	CTG-CCG	Leu-Pro
			A513T	CAA-CTA	Gln-Leu
			A513C	CAA-CCA	Gln-Pro
			G516T	GAC-GTC	Asp-Val
			A526G	CAC-CGC	His-Arg
			C526T	CAC-TAC	His-Tyr
			A526T	CAC-CTC	His-Leu
			C531T	TCG-TTG	Ser-Leu
			C531G	TCG-TGG	Ser-Trp
			C526G	CAC-GAC	His-Asp
			T533C	CTG-CCG	Leu-Pro
Isoniazid	*katG*	68	G315C	AGC-ACC	Ser-Thr
			C315G	AGC-AGG	Ser-Arg
			G315T	AGC-ATC	Ser-Ile
			A315G	AGC-GGC	Ser-Gly
			A315C	AGC-CGC	Ser-Arg
			G315A	AGC-AAC	Ser-Asn
			T328C	TGG-CGG	Trp-Arg
			G328T	TGG-TGT	Trp-Cys
Streptomycin	*rrs*	43	A1401G	A-G	A-G
			A514C	A-C	A-C
			C517T	C-T	C-T
			C513T	C-T	C-T
			C516T	C-T	C-T
Streptomycin	*rpsL*	85	A43G	AAG-AGG	Lys-Arg
			A43C	AAG-ACG	Lys-Thr
			A88G	AAG-AGG	Lys-Arg
			A88C	AAG-ACG	Lys-Thr
Ethambutol	*embB*	57	A306G	ATG-GTG	Met-Val
			G306C	ATG-ATC	Met-Ile
			G306T	ATG-ATT	Met-Ile
			A306C	ATG-CTG	Met-Leu
			G306A	ATG-ATA	Met-Ile
			A306T	ATG-TTG	Met-Leu
			G406C	GGC-GCC	Gly-Ala
			G406A	GGC-GAC	Gly-Asp
			A354C	GAC-GCC	Asp-Ala
			A497G	CAG-CGG	Gln-Arg

Fabrication of the Microfluidic Chip

A customized microfluidic chip with 112 reaction chambers was used for 37 gene mutation sites responsible for resistance against first-line anti-TB drugs using the KASP principle. Once the samples were infused into the microfluidic chip, the entire process, including sample distribution, reaction chamber sealing, PCR and results detection, could be fully sealed, eliminating potential contamination.

The microfluidic chip was injection-molded with polypropylene of dimensions 75 mm long, 25 mm wide, and 2 mm thick. As shown in Figure S1, four infusing channels, each connected with 28 reaction chambers, could be used to simultaneously detect as many as 112 targets (including control) on a chip. To detect the 37 gene mutation sites of TB, the primer pairs were pre-spotted in the corresponding reaction chambers of the chip (Figure 1). For each target, two specific upstream primers (2.5 μM) and the shared downstream primer (0.5 μM) were loaded in the reaction chamber (final volume: 0.14 μL/chamber) with an automated spotter (PersonalArrayer-16, CapitalBio, Beijing). The primers were air-dried at room temperature, the surface of the chip was then sealed with heat-sealing film (LGC), and the sealed chip was stored at 4°C before use.

To ensure a more comprehensive coverage of resistance-associated mutation sites, we designed 37 pairs of specific primers that can adequately target the essential gene segments related to drug resistance (Figure 2A). Each site was amplified in the presence of three primers, yielding two bands with 3′-end allele-specific primers. One of the bands possessed a nucleotide sequence bound to the FAM probe, and the other had a nucleotide sequence bound to the HEX probe at the 5′-end. After amplification, the different fluorescence of the product was analyzed using scanning software. Homozygous mutants generated only green fluorescence, homozygous wild-type genes yielded only red fluorescence, while heterozygotes produced mixed red and green fluorescence.

Multiple tests were performed to establish the optimal measurement conditions of the detection system and to determine the acceptable range of variation for the standard protocol. First, varying concentrations and ratios of different types of primers were analyzed to establish optimal conditions ensuring the highest specificity and signal-to-noise ratio. Second, the sensitivity of the method was assessed by testing a range of DNA concentrations in the samples. The concentration of DNA was 1×10^{0} copies/μL to 1×10^{6} copies/μL, with each concentration pro-spotted in three chambers for PCR amplification.

Microfluidic System for Detecting Mutations

The template, including 5 μL of the extracted genomic DNA, 22.5 μL of 2× KASP PCR master mix, and 17.5 μL of ddH$_2$O, was injected into the infusing channel of the chip. Then, the chip was sealed with single-sided tape and centrifuged at 4000 rpm for 2 minutes to distribute the template into the reaction chambers with primer pairs. Afterward, the chip was thermally sealed to
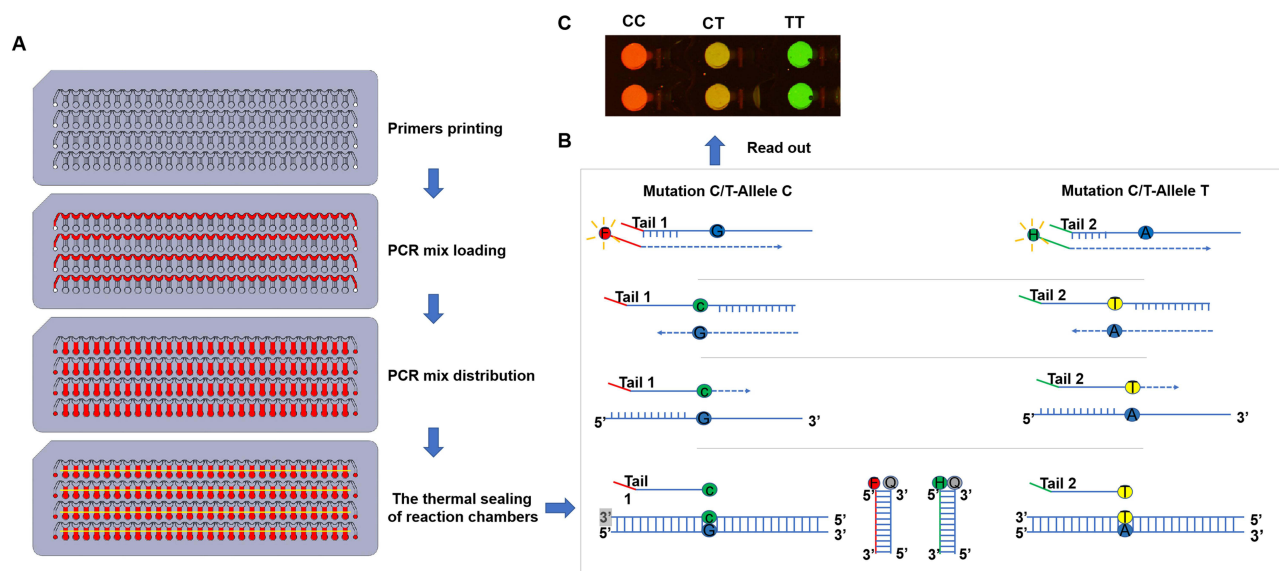

Figure 1 Flowchart of the chip for drug resistance gene detection. (**A**) The corresponding illustrations of each step in the chip sampling process. (**B**) Schematic diagram of the experimental principle. (**C**) The final PCR products analyzed by scanning.
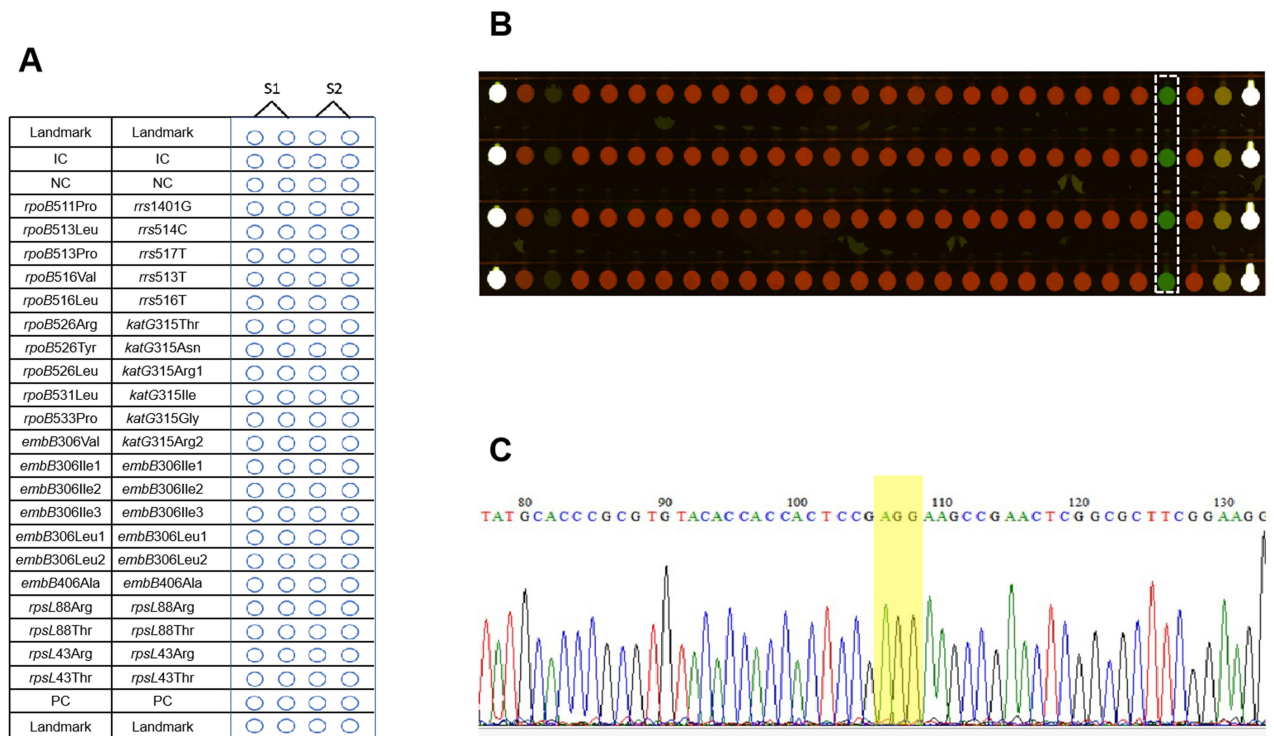
Figure 2 Drug-resistant gene detection results using our chip and Sanger sequencing. (**A**) The layout of primer pairs and controls pre-loaded on the chip. (**B**) The fluorescence images of the reaction chambers for detecting sample No. 47,901 and the genotyping results. (**C**) Sanger sequencing result for sample No. 47,901 and the mutation site is highlighted in yellow in the image.

prevent the medium from evaporating following PCR. The PCR cycles were conducted as follows: an initial step of incubation at 37°C for 5 minutes, followed by denaturation at 95°C for 15 minutes, then cycles consisting of denaturation at 95°C for 20 seconds, annealing at 61°C for 60 seconds and a gradual stepwise cooling from 61°C to 55°C, decreasing the temperature by 0.6°C with each cycle, followed by denaturation at 95°C for 20 seconds and annealing at 55°C for 60 seconds; for a total of 29 PCR cycles. Strain H37Rv was used as a wild-type control, and the plasmid containing mutated DNA was used as a positive control. Once the PCR was completed, the chip was scanned using a LuxScan 10K-D microarray scanner (CapitalBio, Beijing), and the fluorescence data were analyzed and normalized using the instrument's software.

Data Analysis

Genotyping was performed based on the following principles: 1) Median fluorescence intensity of the reaction chamber was calculated using the following formula: (6-carboxyfluorescein [FAM] fluorescence median - median background signal) / (5-hexachlorofluorescein [HEX] fluorescence median - median background signal); 2) The signal was considered to be positive if the signal intensity of the reaction chamber was more than three times higher than that of the negative control; 3) For determining the mutation type, the value of (FAM fluorescence median / HEX fluorescence median) was assessed, whereby a value greater than 0.5 and less than 2.0 indicated a heterozygous mutation, while any other value was interpreted as a homozygous mutation.

Results

Phenotypic Susceptibility Test

Among the 300 TB clinical isolates, 38 strains were identified as RIF-resistant, 64 were INH-resistant, 48 were SM-resistant, 23 were EMB-resistant, 33 were MDR-TB and 20 were fully four-drug-resistant (Table 3).

Table 3 Phenotypic Drug Susceptibility Testing of 300 Clinical TB Isolates

Drug Resistance*	Total, n (%)
Individual drug resistance	
Rifampicin	38 (12.7)
Isoniazid	64 (21.3)
Streptomycin	48 (16)
Ethambutol	23 (7.7)
Resistance pattern	
Multidrug-resistant TB	33 (11)
Extensively drug-resistant TB	20 (6.7)

Note: *An isolate is considered multi-drug-resistant TB (MDR-TB) if it is resistant to at least isoniazid and rifampicin, while an isolate is considered XDR if it is MDR and also resistant to at least one fluoroquinolone and one of the injectable second-line drugs.
Abbreviation: TB, tuberculosis.

Optimization and Analysis of the Drug Resistance Detection System

As shown in Figure 2B, the reaction chambers pre-spotted with the primer pair for *rpsL*43Arg in the chip for detecting sample No. 49,701 showed a significantly different color (green) than other samples (red), indicating homozygous mutation. Moreover, DNA sequencing results showed that it existed 43th codon homozygous mutation of *rpsL* gene (Figure 2C). The results also showed that the 2:2:5 ratio of the two allele-specific primers and the reverse primer provided adequate reaction specificity and maximum fluorescence value. Figure S2 shows that a DNA concentration of less than 1×10^{1} copies/µL did not produce fluorescence signals.

Comparison of Chip Scanning, Phenotypic Drug Sensitivity, and Sequencing Results

RIF-Resistant Strains

The results of microarray scanning indicated that among the 38 isolates with the RIF-resistant phenotype, 29 strains contained mutations in the *rpoB* gene. Notably, none of the 262 strains with the RIF-sensitive phenotype contained *rpoB* gene mutations. The chip scanning results were consistent with the sequencing data. Among the 29 strains harboring a mutated *rpoB* gene, the most frequent mutation was *rpoB*531 (TCG-TTG) detected in 24 (63.15%) strains, followed by *rpoB*526 (CAC-CGC) mutation detected in 3 strains (7.89%), and *rpoB*522 (TCG-CAG) and *rpoB*526 (CAC-TAC) mutations detected in 1 strain (2.63%) each. The sensitivity and specificity of the chip system for detecting RIF resistance were 76.32% and 100%, respectively (Tables 4–7).

INH-Resistant Strains

The results of microarray scanning indicated that among the 64 isolates with the INH-resistant phenotype, *katG* gene mutations were detected in 39 isolates. No mutations were detected in INH-sensitive strains. Further, the chip scan results were consistent with the sequencing results. Among the *katG* mutant strains, there were 33 strains (84.61%) with the *katG*315 (AGC-ACC) mutation, 3 isolates (4.68%) with the *katG*315 (AGC-AAC) mutation, 2 strains (3.12%) with the *katG*315 (AGC-GGC) mutation, and 1 strain (1.56%) with the *katG*315 (AGC-ATC) mutation. The sensitivity and specificity of the chip system for detecting INH resistance were 60.93% and 100%, respectively (Tables 4–7).

SM-Resistant Strains

According to the chip scan results, among the 48 strains with the SM-resistant phenotype, a mutated drug resistance gene was present in 32 isolates. The *rpsL*43 (AAG-AGG) mutation was detected in 2 SM-sensitive strains. The chip scanning results were consistent with the sequencing results. Among the SM-resistant strains, 26 (54.16%) carried the *rpsL*43

Table 4 Evaluation Between Genetic and Phenotypic Characteristics of 20 XDR-TB Isolates and 127 Pan-Sensitive Isolates

Drug	Locus	CHIP	Sanger Sequencing		Sensitivity (%)	Specificity (%)
			Wt	Mt		
RIF	*rpoB*	Wt Mt	108 0	0 19	100	100
INH	*katG*	Wt Mt	119 0	0 8	100	100
SM	*rrs*	Wt Mt	86 0	0 41	100	100
	rpsL	Wt Mt	93 0	0 34	100	100
EMB	*embB*	Wt Mt	111 0	0 26	100	100
XDR-TB			1	19		

Abbreviations: XDR-TB, extensively drug-resistant tuberculosis; RIF, rifampicin; INH, isoniazid; SM, streptomycin; EMB, ethambutol; Wt, Wild type; Mt, Mutation type.

Table 5 Correlation Between Genotypic and Phenotypic Drug Susceptibility Testing

Drug	CHIP	MGIT 960		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		R	S				
RIF	R S	29 9	0 262	76.32	100	100	96.68
INH	R S	39 25	0 236	60.93	100	100	90.42
SM	R S	32 16	2 250	66.66	99.20	94.12	93.98
EMB	R S	16 7	0 277	69.57	100	100	97.54

Abbreviations: RIF, rifampicin; INH, isoniazid; SM, streptomycin; EMB, ethambutol; R, resistant; S, sensitive; PPV, positive predictive value; NPV, negative predictive value.

Table 6 Evaluation Between Phenotypic and Genetic Characteristics of 300 Clinical Isolates*

Drug	Locus	CHIP	Phenotypic DST		Accuracy (%)	Sanger Sequence	Phenotypic DST		Accuracy (%)
			R	S			R	S	
RIF	*rpoB*	Mt Wt	29 9	0 262	97	Mt Wt	29 9	0 262	97
INH	*katG*	Mt Wt	39 25	0 236	92	Mt Wt	39 25	0 236	92
SM	*rpsL/rrs*	Mt Wt	32 16	2 250	94	Mt Wt	32 16	2 250	94
EMB	*embB*	Mt Wt	16 7	0 277	98	Mt Wt	16 7	0 277	98

Note: *The diagnostic performance of the chip in comparison with DNA sequencing and drug susceptibility testing was determined after the resolution of natural polymorphisms and silent mutations.
Abbreviations: RIF, rifampicin; INH, isoniazid; SM, streptomycin; EMB, ethambutol; DST, drug-susceptibility testing; Wt, Wild type; Mt, Mutation type; R, resistant; S, sensitive.

Table 7 Comparison of the Chip Results, Phenotypic DST and Sanger-Sequencing Results

CHIP	Phenotypic DST	Number of Isolates	Sanger Sequencing	Number of Isolates
RIF	**RIF**		*rpoB*	
Resistant	Resistant	n=29	S531L (TCG→TTG) H526Y (CAC→TAC) H526R (CAC→CGC) S522Q (TCG→CAG)	24 1 3 1
Susceptible **INH**	Resistant **INH**	n=9	None *katG*	9
Resistant	Resistant	n=39	S315T (AGC→ACC) S315N (AGC→AAC) S315I (AGC→ATC) S315G (AGC→GGC)	33 3 1 2
Susceptible **SM**	Resistant **SM**	n=25	None *rpsL*	25
Resistant	Resistant	n=32	K43R (AAG→AGG) K88R (AAG→AGG) S513S (AGC→AGT)	26 3 3
Susceptible **EMB**	Resistant Susceptible **EMB**	n=16 n=2	None K43R (AAG→AGG) *embB*	16 2
Resistant	Resistant	n=16	M306I (ATG→ATA) M306L (ATG→CTA)	14 2
Susceptible	Resistant	n=7	None	7

Abbreviations: DST, drug-susceptibility testing; RIF, rifampicin; INH, isoniazid; SM, streptomycin; EMB, ethambutol; None, mutation not detected.

(AAG-AGG) mutation, 3 (6.25%) the *rpsL*88 (AAG-AGG) mutation, and 3 (6.25%) the *rrs*513 (AGC-AGT) mutation. The sensitivity and specificity of the chip system for detecting SM resistance were 66.66% and 99.2%, respectively (Tables 4–7).

EMB Resistant Strains

The results of microarray scanning indicated that among the 23 isolates with the EMB-resistant phenotype, *embB* gene mutations were detected in 16 strains. No mutations of the *embB* gene were detected in EMB-sensitive strains. The results of microarray scanning were consistent with the sequencing results. Among the *embB* mutants, 14 isolates (60.86%) had *embB*306 (ATG-ATA) mutation, and 2 (8.69%) had *embB*306 (ATG-CTA) mutation. The sensitivity and specificity of the chip system for detecting EMB resistance were 69.56% and 100%, respectively (Tables 4–7).

Thus, our results indicated that the proposed chip detected all drug-resistant mutations found by Sanger sequencing, maintaining 100% sensitivity and specificity (Table 4). It was also consistent with DST, both in terms of detecting the presence of resistance and the types of mutations present (Table 6 and Table 7). In regard to detection accuracy, the chip and Sanger sequencing had similar accuracies in comparison to phenotypic DST, ranging from 92% to 98% across all drugs (Table 6). In addition, our proposed chip exhibited high positive predictive value (PPV) and negative predictive value (NPV), indicating high accuracy in predicting resistance (or a lack of resistance), and as demonstrated in Table 5, the PPV was 100% for all drugs, while the NPV ranged from 90.42% to 97.54%. Lastly, unlike phenotypic DST, which only provides information regarding overall resistance, both our proposed chip and Sanger sequencing provided detailed

mutation information, which could be particularly valuable for tracking the spread of specific resistance alleles and developing targeted treatments (Table 7).

Discussion

In this present study, we demonstrated the effectiveness of a microfluidic chip and allele-specific PCR assay for rapidly detecting drug resistance-associated mutations in *M.TB* by assessing different mutations and using different methods compared to previous related studies. Our results indicated that the proposed chip could perform at a similar level to Sanger sequencing but provides results more quickly and at a potentially lower cost, making it a promising alternative for drug susceptibility testing of *M.TB*. Additionally, compared to phenotypic DST, the chip also provides molecular-level information, which is useful for understanding the genetic basis of drug resistance.

Previously, Mohajeri et al investigated the distribution and drug resistance of the *M.TB* Beijing genotype in western Iran,[21] using PCR-based methods to detect the Beijing genotype and performed phenotypic drug susceptibility testing to evaluate drug resistance. They reported that their results significantly differed from previously reported mutation frequencies for codon 526 (CAC to GAC) among Italian isolates (40.1%) and Greek isolates (17.6%). However, the reported mutations differed from ours, making direct comparison difficult. Further, Sadri et al assessed the frequency of mutations associated with isoniazid and rifampicin resistance in *M.TB* strains isolated from patients in western Iran.[22] They also used PCR-based methods to detect mutations in the *katG, inhA* and *rpoB* genes associated with drug resistance. Although some of the mutations they evaluated were similar to those examined in our current study, it is important to note that the methods employed in our research differed. Specifically, our engineered microfluidic chip detection system displayed significant potential with its high sensitivity and specificity. It demonstrated superior accuracy in detecting drug resistance compared to traditional assays and sequencing methods that depend on bacterial culture, thereby highlighting the potential of this innovative detection system as a more precise and efficient method for evaluating drug sensitivity in TB cases.

In the samples included in the present study, the mutation rate of the *rpoB* gene in the RIF-resistant strain was approximately 76%, and the high-frequency mutation sites in *rpoB*531 and *rpoB*526 were consistent with those previously reported.[23] Specifically, the presence of high-frequency mutation sites in *rpoB*531 and *rpoB*526, which are commonly associated with RIF resistance, further validates the reliability of our proposed chip assay in identifying these mutations. Further, mutations in the *katG* gene, which is associated with INH resistance, were found to be linked to high levels of resistance to this important anti-TB drug. The proposed chip assay demonstrated a specificity of 100% in detecting INH-resistant TB cases, while the sensitivity was observed to be 60.93%, indicating room for improvement in accurately detecting INH resistance. In addition, it should be noted that the frequency of INH-resistant TB is influenced by the specific location of mutations within the *katG* gene, suggesting that the lower sensitivity observed in the chip assay for INH resistance detection in this study could be attributed to the particular distribution of mutations within the gene, which may not have been fully captured by our proposed assay design.[24] Thus, while these findings underscore the relevance of the microfluidic chip assay in identifying drug resistance mutations in TB, with *rpoB* mutations associated with RIF resistance being detected with high sensitivity and specificity, the sensitivity for detecting INH resistance was relatively lower, and the role of mutation distribution within *katG* should be further investigated to improve the sensitivity of the chip assay in further improving its accuracy, especially in regard to INH-resistant TB cases.

The high prevalence of drug-resistant mutations in this study might have been attributed to the high levels of drug-resistant TB within the region from which the isolates were collected. Importantly, our department specializes in treating challenging cases, usually referred from lower-tier hospitals that struggle to effectively manage drug-resistant TB, thereby, to a certain extent, contributed to the higher prevalence of drug-resistant mutations observed in the study population. In addition, several other factors might have also contributed to the high levels of drug-resistant TB, such as poor treatment adherence (can lead to the development of drug resistance), inappropriate treatment regimens (either due to suboptimal drug choices or inadequate treatment duration, can also contribute to the emergence of drug-resistant strains), and the circulation of drug-resistant strains within the community, which might have contributed to the overall prevalence of drug-resistant mutations. Thus, to gain a more comprehensive understanding of the dynamics and underlying factors contributing to these observations, further studies are needed into factors such as treatment practices, patient

adherence, healthcare infrastructure, and the epidemiology of drug-resistant TB in the region, which might aid in devising targeted interventions to effectively manage and control drug-resistant TB in the affected population.

In China, the resistance rate of SM is very high and is mostly related to its long-standing and frequent use as a first-line anti-TB drug. The TB database shows that the two-point mutations *rpsL*43 and *rpsL* 88 are responsible for 75% of cases of SM-resistant TB, and the sensitivity of the chip developed in this study for detecting SM resistance was 66.66%. EMB is typically used in combination with other first-line anti-TB drugs such as RIF, INH or pyrazinamide, and the resistance rate for EMB is generally lower compared to other first-line drugs. Although the *embB*306 codon is a mutation hotspot for *M.TB* resistant to EMB, the reliability of *embB*306 as a marker for identifying EMB resistance remains controversial. It was reported that this mutation was only present in 28–68% of EMB-resistant TB cases, with the sensitivity of molecular diagnostic methods for detecting EMB resistance ranging from 60–80% and specificity ranging from 93–100%.[25–27] These values, characterized by high specificity but relatively weaker sensitivity, are consistent with the results obtained from our developed chip assay in our present study.[28] Collectively, these findings highlight the specific challenges and characteristics of resistance rates for different drugs, especially in China. Streptomycin resistance is prevalent due to its historical usage, while EMB resistance, despite having *embB306* as a potential marker, presents controversies and limitations in its detection. Our proposed chip assay demonstrated consistency with previous reports in terms of sensitivity and specificity for detecting these drug resistances, indicating its suitability and alignment with existing diagnostic methods.

A rapid, sensitive and specific genetic method for identifying resistance to TB medication is important to improve the treatment outcomes of TB patients and inhibit the development of acquired drug resistance.[15] This study focused on designing and developing a microfluidic chip-based detection system for common drug resistance-related mutations of *M. TB*. Our proposed method demonstrated an average accuracy of 95% (range: 92–98%) for detecting resistance-linked mutations, a result superior to that of tests based on liquid culture. It should be noted that the variations in performance observed in detecting different drug-resistance genes may not solely be attributed to limitations in the methodology used. These discrepancies may also arise from a lack of correlation between drug-resistance phenotype and genotype, and comparing the assay results with data obtained through Sanger sequencing can provide further evidence to support this possibility. Further, although this study did not include specific cost information for each test method, the potential cost-effectiveness of the chip method compared to Sanger sequencing can be inferred based on the current nature of these technologies and their typical usage. For instance, our proposed chip method seems capable of testing multiple samples simultaneously, increasing its throughput compared to Sanger sequencing, which generally processes one sample at a time; thereby leading to greater testing efficiency, allowing more tests to be conducted in a shorter time frame and potentially lowering the cost per test. Also, the microfluidic chip detection system appears to be simpler to operate and faster in producing results than Sanger sequencing, reducing the person-hours required to process and interpret results, hence reducing labor costs. Lastly, given the high degree of sensitivity, specificity and accuracy of the chip method, there might be less need for additional confirmatory testing, which is often required when results are ambiguous, thereby resulting in further cost savings. However, it is important to note that the real cost-effectiveness would need to be evaluated in a detailed cost-analysis study comprising all the direct and indirect costs associated with both methods (ie, reagents, equipment, labor, etc.) and that the cost-effectiveness could vary depending on the specific settings and scale of use.

Several limitations of the newly developed microfluidic chip detection system should be acknowledged. First, the system requires specialized equipment, including a flatbed PCR instrument, heat sealer, blocker and scanner compatible with the chip. Additionally, manual processing of scan data is necessary. Second, the fixed 3′-end of the competitive allele-specific primer limits the flexibility in selecting optimal PCR primers. Third, reliance on high-quality *M.TB* DNA may introduce errors in complex samples containing a mixture of TB and non-TB mycobacteria. Lastly, a comparison of the efficacy and applicability of the proposed method with other widely used existing methods (ie, GeneXpert, LPA, etc.) was not performed and should be evaluated in future studies. Collectively, the preliminary efforts in this study focused on enhancing the usability of the chip, streamlining chip processing steps, optimizing reaction systems to reduce reliance on sample quality and validating its potential clinical applicability.

In conclusion, the microfluidic chip detection system outperformed traditional drug sensitivity assays and sequencing methods based on bacterial culture due to its higher accuracy in detecting TB drug sensitivity, especially in screening mutations related to the resistance of first-line anti-TB drugs. Our proposed detection system offers a range of advantages, including flexibility in the number of detection sites or samples, ease of operation, and protection against aerosol contamination. Consequently, it represents a more convenient and efficient detection tool for the clinical diagnosis of TB drug resistance.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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