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RESEARCH ARTICLE

ANALYSIS OF GENE MUTATIONS IN GEOGRAPHICALLY RELATED DRUG-RESISTANT TUBERCULOSIS IN HENAN PROVINCE, CHINA, USING THE REVERSE DOT BLOT HYBRIDIZATION

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ABSTRACT

Objective: Drug-resistant tuberculosis (TB) exhibits large geographically related differences; however, little is known about the current mutation profiles of clinical TB isolates in Henan Province, China.

Methods: Seventeen probes for rifampin (RIF) and isonicotinylhydrazine (INH) were hybridized with PCR products to detect drug resistance.

Results: Overall, 138 patients were examined using reverse dot blot hybridization (RDB). Of these, 126 samples were successfully detected. H526Y and S531L rpoB mutations and S315T katG mutation were the most frequently observed in the strains with high- or intermediate-level of drug resistance. This is the first study to show that resistance to RIF and INH is more prevalent than just resistance to RIF (64.58%) or INH (66.67%) in men. There were more drug-resistant cases (approximately 50%) in patients aged 31–59 years than in those aged ≤30 years in Henan Province. The proportion of patients with multidrug-resistant TB was 21.4%, which is higher than that in most other provinces in China.

Conclusion: The detection of rpoB, katG, and inhA mutations in INH- and RIF-resistant pulmonary TB and identification of differences between geographically related drug-resistant TB strains are necessary to prevent the progress of resistant strains and improve TB control in Henan Province. RDB is not only a rapid, sensitive, and low-cost method for the early detection of gene mutations, but can also be used to analyze specific mutations of clinical drug-resistant TB strains.

KEYWORDS: Reverse dot blot hybridization method; Drug-resistant tuberculosis; Molecular characterization; geographically related drug-resistant tuberculosis; Laboratory indexes

1. BACKGROUND

Tuberculosis (TB) is a serious infectious disease caused by *Mycobacterium tuberculosis* bacteria and a leading cause of death globally. According to a World Health Organization global progress report on TB elimination, more than 0.5 million patients with drug-resistant TB in 2018 were new cases [1]. Drug-resistant TB is more difficult to treat and incurs higher costs than TB [1]. Multidrug-resistant TB (MDR-TB) is defined as a strain that has developed resistance to at least two drugs. These strains persist as sources of infection over time, and infections caused by them are difficult to treat [2, 3]. Therefore, the analysis and treatment of MDR-TB using a high-performance, simple, and low-cost method are necessary to improve TB control. The current method for detecting drug-resistant TB is based on the Löwenstein–Jensen standard culture-based drug sensitivity testing system (LJS). However, the time required for the detection of drug-resistant TB after sputum collection is approximately 2 months, which is a major drawback of this method. Drug-resistant TB can be diagnosed more quickly through nucleic acid amplification, 15-loci mycobacterial interspersed repetitive unit-variable number of tandem repeats, and immunological methods. Nonetheless, these methods are not usually recommended, because they require expensive equipment, are difficult to operate, have poor resolution, or do not provide accurate results [3-5].

Drug-resistant TB exhibits large geographically related differences [6-10]. In Henan Province, the drug resistance status in TB was defined approximately 10 years ago [11]. Methods to detect drug resistance include spoligotyping and 26-locus mycobacterial interspersed repetitive units-variable number of tandem repeats, although these have drawbacks [12]. Therefore, the development of a hybridization method that can rapidly evaluate the epidemic and genetic characteristics of drug-resistant TB in a low-cost manner is essential to control TB and show the current mutation profiles of drug-resistant TB in Henan Province, not that 10 years ago. Single nucleotide polymorphism analysis is an appropriate method to detect drug resistance [13] because drug-resistant TB is caused by mutations in relatively limited regions. Additionally, point mutations are major inducers of drug resistance in TB [13]. Thus, in this study, DNA markers related to resistance to rifampin (RIF) (511, 516, 522, 526, 531, and 533) [10, 14-19], *katG* (315), and the promoter region of the *mabA* (fabG1)-inhA (-15) operon for isonicotinylhydrazine (INH) [10, 15-21] were used; the markers were chosen based on previous research and importance of drug resistance. The detection of *rpoB, katG*, and *inhA* mutations in INH- and RIF-resistant pulmonary TB and identification of differences in geographically related drug-resistant TB are necessary to prevent the progress of resistant strains and improve TB control in Henan Province. Based on the mutations in these genes, a reverse dot blot hybridization (RDB) method was designed to simultaneously identify mutants in a simple, rapid, and sensitive manner, and thus define the epidemiological profiles of geographically related drug-resistant TB strains in Henan Province.

2. METHODS

2.1 Materials and Reagents

Taq DNA polymerase was purchased from MBI Fermentas (Vilnius, Lithuania), and membrane strips were purchased from Pall Corporation (New York,NY,, USA). Primers, probes, and DNA extraction buffer were provided by DAAN Gene Co., Ltd. of Sun Yat-sen University (Guangzhou, China).

2.2 Design of Primers and Probes

The complete genomic DNA sequence of *M. tuberculosis* was obtained from GenBank (accession number CP000611). The PCR primers (Table 1) and probes (Table 2) were designed using Oligo (6.31) Primer Analysis Software. Reverse primers were labeled with biotin at the 5′ end. A probe (IS1081) specific for a highly conserved *M. tuberculosis* sequence that should be positive for all TB strains was used.

TABLE 1- *List of biotin-labeled primers for PCR-based reverse dot blot hybridization*

Gene	Primer (sequence 5' → 3')	Product size (bp)
inhA F	(5′-CGCTGCCCAGAAAGGGA-3′)	248
inhA R	Biotin- (5′-CCCCGGTTTCCTCCGGT-3′)	
katG F	(5′-GCTCGGCGATGAGCGTT-3′)	433
katG R	Biotin-(5′-GCTCTTCGTCAGCTCCCA-3′)	
rpoB F	(5′-CGGCATGTCGCGGATGG-3′)	245
rpoB R	Biotin-(5′-CGTCGCGGACCTCCAGC-3′)	
*IS1081 F	(5′-TGCTCGGTGCTGTGGATT -3′)	339

*IS1081 R	Biotin-(5′-GTTGCGCTGATTGGACC-3′)	

F, forward, R: reverse

*IS1081; TB-specific probe used as a positive control for all isolated TB strains.

***TABLE2 -** List of probes used in this study*

Probe name	Probe (sequence 5′ 3′)	Target gene
511 W	(5′-CCAGCTGAGCCAATTCA-3′) →	rpoB
516 W	(5′-TGGACCAGAACAACCCG-3′)	
516 M	(5′-AGCCAATTCATGGTCCAG-3′)	
522 W	(5′-GCTGTCGGGGTTGAC-3′)	
522 M	(5′-CCGCTGTTGGGGTTG-3′)	
526 W	(5′-CCCACAAGCGCCGACTG-3′)	
526 M	(5′-GACCTACAAGCGCCGAC-3′)	
526 M	(5′-TTGACCGACAAGCGC-3′)	
526 M	(5′-CCCGCAAGCGCCGAC-3′)	
531 W	(5′-TGTCGGCGCTGGGG-3′)	
533 W	(5′-TGTCGGCGCTGGGG-3′)	
531 M	(5′-TGTTGGCGCTGGGG-3′)	
533 M	(5′-GTCGGCGCCGGGG-3′)	
315 W	(5′-CAGCGGCATCGAGGT-3′)	katG
315 M	(5′-ACCACAGGCATCGAG-3′)	
315 M	(5′-ACCACCGGCATCGAG-3′)	
15 W	(5′-GCGAGACGATAGGTTG-3′)	inhA
15 M	(5′-GCGAGATGATAGGTTGT-3′)	
*IS1081	(5′-CTCTCGACGTTCAT-3′)	IS1081

W: wild type; M: mutant

*IS1081: positive control

2.3 Sample Preparation

In total, 138 clinical samples (sputum) were obtained from the Department of TB Medicine, the First Affiliated Hospital of Xinxiang Medical University (supplementary information), Xinxiang, Henan Province between October 2018 and September 2019, according to the regulations of the World Health Organization. TB infection was diagnosed based on the diagnostic criteria for pulmonary TB (WS 288—2017): radiography, smear of sputum, tuberculin testing, and culture based on LJS. All patients were treated with anti-TB drugs. DNA sequencing analysis confirmed all clinically suspected resistant samples.

2.4 DNA Extraction

Briefly, 50 μL of each sputum sample was centrifuged at 10,000 × *g* for 5 min. The pellet was resuspended in 25 μL of DNA extraction buffer [0.1 M Tris-HCl (pH 8.0) + 5 mM EDTA] and centrifuged at 10,000 × *g* for 5 min. Then, the sample was lysed at 100 °C for 10 min. Finally, the lysate was centrifuged at 10,000 × *g* for 5 min, and the supernatant containing the extracted DNA was stored at -20 °C.

2.5 Preparation of DNA Samples from Cultured Bacteria

Bacteria were selected from pure colonies on LJS and 150 µL was collected from each culture and centrifuged for 3 min at 5,000 × *g*. The bacteria were lysed as described in section 2.4.

2.6 Preparation of DNA from Sputum Samples Using the Improved Condensing Method

Sputum samples are usually collected at a single time-point; however, in this study, sputum samples from all patients were collected several times during a 24-h period for the improved condensing method. All sputum samples collected during 24 h for each patient were homogenized with exsiccated sodium carbonate (1.8%). The samples were incubated at 100 °C for 30 min and then centrifuged at 3,000 × *g* for 30 min. DNA was extracted from the pellet using the method described in section 2.4.

2.7 PCR Conditions

The extracted DNA (2 µL) was amplified using multiplex PCR. The reaction mixture (50 µL) contained 5× PCR buffer (10 µL), 20 mmol/L of each deoxynucleoside triphosphate (0.5 µL), 5 U/L Taq DNA polymerase (0.6 µL), and 10 pmol/L of each primer (2 µL). Deoxyuridine triphosphate, but not deoxythymidine triphosphate, and 0.5 U uracil-DNA glycosylase were added to the PCR system to prevent contamination. The reaction mixtures were amplified using the Biosystems 2720 Thermal Cycler (Waltham, MA, USA). The PCR cycling conditions were as follows: 50 °C for 3 min, followed by the initial denaturation step at 95 °C for 15 min, 45 cycles of 94 °C for 30 s, 68 °C for 1 min 45 s, and a final extension step at 68 °C for 7 min. We amplified the targets using four primer pairs simultaneously. Targeted DNA was separated via 2.0% agarose gel electrophoresis and stained with ethidium bromide (Fig. 1).

2.8 RDB Analysis

For RDB analysis, the hybrid membrane strip was activated using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride. Five wild-type and seven mutant probes for RIF and two wild-type and three mutant probes for INH (Table 2), which were synthesized and immobilized on nylon membrane strips, were selected with PCR products from TB clinical isolates containing mutations at different codons using RDB. The TB motif probes, color control probe, and TB-specific probe IS1081 were blotted on the membrane. The membranes were incubated at 60 °C for 10 min followed by incubation at 4 °C for up to 6 months.

2.9 Hybridization and Signal Detection

The RDB assay was designed based on the reverse hybridization principle. The process of reverse hybridization is observed as signals on membrane-bound capture probes. A series of oligonucleotide probes based on the rpoB, katG, and inhA sequences of TB were synthesized and then selected with PCR products from clinical isolates containing related gene mutations at different codons using RDB. The oligonucleotide probes for detecting different mutations are listed in Table 2.

The PCR products were denatured at 95 °C for 10 min and cooled on ice for 10 min. Each PCR product (50 µL) was mixed with 8 mL (± 1 mL) hybridization solution (0.3 M NaCl + 0.03 M sodium citrate + 0.1% SDS) and centrifuged at 100–150 × *g* and 60 °C for 60 min. Unhybridized PCR products were removed through washing with hybridization solution. PCR products and the color control probe were bound to the biotin group with peroxidase. The unbound peroxidase was removed by washing the membrane for 3 min with a rinsing solution (0.075 M NaCl + 0.0075 M sodium citrate + 0.1% SDS) twice. Then, the samples were stained with TMB substrate (2 mL TMB + 0.1 M sodium citrate + 2 µL 30% H_2O_2). All detections were standardized using positive and negative controls. A GT-8700F standard OA scanner (Epson, Tokyo, Japan) was used to scan blue hybridization signals on the membrane.

2.10 Statistical Analysis

Statistical analyses of drug resistance were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Chi-square test or Fisher's exact probability test was used to compare the proportions of different groups. Differences were considered statistically significant at P-value less than 0.05.

3. RESULT

3.1 PCR Production

The PCR strategy was designed to amplify the targets of three drug resistance-related genes in TB. The sputum samples were collected from patients and the extracted DNA was used to amplify the targets using four primer pairs. The DNA length of the targets ranged from 245–433 bp (Table 1). The results of electrophoresis are shown in Figure 1.

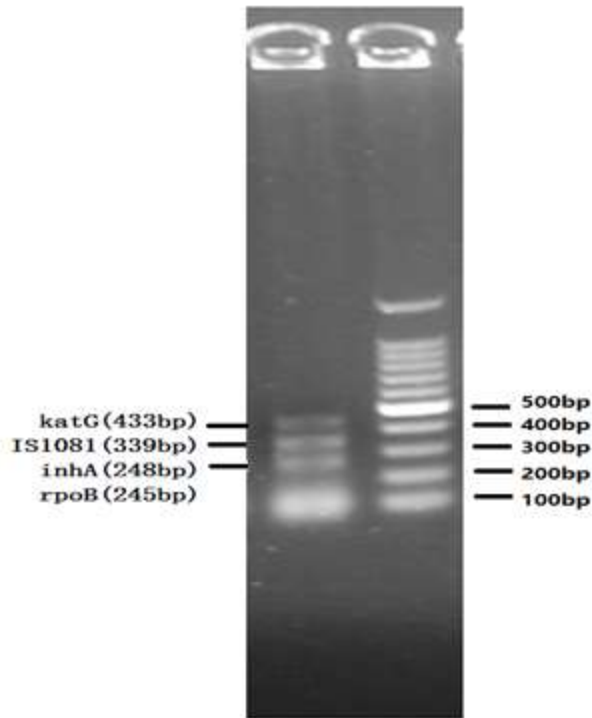

FIGURE 1 - *Agarose gel electrophoresis for PCR product shows the targets of drug resistance-related gene in TB.*

The DNA length of the targets ranged from 245–433 bp.

3.2 Mutation Pattern of TB Isolates

The mutation patterns of TB isolates, identified using the probes listed in Table 2, are shown schematically in Figure 2.

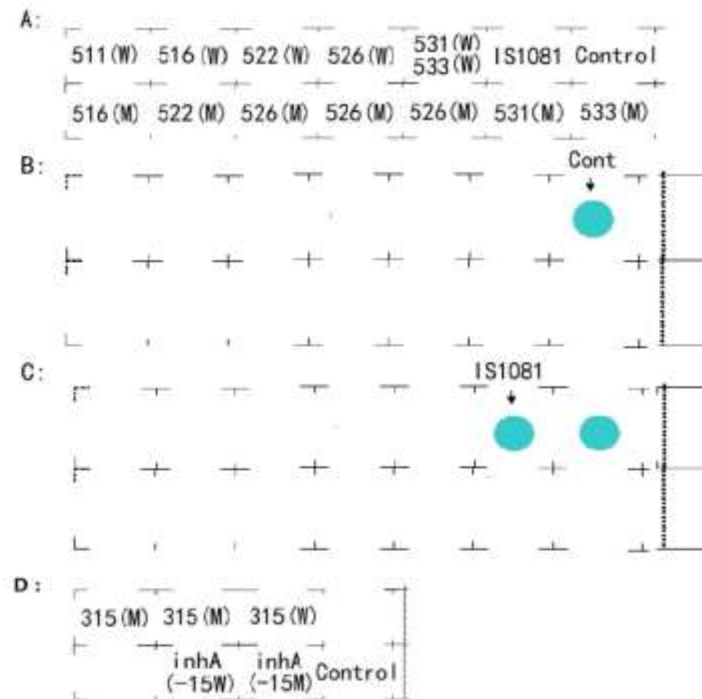

FIGURE 2 - *Mutation pattern of RDB is shown schematically*

(A) Arrangements on the membrane for probes of *rpoB*. W, wild type; M, mutant type; **(B)** Control (blue blob marked with an arrow) to confirm the validity of hybridization experiments. **(C)** The probe of IS1081 used to obtain signal showing TB isolates. **(D)** Arrangements on the membrane for probes of *katG* and *inhA*. W, wild type; M, mutant type;

3.3 Sequencing and RDB Testing of 138 Isolates

To evaluate the suitability of application of RDB in identifying mutations, samples were collected from 138 patients. They were confirmed to have TB by sequencing (Fig. 3). Of the 138 samples, 126 samples (91.30%) were successfully detected using RDB. Sixty drug-resistant strains were identified (47.62%) (Table 3). Among the 60 resistant isolates, 48 were resistant to RIF, 39 were resistant to INH, and 66 (52.38%) were wild-type. The sensitivity was 98.41% [2 *katG* (315 M) was inconsistent with the probes] and specificity was 100% in the 126 isolates successfully detected using DNA sequencing analysis.

TABLE 3 - Reverse dot blot hybridization testing of isolates from the Henan province

	RIF		INH				RIF+INH
	R	S	R			S	R
			katG	inhA	Both		
Wild type	0	78	0	0	0	87	0
Mutant types	48	0	33	18	12	0	27
Total	48	78	33	18	12	87	27

R, resistant; S, susceptible; RIF, rifampin; INH, isonicotinylhydrazine

Among the 60 resistant isolates (Table 4), S531L was the most prevalent *rpoB* resistance mutation, which was present in 21 of the 48 isolates (43.75%), followed by the H526Y mutation, which was present in 12 of the 48 isolates (25.00%). In total, 92.50% of mutations occurred in relatively limited regions of *rpoB* in previous studies. The *katG* codon 315 mutation was the most prevalent INH resistance mutation and was present in 33 isolates (84.62%). The CAC→TAC (H526Y) and TCG→TTG (S531L) *rpoB* mutations were most frequently observed in the strains with high- or intermediate-level RIF resistance. The AGC→ACC (S315T) *katG* mutation was most frequently observed in the strains with high- or intermediate-level INH resistance.

TABLE 4 - *Phenotypes of 126 isolates*

Drug susceptibility	Codon no.	No. of isolates
RIF resistance	rpoB(516)	5
	rpoB(522)	10
	rpoB(526)	12
	rpoB(531)	21
INH resistance	katG(315)	33
	inhA(-15)	18

INH, isonicotinylhydrazine; RIF, rifampin

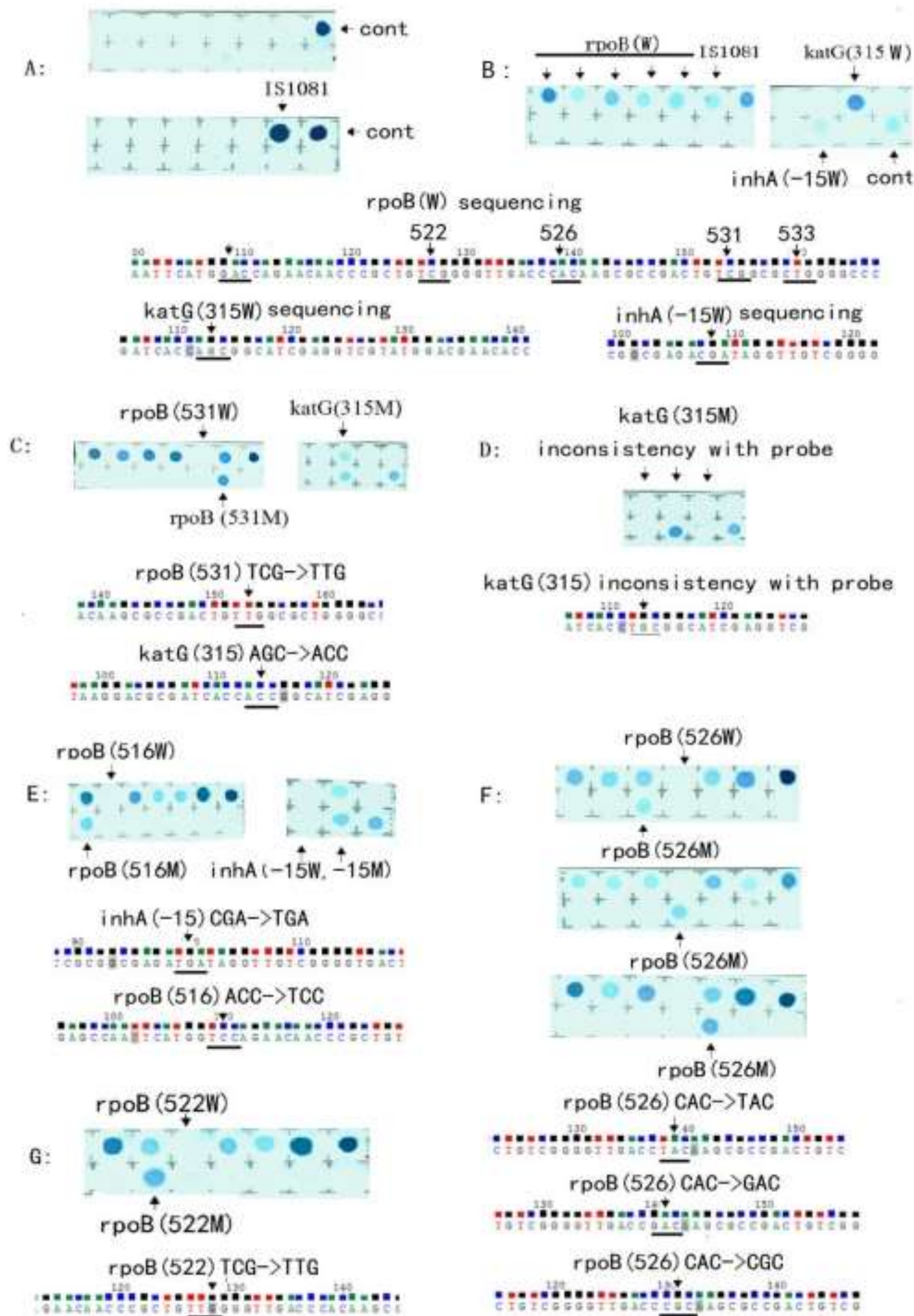

FIGURE 3 - RDB and sequencing of clinical samples

(A) Control (blue blob marked with an arrow) to confirm the validity of hybridization experiments; the probe of IS1081 showing TB samples. **(B)** All wild-type probes used to obtain signals to determine whether samples were wild-type isolates. **(C)** Mutation of *rpoB* (531) and *katG* (315); *rpoB* (531W) and *katG* (315W) did not produce a signal. **(D)** Mutation patterns of clinical isolates and designed probe are different. *katG* (315W) and *katG* (315M) did not produce a signal. **(E)** Mutation of *rpoB* (516) and *inhA* (-15); *rpoB* (516W) and *inhA* (-15W) did

not produce a signal. **(F)** Three different mutation patterns of *rpoB* (526); *rpoB* (526W) did not produce a signal. **(G)** Mutation of *rpoB* (522); *rpoB* (522W) did not produce a signal.

3.4 Characteristics of Patients with TB and Drug Resistance Profiles of Isolates

We investigated patient characteristics to analyze the relationship between the epidemiological profiles of patients and drug-resistant TB (Table 5) in Henan Province, China. The male to female drug resistance ratio was 36 (60.00%) to 24 (40.00%). The proportion of patients with MDR-TB in this study was 27 in total 126 cases (21.43%). Drug-resistant isolates were more likely to be observed in male patients than in female patients, and they exhibited resistance to both drugs (77.78%). According to the age group, MDR cases were most prevalent in patients aged 31–59 years (approximately 50%), followed by those aged ≤ 30 years (approximately 30%).

TABLE 5- *Information related to drug-resistant tuberculosis*

Factors	Total patients	RIF R (%)	INH R (%)	RIF+INH R (%)
Sex	Male (36)	31 (64.58%)	26 (66.67%)	21 (77.78%)
	Female (24)	17 (35.42%)	13 (33.33%)	6 (22.22%)
Age group	≤30 years (21)	17 (35.42%)	12 (30.77%)	8 (29.63%)
	31–59 years (29)	23 (47.92%)	20 (51.28%)	14 (51.85%)
	≥60 years(10)	8 (16.67%)	7 (17.95%)	5 (18.52%)

R, Resistant; RIF, rifampin; INH, isonicotinylhydrazine

4. DISCUSSION

Drug resistance could become a prevalent problem if an effective method for the identification of drug-resistant bacteria is not developed and implemented in public health policies.Based on previous research limitations, such as showing the drug resistance status many years ago [11] and using different detection methods which is showing mutation but cannot determine the mutation pattern which related with the level of drug resistance compared with RDB [12].In view of the restrictions of the currently available detection methods, it would be useful to develop a new detection method for rpoB, katG, and inhA mutations in INH- and RIF-resistant pulmonary TB and identification of differences between geographically related drug-resistant TB to prevent the progress of resistant strains and improve TB control in Henan Province, China..

RDB, which was first described in 1989, is currently used to detect bacterial DNA mutations [22]. The combination of hybridization and multi-PCR is an ideal system for the diagnosis of TB. Accordingly, in the present study, we designed and optimized a multi-PCR system for the diagnosis of TB drug resistance. First, regarding sensitivity, this system was able to successfully amplify each sample containing only one or two TB isolates. Among the 138 patients selected, 126 samples (91.30%) were successfully amplified using PCR. Second, this system is highly specific, because the probe (IS1081) used was designed with a highly conserved sequence specific for TB. Moreover, the developed system is efficient, as four primers were designed to simultaneously amplify three drug resistance-related genes. Further, the hybridization and signal detection system was optimized. The five wild-type and seven mutant probes for RIF and two wild-type and three mutant probes for INH were designed in-house to analyze relevant gene mutations in TB (Fig. 3A–3G). The sensitivity of 126 isolates was 98.41% [only 2 (*katG* (315 M)], which was inconsistent with that of the probes (Fig. 3A–3G), indicating that most drug-resistant isolates can be detected using these probes. Moreover, this system was efficiently designed: multiple isolates (126 isolates in this study) were simultaneously analyzed because all probes were immobilized on one nylon membrane strip (high efficiency) and were rapidly detected (~5 h). Therefore, the combination of RDB and PCR for the detection of TB can reduce false-positive and false-negative results using improved condensing method. Its efficiency for specific hybridization was 100% in this study. Furthermore, this technique is inexpensive and thus suitable for hospitals with inadequate resources.

In this study, 138 clinical isolates were collected. The male to female drug resistance ratio was 36 (60.00%) to 24 (40.00%), indicating that men are more susceptible to drug-resistant TB. A national survey showed a similar TB incidence rate in male patients (67.4%) in China [7]. To the best of our knowledge, this is the first study to report that dual resistance to RIF and INH is more prevalent than resistance to RIF or INH alone (Table 5). This shows that the TB control program in Henan Province is currently grim. Additionally, the high incidence of drug-resistant TB in patients aged 31–59 years indicates the loss of working

hours for many skilled laborers in industries in Henan Province, China.

The incidence of drug-resistant TB differs according to the location of epidemiological investigation [15, 16, 23, 24]. Information related to drug-resistant TB in Henan Province has never been reported. In this study, the proportion of patients with MDR-TB (21.4%) was higher than that of the 2017 global average (18.0%) and in most cities in China, including Beijing (16.4%) [6], Hunan (11.1%) [8], Jiangxi (19.8%)[9], Xinjiang (13.2%) [25], Fujian (9.5%) [10], and Shandong (10.8%) [26]. Our result is similar to that reported for Guizhou (20.7%) [7]. Previous studies have shown that 83.6%–94.2% of mutations occur in relatively limited regions of *rpoB* [25-28]. The result of the present study was in this range. Overall, these findings indicate that there are differences in the epidemiology of drug-resistant TB among regions, and the current TB control program in Henan Province is not very effective.

There are several reasons for the differences in epidemiology of drug-resistant TB among regions: (1) Different genotypes can explain the variability in drug-resistant TB in different countries and districts. Previous studies have shown that Beijing family strains are associated with drug resistance and have a greater likelihood of developing MDR-TB [8, 9, 27, 28]. (2) The implementation of a well-functioning local TB control program related to the epidemiology of drug-resistant TB in certain regions. For example, TB education and psychological support to patients with drug-resistant and MDR-TB without charging any fee through funding such as “National Science and Technology Major Projects” and “Global Fund” [10 7]. (3) A favorable social environment for better management and control of TB plays an important role in the overall decline of MDR-TB in different regions; this includes the establishment of a strong relationship with other departments at the hospital and news/social media to improve public awareness of TB and educate the general public [7]. (4) The mutated profiles of TB isolates from different areas might have somewhat geographically related differences. Our study also showed that the relationship between drug resistance and mutations in TB in Henan Province is different from that in Hunan [8],Fujian [10], and Jiangxi [9]. (5) Differences in genetic background, drug metabolism, and lifestyle among different ethnic groups could be another reason for the difference in epidemiology. A previous study reported that the prevalence of MDR-TB in Xinjiang was higher than that in Han people [25].

Sputum smearing appears to have low sensitivity (67.0% [29]–71.0% [30]) when used directly on clinical specimens; at least 1–9 acid-fast bacilli per 10 fields of sputum smearing in carbol fuchsin staining were required in other studies [31]. However, the sensitivity of our method was 91.30%. The reason for the different sensitivities is that we used an improved condensing method for the first time to collect samples. This method may hugely improve the rate of positive results that go unreported.

To the best of our knowledge, this is the first report showing the relationship between drug resistance and mutations in TB in Henan Province, China. According to the results of RDB (Fig 3A–3G) and LJS, the mutations in codons CAC→TAC (H526Y) and TCG→TTG (S531L) of *rpoB* gene were most frequently observed in the strains with high- or intermediate-level RIF resistance. The mutation in codon 315 AGC→ACC (S315T) of *katG* was most frequently observed in the strains with high- or intermediate-level INH resistance. The above findings were consistent between RDB and sequencing and were confirmed using LJS, indicating that one can determine the extent of drug resistance and choose the most appropriate drugs for clinical treatment based on the RDB results.

There are some limitations to this study. First, one drug-resistant sample (1.7%) was confirmed using the culture-based method and not with a mutant probe (Fig. 3D) using RDB. Additionally, 12 clinical isolates (8.7%) were not detected using the RDB method. The reasons for the difference in results between the culture-based method and RDB method are as follows: (1) Sequence differentiation in the relatively limited regions from which the probes are selected [32-36]. We designed probes according to the hot-spot region and assayed them for performance, which is a possible reason for damaged or missed individual TB isolates. We believe that the results will be more accurate after improvement of the probes and hybridization system. (2) In this study, probes with spots for mutations (e.g., *rpoB* 509 and *rpoB* 517) were not prepared. (3) The mutation patterns of some clinical isolates and designed probes were different; therefore, *katG* (315W) and *katG* (315M) did not present a signal [10, 14-19]. More suitable primers and probes are required to improve the sensitivity of our system in the future.

5. CONCLUSIONS

In Henan Province, the drug resistance status in TB was defined approximately 10 years ago and using different detection methods which is showing mutation but cannot determine the mutation pattern which related with the level of drug resistance compared with reverse dot blot hybridization. This study revealed that reverse dot blot hybridization can be useful for detecting INH- and RIF-resistant TB in a robust, rapid, simple, and cost-effective manner. Our study provides important insights into the analysis of Drug-resistant tuberculosis (TB) exhibits large geographically related differences.

Abbreviations:

RDB, Reverse Dot Blot hybridization;

RIF, Rifampicin;

INH, Isonicotinylhydrazine;

TB, Tuberculosis;

LJ S, Löwenstein-Jensen standard culture-based drug sensitivity testing system;

MDR-TB, Multi-Drug resistant tuberculosis;

SNP, Single nucleotide polymorphism;

NICPBP, National institute for the control of pharmaceutical and biological products;

EDC, N-(3-imethylaminopropyl)-N-ethylcarbodiimide hydrochloride;

POD, Peroxidase;

TMB, 3,3',5,5'-tetramethylbenzidine (TMB);

AFB, Acid-fast bacilli;

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Data Availability

All the data analyzed throughout this research are included in this published article.

Author Contributions

TM-C, GA-Z and LY-J conceived and designed the study.

MZ- X, J- L, X-W, WY- B, FG-K , F-L, M-I-S, JW- C performed the experiments.. MZ- X and J- L wrote the paper.

MZ- X, J- L, GA-Z, LY-J and M-I-S reviewed and edited the manuscript.

All authors (1) made substantial contributions to the conception or design of the work, or to the acquisition, analysis, or interpretation of data for the work; (2) participated in drafting the work or revising it critically for important intellectual content; (3) approved the final version to be published; and (4) agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

Ethics approval and consent to participate

Approval for this study was obtained from the Medical Ethics Committee of Scientific Research Project of the First Affiliated Hospital of Xinxiang Medical University ,Written informed consent was obtained from each participant according to the Federal and Institutional Guidelines **.**Written informed consent was obtained from a parent or guardian for participants under 16 years old.

Competing interests policy

The authors have declared that no competing interests exist.

Conceived and designed the research Funding

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References

1. Wang X, Jiao J, Xu W, Chai X, Li Z, Wang Q: A simple, rapid and economic method for detecting multidrug-resistant tuberculosis. The Brazilian Journal of Infectious Diseases: An Official Publication of the Brazilian Society of Infectious Diseases 2013, 17:667-671.
2. Shi J, Zheng D, Zhu Y, Ma X, Wang S, Li H, Xing J: Role of MIRU-VNTR and spoligotyping in assessing the genetic diversity of Mycobacterium tuberculosis in Henan Province, China. BMC Infectious Diseases 2018, 18:447.
3. Harding E: WHO global progress report on tuberculosis elimination. The Lancet Respiratory medicine 2020, 8:19.
4. Zhu L, Yang YZ, Guan HY, Cheng SM, Jin YY, Tan WG, Wu QF, Liu XL, Zhao MG, Lu ZH: Trends in drug-resistant tuberculosis after the implementation of the DOTS strategy in Shenzhen, China, 2000-2013. International Journal of Tuberculosis & Lung Disease the Official Journal of the International Union Against Tuberculosis & Lung Disease 2017, 21:759.
5. Kargarpour KM, Sadegh HR, Farmanfarmaei G, Masoumi M, Fateh A, Javadi G, Rahimi JF, Vaziri F, Siadat SD: Evaluation of the impact of polyclonal infection and heteroresistance on treatment of tuberculosis patients. Scientific Reports 2017, 7:41410.
6. Hu Y, Wu L, Xu B: The longitudinal trends of multidrug and extensively drug resistant tuberculosis in China. International Journal of Mycobacteriology 2015, 4:87-88.

7. Kazemian H, Kardan-Yamchi J, Bahador A: Efficacy Of Line Probe Assay In Detection Of Drug-Resistant Pulmonary Tuberculosis In Comparison With GeneXpert And Phenotypic Methods In Iran And Genetic Analysis Of Isolates By MIRU-VNTR. 2019, 12:3585-3593.

8. Liu Y, Jiang X, Li W, Zhang X, Wang W, Li C: The study on the association between Beijing genotype family and drug susceptibility phenotypes of Mycobacterium tuberculosis in Beijing. Scientific reports 2017, 7:15076.

9. Lan Y, Li Y, Chen L, Zhang J, Zhang H: Drug resistance profiles and trends in drug-resistant tuberculosis at a major hospital in Guizhou Province of China. Infection and drug resistance 2019, 12:211-219.

10. Zhao LL, Chen Y, Chen ZN, Liu HC, Hu PL, Sun Q, Zhao XQ, Jiang Y, Li GL, Tan YH, Wan KL: Prevalence and molecular characteristics of drug-resistant Mycobacterium tuberculosis in Hunan, China. Antimicrobial agents and chemotherapy 2014, 58:3475-3480.

11. Yuan X, Zhang T, Kawakami K, Zhu J, Zheng W, Li H, Deng G, Tu S, Liu W: Genotyping and clinical characteristics of multidrug and extensively drug-resistant tuberculosis in a tertiary care tuberculosis hospital in China. BMC infectious diseases 2013, 13:315.

12. Zhao LL, Huang MX, Xiao TY, Liu HC, Li MC, Zhao XQ, Liu ZG, Jiang Y, Wan KL: Prevalence, risk and genetic characteristics of drug-resistant tuberculosis in a tertiary care tuberculosis hospital in China. Infection and drug resistance 2019, 12:2457-2465.

13. Wang X, Jiao J, Xu W, Chai X, Li Z, Wang Q: A simple, rapid and economic method for detecting multidrug-resistant tuberculosis. The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases 2013, 17:667-671.

14. Shi J, Zheng D, Zhu Y, Ma X, Wang S, Li H, Xing J: Role of MIRU-VNTR and spoligotyping in assessing the genetic diversity of Mycobacterium tuberculosis in Henan Province, China. BMC infectious diseases 2018, 18:447.

15. Bergval IL, Vijzelaar RN, Dalla Costa ER, Schuitema AR, Oskam L, Kritski AL, Klatser PR, Anthony RM: Development of multiplex assay for rapid characterization of Mycobacterium tuberculosis. Journal of clinical microbiology 2008, 46:689-699.

16. Cao Z, Lan Y, Chen L: Resistance To First-Line Antituberculosis Drugs And Prevalence Of pncA Mutations In Clinical Isolates Of Mycobacterium tuberculosis From Zunyi, Guizhou Province Of China. 2019, 12:3093-3102.

17. Jhanjhria S, Kashyap B, Gomber S, Gupta N, Hyanki P, Singh NP, Khanna A, Sharma AK: Phenotypic isoniazid resistance and associated mutations in pediatric tuberculosis. The Indian journal of tuberculosis 2019, 66:474-479.

18. Jabbar A, Phelan JE: Whole genome sequencing of drug resistant Mycobacterium tuberculosis isolates from a high burden tuberculosis region of North West Pakistan. 2019, 9:14996.

19. Sinha P, Banerjee T, Srivastava GN, Anupurba S: Rapid detection of drug-resistant Mycobacterium tuberculosis directly from clinical specimens using allele-specific polymerase chain reaction assay. The Indian journal of medical research 2019, 150:33-42.

20. Imperiale BR, Di Giulio AB, Mancino MB, Zumarraga MJ, Morcillo NS: Surveillance and characterization of drug-resistant Mycobacterium tuberculosis isolated in a reference hospital from Argentina during 8 years' period. Int J Mycobacteriol 2019, 8:223-228.

21. Li Q, Gao H, Zhang Z, Tian Y, Liu T, Wang Y, Lu J, Liu Y, Dai E: Mutation and Transmission Profiles of Second-Line Drug Resistance in Clinical Isolates of Drug-Resistant Mycobacterium tuberculosis From Hebei Province, China. Frontiers in microbiology 2019, 10:1838.

22. Hang NTL, Hijikata M, Maeda S, Thuong PH, Ohashi J, Van Huan H, Hoang NP, Miyabayashi A, Cuong VC, Seto S, Van Hung N, Keicho N: Whole genome sequencing, analyses of drug resistance-conferring mutations, and correlation with transmission of Mycobacterium tuberculosis carrying katG-S315T in Hanoi, Vietnam. 2019, 9:15354.

23. Pitso L, Potgieter S, Van der Spoel van Dijk A: Prevalence of isoniazid resistance-conferring mutations associated with multidrug-resistant tuberculosis in Free State Province, South Africa. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde 2019, 109:659-664.

24. Saiki RK, Walsh PS, Levenson CH, Erlich HA: Genetic Analysis of Amplified DNA with Immobilized Sequence-Specific Oligonucleotide Probes. Proceedings of the National Academy of

Sciences of the United States of America 1989, 86:6230-6234.

25. Nimmo C, Doyle R, Burgess C, Williams R, Gorton R, Mchugh TD, Brown M, Morris-Jones S, Booth H, Breuer J: Rapid identification of a Mycobacterium tuberculosis full genetic drug resistance profile through whole genome sequencing directly from sputum. International Journal of Infectious Diseases 2017, 62.

26. Negi S, Rai A, Gupta S, Khare S, Lal S: Characterization of RPO B gene for detection of rifampicin drug resistance by SSCP and sequence analysis. Indian J Med Microbiol 2009, 12:e318-e318.

27. Qi YC, Ma MJ, Li DJ, Chen MJ, Lu QB, Li XJ, Li JL, Liu W, Cao WC: Multidrug-resistant and extensively drug-resistant tuberculosis in multi-ethnic region, Xinjiang Uygur Autonomous Region, China. PloS one 2012, 7:e32103.

28. Deng Y, Wang Y, Wang J, Jing H, Yu C, Wang H, Liu Z, Graviss EA, Ma X: Laboratory-based surveillance of extensively drug-resistant tuberculosis, China. Emerging infectious diseases 2011, 17:495-497.

29. Solomon G, Ramona G, Alexandra P, Tuija K, Emmi A, Judith B, Sven H, Victoria R, Gunilla KL: Drug resistant Mycobacterium tuberculosis of the Beijing genotype does not spread in Sweden. Plos One 2010, 5:e10893.

30. Tanveer M, Hasan Z, Siddiqui AR, Ali A, Kanji A, Ghebremicheal S, Hasan R: Genotyping and drug resistance patterns of M. tuberculosis strains in Pakistan. BMC infectious diseases 2008, 8:171.

31. Aragón LM, Navarro F, Heiser V, Garrigó M, Español M, Coll P: Rapid detection of specific gene mutations associated with isoniazid or rifampicin resistance in Mycobacterium tuberculosis clinical isolates using non-fluorescent low-density DNA microarrays. J Antimicrob Chemother 2006, 57:825-831.

32. Brossier F, Veziris N, Truffotpernot C, Jarlier V, Sougakoff W: Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of Mycobacterium tuberculosis with low- and high-level resistance. Journal of Clinical Microbiology 2006, 44:3659-3664.

33. Shimizu Y, Dobashi K, Yoshikawa Y, Yabe S, Higuchi S, Koike Y, Mita Y, Utsugi M, Endou K, Takahashi K: Five-antituberculosis Drug-resistance Genes Detection Using Array System. Journal of Clinical Biochemistry & Nutrition 2008, 42:228.

34. Z S, Y C, X Z, J Z, Y L, Y Q, Y L, L N, A G, C L: Characterization of extensively drug-resistant Mycobacterium tuberculosis clinical isolates in China. Journal of Clinical Microbiology 2008, 46:4075.

35. Lopez-Avalos G, Gonzalez-Palomar G, Lopez-Rodriguez M, Vazquez-Chacon CA, Mora-Aguilera G, Gonzalez-Barrios JA, Villanueva-Arias JC, Sandoval-Diaz M, Miranda-Hernández U, Alvarez-Maya I: Genetic diversity of Mycobacterium tuberculosis and transmission associated with first-line drug resistance: A first analysis in Jalisco, Mexico. Journal of Global Antimicrobial Resistance 2017.

36. Gupta AK, Reddy VP, Lavania M, Chauhan DS, Venkatesan K, Sharma VD, Tyagi AK, Katoch VM: jefA (Rv2459), a drug efflux gene in Mycobacterium tuberculosis confers resistance to isoniazid & ethambutol. Indian Journal of Medical Research 2010, 132:176-188.

37. Dhar N, Mckinney JD: Mycobacterium tuberculosis persistence mutants identified by screening in isoniazid-treated mice. Proceedings of the National Academy of Sciences of the United States of America 2010, 107:12275-12280.

38. Li Y, Zeng J, Zhang H, He ZG: The characterization of conserved binding motifs and potential target genes forM. tuberculosisMtrAB reveals a link between the two-component system and the drug resistance ofM. smegmatis. BMC Microbiology,10,1(2010-09-16) 2010, 10:242.