Original Article

Rapid Detection of Rifampin-resistant Clinical Isolates of *Mycobacterium tuberculosis* by Reverse Dot Blot Hybridization*

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Abstract

Objective A PCR-reverse dot blot hybridization (RDBH) assay was developed for rapid detection of *rpoB* gene mutations in 'hot mutation region' of *Mycobacterium tuberculosis (M. tuberculosis)*.

Methods 12 oligonucleotide probes based on the wild-type and mutant genotype *rpoB* sequences of *M. tuberculosis* were designed to screen the most frequent wild-type and mutant genotypes for diagnosing RIF resistance. 300 *M. tuberculosis* clinical isolates were detected by RDBH, conventional drug-susceptibility testing (DST) and DNA sequencing to evaluate the RDBH assay.

Results The sensitivity and specificity of the RDBH assay were 91.2% (165/181) and 98.3% (117/119), respectively, as compared to DST. When compared with DNA sequencing, the accuracy, positive predictive value (PPV) and negative predictive value (NPV) of the RDBH assay were 97.7% (293/300), 98.2% (164/167), and 97.0% (129/133), respectively. Furthermore, the results indicated that the most common mutations were in codons 531 (48.6%), 526 (25.4%), 516 (8.8%), and 511 (6.6%), and the combinative mutation rate was 15 (8.3%). One and two strains of insertion and deletion were found among all strains, respectively.

Conclusion Our findings demonstrate that the RDBH assay is a rapid, simple and sensitive method for diagnosing RIF-resistant tuberculosis.

Key words: *Mycobacterium tuberculosis*; Rifampin-resistance; Reverse dot blot hybridization

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INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), remains one of the most significant fatalities among infectious diseases worldwide. The World Health Organization (WHO) had estimated 8.6 million TB cases worldwide, and 1.3 million deaths attributed to TB in 2012. China is one of the 22 countries with the highest burden, and was responsible for 12% (1 million) of global TB cases in 2012, second only to India in the total number of new TB cases[1].

Moreover, drug-resistant TB, especially multi-drug resistant (MDR) *M. tuberculosis* defined as combined resistance to at least isoniazid (INH) and rifampin (RIF), pose challenges for the control and prevention of this deadly disease. Generally, detection of drug-resistant *M. tuberculosis* is performed by conventional drug sensitivity test (DST) on bacteria isolated from sputum, and this test has been used for decades, but it takes 6-8 weeks to obtain the results[1]. This delay in diagnosis can worsen the disease, and hasten the transmission of TB.

RIF has been used as a critical drug for treating TB since its development in early 1960s. The widespread use of RIF in global TB control has resulted in increasing clinical relapse rates. Previous studies have demonstrated that RIF resistance serves as an excellent indicator for MDR-TB detection as 90% of RIF-resistant strains are also INH-resistant[2]. Collectively, DNA sequencing studies observed that more than 95% of the RIF-resistant *M. tuberculosis* strains were found within an 81 base pairs (bp) hot-spot region (codons 507 to 533) of the gene, *rpoB*, encoding the β-subunit of RNA polymerase[2-4]. Current molecular methods used to screen for RIF resistance-determining region (RRDR) mutations include DNA sequencing, polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP), dideoxy fingerprinting[5], heteroduplex formation, molecular beacon PCR[6], biochip[7], etc. The commercial molecular assays, such as GenoType MTBDR*plus* and Xpert MTB/RIF[8-10], which indirectly identify mutations by lack of probe hybridization to wild-type loci are rapid, safe and sensitive, but their requirement for expensive instruments has limited their clinical application in some poor areas where they are urgently needed. Consequently, establishing a new assay to detect MTBDR is significant for TB prevention and control.

In this study, DNA sequencing was used to analyze the characterization and distribution of *rpoB* gene mutations associated with RIF resistance. Based on that, we developed a PCR-reverse dot blot hybridization (RDBH) assay for rapid identification of the target region in the RRDR of the *rpoB* gene, and evaluated the utility of this assay for primary screening of RIF resistance and MDR-TB as compared to DST and *rpoB* gene sequencing.

MATERIALS AND METHODS

Strains and Clinical Isolates

Based on the RIF-resistant status tested with DST, a total of 300 *M. tuberculosis* isolates were randomly selected from the strain bank of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. These isolates were collected from six different provincial tuberculosis hospitals and tuberculosis control centers, in which 103 were from Xizang Tibetan Autonomous Region, 99 from Hunan province, 42 from Sichuan province, 29 from Xinjiang Uygur Autonomous Region of Henan province, 25 from Anhui province and two from Shanxi province. *M. tuberculosis* (H37Rv) reference strain was obtained from the TB laboratory of the National Institute for Communicable Disease Control and Prevention in Beijing, China.

Bacterial Strains and Phenotypic Drug Susceptibility Testing

The mycobacterial culture, conventional species identification, and DST were performed as previously described[11]. All isolates were initially classified as *M. tuberculosis* by PNB/TCH differential media. The DST for the isolates was performed by the proportion method on Lowenstein-Jensen (L-J) medium using the relevant anti-TB drugs according to the WHO recommendation[11]. The isolates were tested for resistance to critical concentrations of RIF (40 μg/mL), INH (0.2 μg/mL), streptomycin (SM; 4 g/mL), ethambutol (EMB; 2 μg/mL), and para-aminosalicylic acid (PAS; 0.5 μg/mL).

Genomic DNA Extraction

Genomic DNA was extracted from fresh mycobacterial colonies grown on L-J media slants. The bacterial cells were transferred to microcentrifuge tubes containing 200 μL of TE buffer

(10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0), heat-killed at 80 °C for 30 min. After centrifugation for 5 min at 12,000 rpm, the supernatants were recovered and stored at -20 °C before use.

PCR Amplification and Sequencing of the rpoB Gene

A 629-bp fragment of the *rpoB* gene including RRDR was amplified using specific primers (forward primer, biotinylated at the 5' end: 5'-bio-GAG CCC CCG ACC AAA GA-3'; reverse primer: 5'-ATG TTG GGC CCC TCA GG-3'). The PCR was standardized in a final volume of 50 μL containing 200 μmol/L of each dNTP, 1×PCR buffer, 1.5 mmol/L $MgCl_2$, 0.4 μmol/L of each primer, 10-100 ng of genomic DNA and 5U of Taq DNA polymerase. The PCR reaction was performed as follows: 10 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 60 °C, 1.5 min at 72 °C, and 10 min at 72 °C. The PCR products were analyzed by electrophoresis using 2% agarose gels.

The PCR products were sequenced by Shanghai Biological Engineering Company, China, and the results were compared with the homologous sequences of the *M. tuberculosis* (H37Rv) reference strain by BLAST analysis.

RDBH Assay

According to the sequencing results, the most frequent mutation sites identified in our study were codons 531 (48.6%), 526 (25.4%), 516 (8.8%), and 511 (6.6%), which are consistent with previous studies[12]. Therefore, a total of 12 oligonucleotide probes based on the wild-type and mutant genotype *rpoB* sequences of *M. tuberculosis* were designed to screen the most frequent wild-type and mutant genotypes for diagnosing RIF resistance using Oligo Analyzer 3.0 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). The oligonucleotide probe sequences are listed in Table 1. The probes were designed with amino-labeled 5'-terminal (Saibaisheng Company, Beijing, China). The negatively-charged nylon membrane (Biodyne C, Pall Corporation) was activated in a solution of 16% (w/v) 1-ethyl-3-(3-dimethylamninopropyl) carbodiimide (EDAC; BBI, USA) for 15 min at room temperature (RT, 25 °C), and washed in distilled water for 2min at RT. The membrane was placed in a miniblotter (MN45; Immunetics, USA). Then, 10 μL of each oligonucleotide probe was diluted to appropriate concentration in 0.5 mol/L $NaHCO_3$ (pH 8.4), and transferred to the miniblotter slots followed by incubation for 1 min at RT. The membrane was removed from the miniblotter, incubated in freshly prepared 0.1 mol/L NaOH for 10 min, and washed in 2×SSPE (360 mmol/L NaCl, 20 mmol/L Na_2HPO_4, 200 mmol/L EDTA, pH 7.4)/0.1% sodium dodecyl sulfate (SDS) (Sigma) for 5 min at 60 °C. Finally, the membrane was incubated in 20 mmol/L EDTA (pH 8.0) for 15 min at RT, and stored in a well-sealed plastic bag at 4 °C until further use.

Table 1. List of 12 Probes Used for PCR-based Dot Blot Hybridization

Probe	Genotype	Sequence of Probes (5'-3')	Size (bp)	Temperature (°C)	Type	Concentration pmol/150 μL
Wt1	509-514	AGC CAG CTG AGC CAA TTC AT	20	52.3	Wild	12.5
Wt2	514-520	TTC ATG GAC CAG AAC AAC CCG	21	59.8	Wild	12.5
Wt3	521-525	G CTG TCG GGG TTG ACC	16	54.0	Wild	100.0
Wt4	524-529	TTG ACC CAC AAG CGC CGA	18	58.0	Wild	50.0
Wt5	530-534	CTG TCG GCG CTG GGG C	16	58.0	Wild	100.0
Mt1	511 CTG-CCG	AGC CAG CCG AGC CAA TTC AT	20	59.4	Mutant	50.0
Mt2a	516 GAC-GGC	TTC ATG GGC CAG AAC AAC C	21	58.0	Mutant	50.0
Mt2b	516 GAC-TAC	TTC ATG TAC CAG AAC AAC CCG	19	52.9	Mutant	50.0
Mt4a	526 CAC-GAC	TTG ACC GAC AAG CGC CGA	18	58.0	Mutant	100.0
Mt4b	526 CAC-CGC	TTG ACC CGC AAG CGC CG	17	58.0	Mutant	200.0
Mt4c	526 CAC-TAC	TTG ACC TAC AAG CGC CG	17	58.0	Mutant	200.0
Mt5	531 TCG-TTG	CTG TTG GCG CTG GGG C	16	56.0	Mutant	50.0

Before hybridization, the membrane was washed in 2×SSPE/0.1% SDS for 5 min at 50 °C. 50 μL of the amplified PCR product along with 150 μL of 2×SSPE/0.1% SDS was heat-denatured at 100 °C for 10 min, and transferred to an ice bath. The denatured DNAs were added into the channel, which is perpendicular to the immobilized probe rows, and then hybridized for 60 min at 60 °C. After hybridization, the membrane was washed twice for 10 min with 2×SSPE/0.5% SDS at a predetermined optimal hybridization temperature. The membrane was incubated in 20 mL of 2×SSPE/0.5% SDS mixed with streptavidin-AP conjugate (Roche, 11093266910) at 42 °C for 40 min, and then the unbound conjugate was removed by washing twice in 2×SSPE/0.5% SDS buffer for 10 min each at the same temperature. The membrane was washed twice in 2×SSPE for 5 min at ambient temperature, and incubated with 2.5 mL of CDP-Star (Roche) for 4 min. Finally, the membrane was exposed to an X-ray film (IAEA) for 1 h, dried at RT, and then the results were recorded.

Statistical Analysis

The differences in *rpoB* mutations between DNA sequencing and RDBH assay were analyzed by the Pearson's chi-square test. All statistical analyses were performed using SPSS 18.0 software, and $P<0.05$ was considered to be statistically significant.

RESULTS

Conventional Identification of M. tuberculosis Clinical Isolates

Out of the 300 clinical isolates of *M. tuberculosis*, 67 isolates were fully susceptible to RIF, INH, SM, EMB, and PAS, while 233 isolates were resistant to one or more of these anti-tuberculosis drugs. Of the 233 drug-resistant isolates, 181 (60.3%) isolates were resistant to RIF, but only 28 strains were mono-resistant to RIF, while 52 were relatively RIF-sensitive. As shown in Table 2, of these 181 RIF-resistant isolates, 130 (71.8%) were also resistant to INH, i.e. MDR-TB strains. In addition, 112 (61.9%) were resistant to SM, 28 (15.5%) were resistant to EMB, and 28 (15.5%) were PAS-resistant.

DNA Sequencing of the rpoB Gene

As compared to the results of conventional DST, 283 of the 300 samples were correctly identified by DNA sequencing analysis (94.3% accuracy). Among these isolates, 166 (91.7%) of the 181 RIF-resistant strains showed 32 different genotypic mutations, including single-point and multiple-point mutations. A total of 150 (82.9%) isolates were identified as single mutations involving the most frequent mutation sites in codons 531 and 526, while others were distributed in codons 511, 513, 514, 516, 522, and 533 (for details, refer to Table 3). Codon 531

Table 2. Distribution of Drug-resistant Phenotypes among 233 Clinical Isolates of *M. tuberculosis*

No. of Isolates	Phenotypes of Drug Resistance				
	INH*	RIF*	SM*	EMB*	PAS*
RIF-resistance isolates (*n*=181)					
49	R#	R	R	S#	S
34	R	R	S	S	S
28	S	R	S	S	S
23	R	R	R	S	R
19	S	R	R	S	S
14	R	R	R	R	S
5	R	R	S	R	S
5	R	R	R	R	R
2	S	R	S	R	S
2	S	R	R	R	S
RIF-sensitive isolates (*n*=52)					
18	R	S	S	S	S
13	R	S	R	S	S
11	R	S	R	R	S
6	R	S	R	S	R
2	R	S	R	R	R
1	R	S	S	R	R
1	S	S	R	S	S

Note. *INH, isoniazid; RIF, rifampin; SM, streptomycin; EMB, ethambutol; PAS, para-aminosalicylic acid; #R, resistant; S, sensitive.

Table 3. Comparative Analysis between RDBH, DNA Sequencing, and DST

DNA Sequencing		RDBH Assay	DST to RIF		
Nucleotide changes	Amino acid changes		Strains (*n*=300)	R (*n*=181)	S (*n*=119)
531 TCG-TTG	Ser-Leu	Wt5-, Mt5+	88	88	
533 CTG-CCG	Leu-Pro	Wt5-, Mt5-	7	6	1
511 CTG-CCG	Leu-Pro	Wt1-, Mt1+	2	1	1
513 CAA-AAA	Gln-Lys	Wt1-, Mt1-	1	1	
513 CAA-CCA	Gln-Pro	Wt1-, Mt1-	1	1	
514-515 TTC insertion	Phe insertion	Wt1-, Mt1-	1	1	
516 GAC-TAC	Asp-Tyr	Wt2-, Mt2b+	3	3	
516 GAC-GTC	Asp-Val	Wt2-	2	2	
516 GAC-GGC	Asp-Gly	Wt2-, Mt2a+	2	2	
522 TCG-ATG	His-Met	Wt2-	1	1	
526 CAC-GAC	His-Asp	Wt4-, Mt4a+	14	14	
526 CAC-AAC	His-Asn	Wt4-	11	11	
526 CAC-TAC	His-Tyr	Wt4-, Mt4c+	6	6	
526 CAC-CGC	His-Arg	Wt4-, Mt4b+	4	4	
526 CAC-CTC	His-His	Wt4-	3	3	
526 CAC-TGC	His-Cys	Wt4-	2	2	
526 CAC-AGC	His-Ser	Wt4-	1	1	
526 CAC-GGC	His-Gly	Wt4-	1	1	
509 AGC-AG, 510-511 deletion, 512 AGC-C	base C deletion, base AG deletion	Wt1-, Mt1-	1	1	
510 CAG-CAC, 526 CAC-TAC	Gln-His, His-Tyr	Wt4-, Mt4c+	1	1	
511 CTG-CCG, 505 TTC-CTC	Ler-Pro, Phe-Leu	Wt1-, Mt1+	1	1	
511 CTG-CCG, 515 ATG-CTG	Leu-Pro, Met-Val	Wt1-, Wt2-, Mt1+	2	2	
511 CTG-CCG, 512 AGC-ACC, 515 ATG-ATC	Leu-Pro, Ser-Thr, Met-Ile	Wt1-, Mt1+	1	1	
511 CTG-CCG, 516 GAC-TAC	Leu-Pro, Asp-Tyr	Wt1--Wt2-, Mt1+, Mt2b+	2	2	
511 CTG-CCG, 516 GAC-GGC	Leu-Pro, Asp-Gly	Wt1--Wt2-, Mt1+, Mt2a+	2	2	
511 CTG-CCG, 526 CAC-AAC	Leu-Pro, His-Asn	Wt1-, Wt4-, Mt1+	1	1	
516 GAC-GGC, 533 CTG-CCG	Asp-Gly, Leu-Pro	Wt2-, Wt5-, Mt2a+	1	1	
516 GAC-GCC, 533 CTG-CCG	Asp-Ala, Leu-Pro	Wt2-, Wt5-	1	1	
516 GAC-TAC, 526 CAC-CAA	Asp-Tyr, His-Gln	Wt2-, Wt5-, Mt2b+	1	1	
516 GAC-TTC, 526 CAC-AAC	Asn-Phe, His-Asn	Wt2-, Wt4-	1	1	
516 GAC deletion, 541 GAG-GGG	Asn deletion, Glu-Gly	Wt2-	1	1	
No mutation	no mutation	Wt1+-Wt5+	134	17	117

(TCG-TTG) was the most frequent site of mutations conferring RIF resistance on these strains, which accounted for 48.6%. Codon 526 was the most diverse site of mutation, with eight different amino acid substitutions-GAC (Asp), AAC (Asn), TAC (Tyr), CGC (Arg), CTC (His), TGC (Cys), AGC (Ser), and GGC (Gly), with two isolates CAC (His)-CTC (His) of synonymous mutations to be covered. Furthermore, combined mutations were observed in 16 (8.8%) RIF-resistant isolates. In addition to point mutations, we also detected two deletion mutations and one insertion mutation, including codon 516 GAC (Asp) deletion; 509 AGC (Ser) absence of base C combined codon 510, 511 deletion and only C left in 512 AGC, besides, TTC (Phe) insertion between codons 514 and 515. 117 of the 119 RIF-sensitive strains did not show any mutation in RRDR of *rpoB* gene (98.3% specificity). The PPV and NPV were 98.8% and 88.6%, respectively.

In addition, 10 (6.54%) out of 153 strains resistant to RIF did not show any mutation in the fragment of *rpoB* gene, and four isolates had no mutation among the 28 RIF mono-resistant strains based on DNA sequencing. Moreover, we found no mutation in 67 isolates fully susceptible to RIF, INH, SM, EMB, and PAS, while two of the 52 relatively RIF-sensitive samples (Table 2) that were resistant to INH and SM were found to have mutations in 511 CTG (Leu)-CCG (Pro) and 533 CTG (Leu)-CCG (Pro). Based on these data, there appeared to be no correlation between the mutation rates of RIF mono-resistant strains and poly-resistant strains that were resistant to RIF and other drugs (χ^2=1.40, P>0.05). Similarly, there was no correlation between the mutation rates of fully drug-sensitive strains and relatively RIF-sensitive strains (χ^2=0.81, P>0.05) (Tables 4 and 5).

RDBH Assay

Five Wt probes (Wt1-Wt5) and seven Mt probes (Mt1, Mt2a, Mt2b, Mt4a, Mt4b, Mt4c, Mt5) (Table 1, Figure 1) were used to detect the mutations in the RRDR of *M. tuberculosis*. We tried three hybridization-based melting temperatures (50 °C, 55 °C, and 60 °C) (Table 1) of the designed oligonucleotides, and found that non-specific hybridization and background noise were absent at 60 °C. Therefore, this temperature was selected as the optimal hybridization temperature. A clear and identifiable signal from the given probes was recorded as 'positive'. When all the Wt probes reacted positively and the Mt probes were negative, the experimental strain was deemed to be susceptible to RIF; lack of hybridization signal for one or more Wt probes demonstrated that the strains were a mutant genotype. Furthermore, when one of the Mt probes was positive (Mt+) and the corresponding Wt probe signal was absent (Wt-), it indicated the presence of a mutation in the target codon.

167 strains were considered to be RIF-resistant by RDBH assay, majority of the mutations (120, 71.9%) were directly determined by four Mt probes (Mt5, Mt4a, Mt4c, and Mt4b), and the rate rose to 83.2% if an uncertain mutant type was involved (Table 6).

Table 4. Comparison between RIF Mono-resistant Strains and Strains Resistant to RIF and Other Drugs

Drug-resistance	DNA Sequencing		Total
	With mutation	Without mutation	
Mono-resistance	24	4	28
Resistant to RIF and others	143	10	153
Total	167	14	181

Note. χ^2=1.40, P>0.05.

Table 5. Comparison between Fully Susceptible and Relatively RIF-sensitive Strains

Drug-resistance	DNA Sequencing		Total
	With mutation	Without mutation	
Fully susceptible	0	67	67
Relatively RIF-sensitive	2	50	52
Total	2	117	119

Note. χ^2=0.81, P>0.05.

As compared to the results of DST, 165 (91.2%) out of 181 strains were identified as RIF-resistant, of which the mutation type could be accurately determined in 132 (72.9%) samples, and we were unable to identify the mutation type of 33 (18.2%) isolates due to lack of hybridization signal in both wild-types and mutant types. Of the 119 RIF-sensitive isolates, a total of 117 (98.3%) isolates

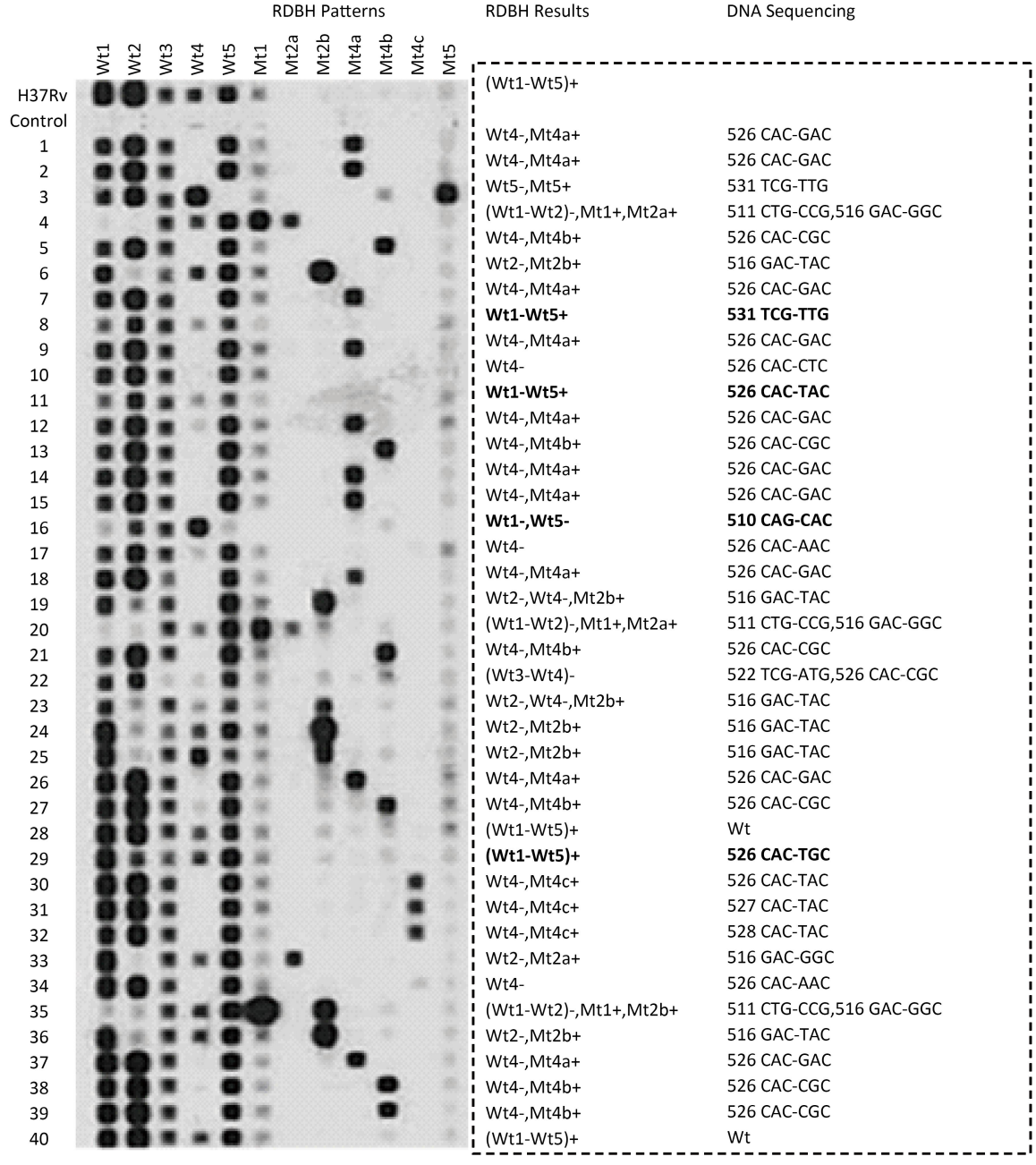

Figure 1. Detection and comparative analysis between blot hybridization map and DNA sequencing. H37Rv: positive control; Control: negative control; 1-40: RIF-resistant clinical isolates; Wt-: react negatively in Wt probe; Wt+: react positively in Wt probe; Mt+: react positively in Mt probe. Boldface: inconsistent results between RDBH assay and DNA sequencing.

hybridized only with Wt probes, and the remaining two had no Wt signal, one of them had strong signal in probe Mt1 and the other was negative for both Wt5 and Mt5 probes (Table 7).

Among the 168 *M. tuberculosis* strains that had shown alterations in the RRDR of the *rpoB* gene based on DNA sequencing, 164 (97.6%) showed same results by the RDBH (Table 8). However, the RDBH failed to identify four isolates that were phenotypically RIF-resistant, of which two harbored mutation in 531 (TCG-TTG), and the other two had a mutation at codon 526 where CAC (His) was replaced by TAC (Tyr) or TGC (Cys). Besides, three out of 129 RIF-sensitive strains confirmed by DNA sequencing showed mutations by RDBH, of which two had interpretable signal in probe Mt5 (531TCG-TTG) while Wt5 (531 Ser) reacted negatively, the remaining one showed no hybridization signal in both Wt5 and Mt5 but harbored a rare mutation in 533 CTG-CCG by sequencing. Other disagreements between DNA sequencing and RDBH were as follows: codon 526CAC in three strains was replaced by TGC, CGC, and TAC showed 526 (CAC-GAC) by RDBH; some rare mutations such as 533 (CTG-CCG), 510 (CAG-CAC), 512 (AGC-ACC), 515 (ATG-ATC) in three combined mutation isolates were not correctly detected by RDBH. In conclusion, the accuracy, PPV and NPV of RDBH were 97.7%, 98.2%, and 97.0%, respectively, when compared to DNA sequencing.

Table 6. Distribution of *rpoB* Gene mutations in 167 Clinical Isolates of *M.tuberculosis* by RDBH Assay

RDBH Assay	Mutation Type	No. of Isolates	Constituent Ratio (%)
Wt5-, Mt5+	531TCG-TTG	89	53.29
Wt4-, Mt4a+	526CAC-GAC	17	10.18
Wt4-	524-529 uncertain mutant type	10	5.99
Wt5-, Mt5-	530-534 uncertain mutant type	9	5.39
Wt4-, Mt4c+	526CAC-TAC	8	4.79
Wt4-, Mt4b+	526CAC-CGC	6	3.59
Wt1-, Mt1+	511CCG-CTG	4	2.40
Wt1-, Mt1-	509-514 uncertain mutant type	3	1.80
Wt2-, Mt2b+	516GAC-TAC	3	1.80
Wt2-	514-520 uncertain mutant type	3	1.80
Wt1-, Wt2-, Mt1+	511CTG-CCG+514-520 uncertain mutant type	3	1.80
Wt2-, Mt2a+	516GAC-GGC	2	1.20
Wt1-Wt2-, Mt1+, Mt2b+	511CTG-CCG+516GAC-TAC	2	1.20
Wt1-, Wt2-, Mt2a+	511CTG-CCG+516GAC-GGC	2	1.20
Wt3-	521-525 uncertain mutant type	1	0.60
Wt1-, Wt4-, Mt1+	511CTG-CCG+524-529 uncertain mutant type	1	0.60
Wt1-, Wt4-	509-520 uncertain mutant type	1	0.60
Wt2-, Wt4-, Mt2b+	516GAC-TAC+524-529 uncertain mutant type	1	0.60
Wt2-, Wt4-	514-520, 524-529 uncertain mutant type	1	0.60
Wt2-, Wt5-, Mt2a+	516GAC-GGC+530-534 uncertain mutant type	1	0.60
Total		167	100

Table 7. Analysis of RDBH and DST

RDBH Assay	DST Method		Total
	With mutation	Without mutation	
With mutation	165	2	167
Without mutation	16	117	133
Total	181	119	300

Table 8. Analysis of RDBH and DNA Sequencing

RDBH Assay	DNA Sequencing		Total
	With mutation	Without mutation	
With mutation	164	3	167
Without mutation	4	129	133
Total	168	132	300

DISCUSSION

It is well known that the mutation of *rpoB* gene, which encodes the β-subunit of the RNA polymerase, is the predominant mechanism for conferring RIF resistance. A previous study showed that about 90%-95% RIF-resistant strains had *rpoB* gene mutation, especially in the hot-spot region corresponding to codons 507-533[13-14]. This provided evidence to identify drug susceptibility by analyzing the genotypes of clinical isolates. Many studies have emphasized the importance of DNA sequencing analysis because it is intuitive, reliable and rapid. However, it is not feasible in most clinical laboratories in developing countries, since it requires an expensive automated sequencer[14-15]. Recently, Xpert MTB/RIF assay, a hemi-nested real-time PCR based method to detect the amplified specific target fragment of *rpoB* gene was recommended by the WHO for rapid diagnosis of drug-resistant *M. tuberculosis* clinical isolates[16-17]. Additionally, two novel commercial genotypic tests, INNO-LiPA Rif.TB (Innogenetics, Belgium) and Genotype MTBDR*Plus* (Hain Diagnostika, Germany) have been developed for the identification of mutations in the *rpoB* hot-spot region. These tests are based on reverse hybridization, which is seen as signals on membrane-bound capture probes. LiPA can detect the presence of resistance to RIF, while the GenoType MTBDR*Plus* assay can be used for the simultaneous detection of resistance to INH and RIF[9]. A systematic review and meta-analysis indicated that the pooled sensitivity and specificity of LiPA and MTBDR*Plus* were 94.1%, 95.9% and 98.8%, 98.0%, respectively[18]. Although the two tests showed high sensitivity and specificity, the kits are not affordable in some poor countries, where they are desperately needed. In contrast, our RDBH assay can be easily performed in laboratories where a PCR Amplifier is available instead of special instruments. Above all, the RDBH assay is simple, efficient, and accurate, which shows a promising prospect in clinical application.

As shown in this study, 165 of the 181 RIF-resistant and 117 of the 119 RIF-sensitive strains were correctly identified by RDBH as compared to conventional tests, with 91.2% sensitivity and 98.3% specificity. As compared to the results of DNA sequencing, the RDBH assay successfully detected 97.6% of the RIF-resistant isolates with alterations. Only three samples, which showed mutations by sequencing, were considered as WT by the RDBH. In agreement with previous reports[19], the most frequently observed mutations were Ser531Leu (48.6%, 88/181). This frequency was higher than the data in United States (32%, 69/124)[20], and lower than in Brazil (64%, 48/74) and Syria (56.5%, 39/69)[21-22]. The combined mutation rate of codons 531 and 526 in our study was 74.0% (134/181), which was higher than the published finding by Hairong Huang[23]. This demonstrated that 531 and 526 were the predominant mutable codons in RIF-resistant strains. We also found two novel mutation types outside the RRDR, i.e. 505 TTC-CTC and 541 GAG-GGG, and similar findings were reported in China[12,24]. Also, it is notable that 511 alterations were involved in seven combined mutations. It was suggested that 511 Leu could easily mutate after the other codons change, or alternatively, when 511 Leu was substituted, the other amino acids would become unstable[23]. Furthermore, a rare mutation 511 CTG-CCG was simultaneously found by DNA sequencing and RDBH, although it showed RIF-sensitivity by DST. In addition, a 533 CTG-CCG mutation was also detected by sequencing and expressed no hybridization signal at the Wt5, but it was considered to be RIF-sensitive by DST. This was because codon 533 was involved in the sequencing of Wt5, and blocked the specific base pairing. This phenomenon was also observed by Eunjin Cho et al.[25]. These inconsistent results implied that the limitation of redesigned specific probe hinders the detection of mutation in target region. Besides, DST could not detect two isolates in our test, which were found to have mutation by the other two assays. Additionally, another contradictory result by different methods confirmed that some mutations are outside the RRDR or have other resistance mechanisms, such as the efflux pump, which was suggested to play a role in RIF-resistant clinical strains of *M. tuberculosis* with no mutation in the RRDR[26]. Therefore, the negative results should be considered together with the clinical presentation to form an overall judgment. Our data is similar to the study by Maschmann Rde A et al., which showed that the RDBH assay for the *rpoB* gene reached 92.3% and 90.6% sensitivity, and 98.1% and 100% specificity when compared with DST and sequencing, respectively[27]. Moreover, 167 isolates showed mutations by RDBH, which mainly consist of 58.7% (98/167) with no signal in probe Wt5 (89 isolates had strong signal in probe Mt5), 24.6% (32/167) with no signal in probe Wt4 (526 CAC replaced by GAC, TAC, CGC), and 4.2% (4/167) with clear spot at Mt2. The

results were similar to the study on molecular characterization of *rpoB* gene from Sichuan and Tianjin, China[12,28]. Currently, the RDBH assay also has the disadvantages: It is not a robotic method, and requires more time (about 6 h) as compared to other automated systems and needs several reagents and buffers from different distributors, eg EDAC, streptavidin-AP conjugate, CDP-Star. Furthermore, we also identified false-negative or non-specific results. Future studies should expand the detection coverage of the probe, and optimize the dot blot conditions by changing the probe concentration and adjusting the hybridization temperature.

In conclusion, it takes only two days from DNA extraction to identification using the RDBH method, which also sharply reduces the DST's experimental time, and improves the efficiency of diagnosis. Given the high sensitivity, specificity and superior consistency of the probe performance with DST, 45 specimens were simultaneously examined with RDBH. Therefore, the RDBH is an efficient, simple, and reliable method, which can be applied as a screening test for detecting RIF-resistance in clinical isolates of *M. tuberculosis*.

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AUTHOR CONTRIBUTIONS

WAN Kang Lin and WU Yi Mou conceived and designed the experiments. ZHAO Xiu Qin, LIU Zhi Guang, and ZHANG Yuan Yuan collected and cultured the clinical strains, and obtained all the information on the patients. GUO Qian, YU Yan, ZHU Yan Ling, LI Gui Lian, and WEI Jian Hao performed the experiments, and analyzed the data. WAN Kang Lin and WU Yi Mou contributed reagents, materials, and analysis tools. WAN Kang Lin and GUO Qian wrote the paper.

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