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Original Article

Reverse dot blot hybridization for detection of *gyrA* mutation for rapid diagnosis of *Mycobacterium tuberculosis* resistant to fluoroquinolones

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Abstract: Rapid detection and timely treatment are of great significance to reduce *Mycobacterium tuberculosis* (*M. tuberculosis)* transmission, especially in cases suffering from the infection of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *M. tuberculosis*. Here we have developed a reverse dot blot hybridization (RDBH) technique for rapid diagnosis of *M. tuberculosis* resistance to fluoroquinolones (FQ). One wild-type and seven mutant oligonucleotide probes were designed to detect the *gyrA* gene common mutations conferring to FQ resistance. A total of 160 clinical *M. tuberculosis* isolates including 83 FQ-resistant and 77 FQ-sensitive strains were analyzed in this study. Compared with the results of culture-based phenotypic drug susceptibility test (DST), the overall sensitivity, specificity and accuracy of this RDBH assay were 71.1% (59/83), 100% (77/77) and 85.0% (136/160), while compared with direct DNA sequencing method, the sensitivity, specificity and accuracy of the RDBH were 93.7% (59/63), 100% (97/97) and 97.5% (156/160), respectively. Therefore, the newly-established RDBH assay is rapid, simple and efficient method to detect fluoroquinolones resistance of M. tuberculosis and can hold the promise for screening FQ-resistance in the clinical samples from the patients with tuberculosis.

Keywords: *Mycobacterium tuberculosis*, fluoroquinolones resistance, reverse dot blot hybridization

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), ranks as the second leading cause of death among infectious diseases worldwide [1]. Rapid case detection and timely treatment are of great significance to reduce *M. tuberculosis* transmission, especially in cases suffering from the infection of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *M. tuberculosis*. MDR *M. tuberculosis* is defined as the strain resistance to at least both isoniazid (INH) and rifampin (RIF), whereas XDR *M. tuberculosis* is MDR with additional resistant to both a fluoroquinolone (FQ) and at least one of the following second-line injectable agents: capreomycin (CAP), kanamycin (KAN), and amikacin (AMK), constitutes an emerging threat for effective TB prevention and control [2]. In 2014, World Health Organization (WHO) reported that an estimated 9% of MDR-TB were XDR-TB worldwide, China alone accounted for 11% of total cases and an estimated 5.7% of new TB cases were MDR-TB [3].

Fluoroquinolones have been demonstrated to have high in vitro activity against *M. tuberculosis* and used as backbone drugs recommended to treat MDR-TB [4]. The main target of FQ in *M. tuberculosis* is DNA gyrase, a $GyrA_2GyrB_2$ tetrameric enzyme consist of two A and two B subunits encodes by *gyrA* and *gyrB* genes, respectively. The mutations in the specific *gyrA* region which is called the fluoroquinolone resistance-determining region (QRDR) are the predomi-

nately mechanism conferring FQ resistance of *M. tuberculosis* [5, 6]. Some researches showed that approximately 50%-91% of FQ-resistant *M. tuberculosis* isolates bore missense mutations in QRDR of *gyrA* (codons 88 to 94) [7-10]. Therefore, it is critical to identify the mutations associated with FQ-resistance as soon as possible for timely adequate adjusting treatments and improving the outcome of patients.

Generally, culture-based tests remain the gold standard for detecting drug-resistant *M. tuberculosis*, but it takes at least 2-4 weeks after the isolate has been cultivated. Therefore, nucleic acid-based drug susceptibility methods are considered more attractive as diagnostic means. Currently, WHO has endorsed the molecular probe assays to detect common mutations conferring resistance to specific anti-tuberculosis drugs [1]. Two commercial detecting system: GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) and GenoType MTBDR*plus* (Hain Life Science GmbH, Nehren, Germany), which indirectly identify mutations by lack of probe hybridization to wild-type loci have been recommended in clinical applications [11-13]. However, the technologies can not detect the resistance to second-line drugs. Although the novel Hain Life Science GenoType MTBDRs*l*molecular kit were evaluated for second-line drugs and EMB resistance-associated mutations, the expensive equipment and consumables has also hindered the widespread use in TB high-burden and low-income countries.

The reverse dot blot hybridization (RDBH) assay has been widely performed in the spoligotyping technique for *M. tuberculosis* molecular genotyping identification [14, 15]. Similarly, the method has been developed to identify the mutations that confer resistance to antibiotics. In this study, we established a RDBH method for simultaneous identification of seven common *gyrA* gene mutations and further blind evaluate the sensitivity and specificity of this assay compared to the results of conventional drug susceptibility test (DST) based on bacterial culture and DNA sequencing [16, 17].

Materials and methods

Clinical isolates

A total of 160 clinical *M. tuberculosis* isolates were randomly obtained from different patients with TB in Fujian, Tibet, Henan, Sichuan, Hunan province of China and *M. tuberculosis* reference strain H37Rv was provided by Tuberculosis Laboratory of the National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC) in Beijing used as the standardization reference of the method.

Phenotypic drug susceptibility testing

All isolates were initially identified as *M. tuberculosis* by PNB/TCH differential media. The drug susceptibility testing with the proportion method on L-J medium was performed according to WHO guidelines by using recommended concentration (2 mg/mL for Ofloxacin) [16]. The critical growth proportion for resistance was 1%.

Genomic DNA extraction

Genomic DNA was extracted from fresh mycobacteria colonies grown on L-J media slants by resuspending in 500 ml TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), then incubated at 95°C for 30 min. After centrifugation for 3 min at 12000 rpm, the supernatants were recovered and stored at -20°C before use.

PCR amplification and sequencing of the gyrA gene

A 314-bp fragment of *gyrA* QRDR was amplified using *gyrA* primers (forward primer, biotinylated at the 5' end: 5'-bio-GGGTGCTCTATGCAATGTTCG-3'; reverse primer: 5'-GCCGTCGTAGTTAGGGATGA-3'). The PCR was standardized in a total reaction volume of 50 mL containing 25 mL 2× Hot Taq MasterMix (Sibino Biotech, Beijing, China), 1 mL (10 mmol/mL) of each primer, 2 mL (20-200 ng/mL) genomic DNA and added DD H_2O to 50 mL. PCR was done as follows: pre-denaturation at 94°C for 5 min, 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, then the final extension 10 min at 72°C.

The PCR products were sent to TsingKe Company (Beijing, China) for sequencing, and then the results were aligned with the homologous sequences of *M. tuberculosis* reference strain H37Rv by using personalized Perl script.

Hybridization and detection

We designed 8 oligonucleotide probes (**Table 1**) based on wild-type (wt) and common mutant type (mt) were immobilized onto the negatively-charged nylon membrane (Biodyne C, Pall

Table 1. The information of eight probes used for detecting *gyrA* gene of *M. tuberculosis*

Probe	Codon	Genotype	Sequence
pwt	90-94	Wild-type	C GCG TCG ATC TAC GAC A
pm1	90	Mutant type (GTG)	TC GTA GAT CGA CAC GTC G
pm2	91	Mutant type (CCG)	C GCG CCG ATC TAC GAC A
pm3a	94	Mutant type (GGC)	GT GCC GTA GAT CGA CGC
pm3b	94	Mutant type (GCC)	G TCG ATC TAC GCC ACC CTG
pm3c	94	Mutant type (TAC)	ATC TAC TAC ACC CTG GTG C
pm3d	94	Mutant type (AAC)	C GCG TCG ATC TAC AAC AC
pm3e	94	Mutant type (CAC)	C GCG TCG ATC TAC CAC AC

Corporation) in parallel lines. 20 mL of each PCR product was diluted in 150 mL 2× SSPE/0.1% SDS and heat-denatured at 100°C for 10 min, then cooled on ice immediately. The denatured single-stranded DNAs were applied on the prepared membrane in the miniblotter slots and hybridized at 60°C for 1 h. The membrane was washed twice each time for 10 min with 2× SSPE/0.5% SDS buffer at 50°C after hybridization. The membrane was subsequently incubated at 42°C for 40 min with 1:2000 diluted streptavidin-AP conjugate in 20 mL 2× SSPE/0.5% SDS, and then the unbound conjugate was removed by washing twice in 2× SSPE/0.5% SDS for 10 min at the same temperature, rinsed once in 2× SSPE for 5 min at ambient temperature. The chemiluminescent detection was under the condition of protection from light accession KPL color-developing agent following the manufacturer's instructions (KPL, Inc) at 42°C for 40 min. Finally, the results were recorded.

A clear and identifiable purple-blue spot was recorded as 'positive'. When the wild-type probes reacted positively and all the seven mutant type probes were negative, the experimental clinical isolate was recorded susceptible to FQ; Only the signal of mutant probes whose color were much stronger than the wild-type probe, the strains were considered as mutant genotypes (**Figure 1**). In order to ensure the quality and repeatability of this research, all the strains were repeated detected by RDBH.

Statistical analysis

A Pearson's chi-square test was used to analyze the sensitivity and specificity of the RDBH assay in comparison with the DST. The consistency analysis on the results of the different methods was used by Kappa identity test. Kappa value was used for evaluating the agreement to compare the two methods, e.g., RDBH and DNA sequencing, respectively. Kappa value below 0.40 revealed weak consistency, values between 0.41-0.60 indicated moderate agreement and values above 0.60 demonstrated good agreement [18]. All statistical analyses were performed using SAS 9.2 software.

Results

Phenotypic drug susceptibility testing of M. tuberculosis clinical isolates

Out of the 160 *M. tuberculosis* clinical isolates, 83 were fluoroquinolone resistant and the remaining 77 strains were identified as fluoroquinolone sensitive by means of conventional proportion method.

DNA sequencing of the gyrA gene

The DNA sequencing indicated that the AGC→ACC alteration at codon 95 was found in all of the clinical *M. tuberculosis* isolates. As compared to the DST results, no mutation was observed in FQ-sensitive strains and 63 (75.9%) of the 83 fluoroquinolone resistant *M. tuberculosis* bear *gyrA* mutations in QRDR. Among these 63 *gyrA* mutation isolates, the point mutations were clustered at codon 90, 91 and 94. Position 94 was the most frequent and diverse site of mutation associated with FQ resistance, with six different amino acid substitutions-GGC (Gly), GCC (Ala), TAC (Tyr), AAC (Asn), CAC (His) and GTC (Val), which accounted for 50.6% of FQ resistant *M. tuberculosis* strains. Asp94Gly, Asp94Ala and Ala90Val were the most prevalent mutation type, totally harbored by 49.4% (n=41) of the isolates. In addition, two double sites mutations-Ala90Val+Asp94Ala, Ser91Pro+Asp94Ala were detected in our research (**Table 2**).

RDBH assay

A total of 83 FQ-resistant and 77 FQ-sensitive clinical *M. tuberculosis* isolates were tested by RDBH assay. The results showed that 59 (71.1%) of the FQ-resistant strains possessed *gyrA* gene mutations in the QRDR and 77 isolates phenotypically sensitive to FQ, including

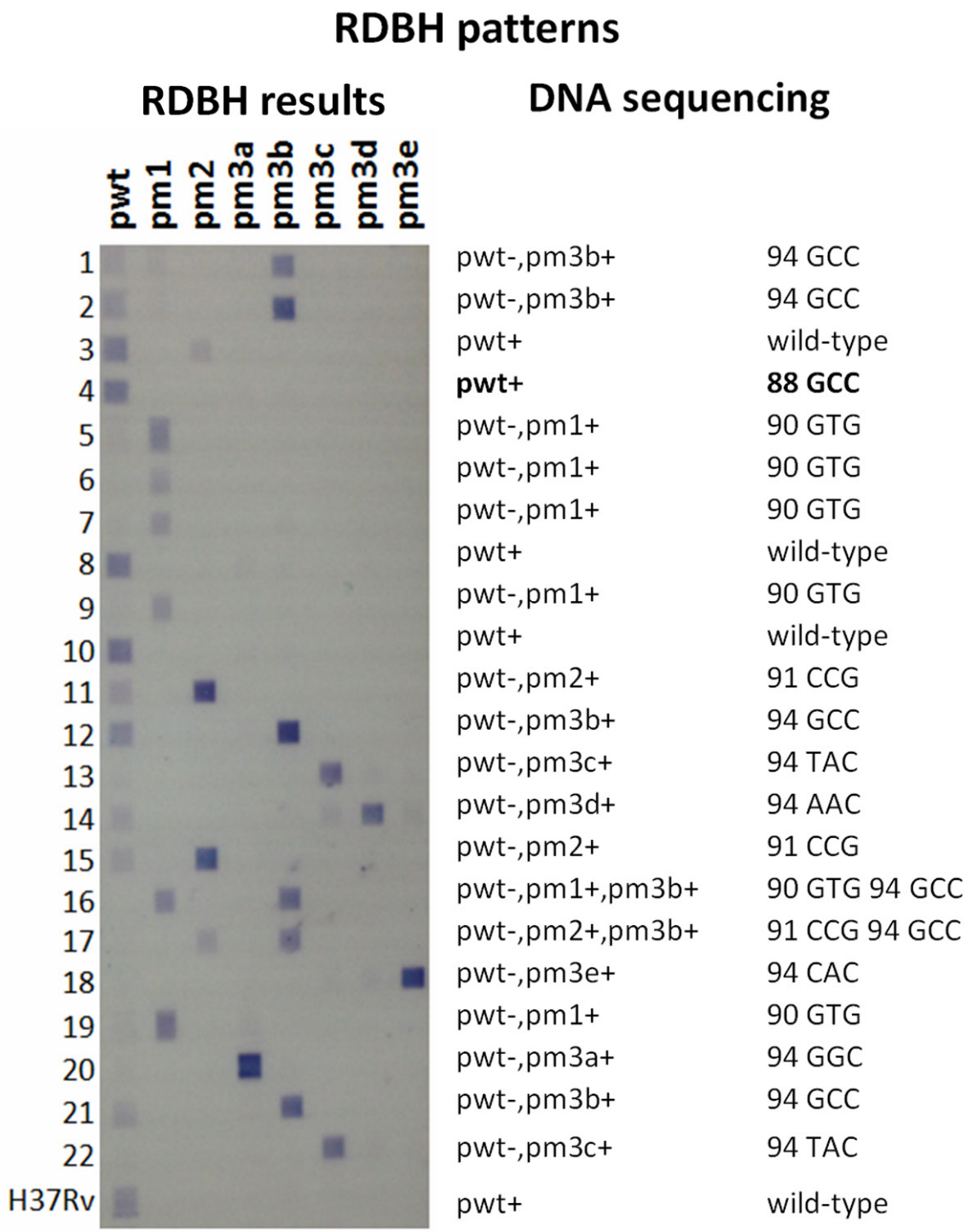

Figure 1. Reverse dot blot hybridization map of *gyrA* mutations conferring FQ resistance. H37Rv: negative control (pwt positive control); 1-22: FQ-resistant clinical isolates; pwt-: absence of wild-type probe spot or the wild-type spot was much weaker than mutant probe; pm+: presence of mutant probe spot; Bold: inconsistent results between RDBH and DNA sequencing.

H37Rv, had no mutations in *gyrA* QRDR. Compared to DST, the sensitivity, specificity, and accuracy of RDBH were 71.1% (59/83), 100% (77/77) and 85.0% (136/160), respectively. The kappa value was 0.70, showing good agreement between RDBH and DST.

Table 2. Distribution of gyrA gene mutations in 63 clinical isolates of *M. tuberculosis*

DNA Sequencing		RDBH assay	No. of isolates	Mutation frequency (%)
Codon	Mutation type			
88	GGC→GCC(Gly→Ala)	pwt+	2	2.4
90	GCG→GTG(Ala→Val)	pwt-, pm1+	11	13.3
91	TCG→CCG(Ser→Pro)	pwt-, pm2+	8	9.6
94	GAC→GGC(Asp→Gly)	pwt-, pm3a+	18	21.7
94	GAC→GCC(Asp→Ala)	pwt-, pm3b+	12	14.5
94	GAC→TAC(Asp→Tyr)	pwt-, pm3c+	3	3.6
94	GAC→AAC(Asp→Asn)	pwt-, pm3d+	2	2.4
94	GAC→CAC(Asp→His)	pwt-, pm3e+	3	3.6
94	GAC→GTC(Asp→Val)	pwt-	2	2.4
90+94	GCG→GTG(Ala→Val) GAC→GCC(Asp→Ala)	pwt-, pm1+, pm3b+	1	1.2
91+94	TCG→CCG(Ser→Pro) GAC→GCC(Asp→Ala)	pwt-, pm2+, pm3b+	1	1.2
Total			63	75.9

Among the 63 *M. tuberculosis* strains that carried mutations in the QRDR of *gyrA* based on DNA sequencing, 57 (90.5%) were recorded the same results and four isolates failed to recognized by the RDBH assay. Among the six missed diagnosis samples, two had a single mutation located outside the target region of Wt probe-codon 88 (GGC→GCC), two had amino acid substitutions at codon 94 where GAC (Asp) was replaced by CAC (His) and GCC (Ala), and another two isolates which sequencing showed 94 GAC→GTC alteration, absent the both Wt and Mt probes hybridization signal in our assay were considered to had a mutation in codon 90-94, but the mutant type was not involved in our designed probes. The sensitivity, specificity and accuracy of RDBH assay for FQ resistance detection compared with DNA sequencing were 93.7% (59/63), 100% (97/97), 97.5% (156/160). The kappa value was 0.95, revealing excellent agreement between RDBH and DNA sequencing.

All the strains detected by RDBH assay repeatedly showed the same outcome.

Discussion

Fluoroquinolones are the potent second-line anti-tuberculosis drugs which have been widely applied to treat TB, especially for MDR-TB. The abuse and excessive use has lead to the increasing emergence of FQ-resistant *M. tuberculosis* in China [7]. Fluoroquinolones, predominately by inhibiting the DNA gyrase of *M. tuberculosis* to block DNA replication and transcription, result in bacterial death. A previous report showed that the missense mutations clustered in a conserved 320 bp nucleotide sequences of *gyrA* gene had been proved main mechanism conferring FQ resistance of *M. tuberculosis* [15]. Otherwise, the mutations found in *gyrB* may convey low levels of phenotypic FQ resistance [19].

In our study, 63 (75.9%) of 83 clinical *M. tuberculosis* isolates with phenotypic FQ resistance were observed *gyrA* QRDR mutations. The frequency of the mutation associated with FQ resistance was similar to the data of Shanghai (75.7%) [20] and France (78.0%) [21], higher than that in Russia (57%) [22] and Korea (69.2%) [23], but lower than the results of America (85.0%) [24]. Therefore, it was inferred that *gyrA* mutation in clinical *M. tuberculosis* isolates might vary in different geographical region and sampling. Our study indicated that the most frequently mutation type was 94 GAC→GGC, followed by 94 GAC→GCC and 90 GCG→GTG, which was responsible for 21.7%, 14.5%, 13.3%, respectively. This observation coincided with the research results from reported by Takiff et al [6], Hu Y [25] and Wang H [26]. Furthermore, we also observed that all clinical strains possessed codon 95 (AGC→ACC) mutation, which showed to be unrelated to fluoroquinolones resistance but considered as a natural polymorphism in *M. tuberculosis*.

The application of the reverse dot blot hybridization assay to investigate the incidence of *M. tuberculosis* resistance was reported in the world in recent years. As shown in our research, 59 of the 83 FQ-resistant and all the FQ-sensitive strains were successfully identified by RDBH as compared to DST, with 71.1% sensitivity and 100% specificity. As compared to the results of DNA sequencing, our assay could correctly detected 97.5% of FQ-resis-

tance. Moreover, the two double-sites mutation isolates were accurately caught by RDBH assay, showing that our method also had good sensitivity in detecting multiple locus mutations. However, the analysis of inconsistent results among DST, DNA sequencing and RDBH assay could be included as follows. Firstly, for the 20 FQ-resistant isolates carried no mutations in the *gyrA* QRDR in this study, we speculated that some mutations attributed to *gyrB* gene, either they were outside the QRDR or the resistance might be caused by other underlying mechanisms, such as drug efflux pumps as well as the decreased permeability of the outer membrane [27, 28]. Besides, we failed to detect the specific mutant type of samples which bear 88 GGC→GCC or 94 GAC→GTC alteration. It implied that certain limitation of probe design made some less frequently mutation type in "hot-spot region" to escape the detection.

The Global Tuberculosis Report 2014 indicated that drug resistance surveillance would expand to more countries and cover more key drugs such as Fluoroquinolones in order to understand and control the prevalence of drug resistance all over the world. Molecular technologies have been being incorporated into drug resistance surveys [29]. Currently, GenoType MTBDRsl was a reliable tool for rapid identification of second-line drugs resistance in laboratory studies, but so far in China, it has not received approval in clinic use. Additionally, the high cost of equipments and consumables as well as the potential requirement for laboratory facilities has limited its clinical application in some developing countries. In contrast, our RDBH technology is a probe-based method that can be easily performed in local laboratories where a PCR amplifier is available. 45 specimens could be examined simultaneously by RDBH, and this can reduce the cost of the assay, making it practical for large sample screening of any suspected MDR-TB patients in clinical laboratory.

To conclude, it takes only 6-8 h to complete RDBH assay post-DNA extraction that sharply reduce and simplify laboratory work [30]. Given the probe performed high sensitivity, specificity and superior agreement with DST and DNA sequencing. A lot of our experiments indicated that the technique has good reproducibility and stability. Therefore, the RDBH assay established in our study is a rapid, simple and efficient method, which can provide a good basis for developing the FQ-resistance detection kit and has a good application prospect being used for the diagnosis and surveillance of the drug resistance of M. tuberculosis to Fluoroquinolones. Furthermore, research is underway to concern if the RDBH assay could detect directly DNA extracted from clinical specimen, which will further accelerate the detection and play a vital role in FQ-resistant tuberculosis.

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Disclosure of conflict of interest

None.

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