Exploratory development of PCR-fluorescent probes in rapid detection of mutations associated with extensively drug-resistant tuberculosis

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Abstract
This study aims to evaluate the clinical value of PCR-fluorescent probes for detecting the mutation gene associated with extensively drug-resistant tuberculosis (XDR-TB). The molecular species identification of 900 sputum specimens was performed using polymerase chain reaction (PCR)–fluorescent probe. The mutations of the drug resistance genes \textit{rpoB}, \textit{katG}, \textit{inhA}, \textit{embB}, \textit{rpsL}, \textit{rrs}, and \textit{gyrA} were detected. The conventional drug susceptibility testing (DST) and PCR-directed sequencing (PCR-DS) were carried out as control. DST demonstrated that there were 501 strains of rifampicin resistance, 451 strains of isoniazid resistance, 293 strains of quinolone resistance, 425 strains of streptomycin resistance, 235 strains of ethambutol resistance, and 204 strains of amikacin resistance. Furthermore, 427 (47.44%) or 146 (16.22%) strains were MDR-TB or XDR-TB, respectively.

The mutations of the \textit{rpoB}, \textit{katG}/\textit{inhA}, \textit{embB}, \textit{rpsL}, \textit{rrs}, and \textit{gyrA} genes were detected in 751 of 900 TB patients by PCR-fluorescent probe method, and the rate of drug resistance was 751/900 (83.44%). No mutant genes were detected in the other 149 patients. Compared with DST, the mutant rates of \textit{rpoB}, \textit{katG}/\textit{inhA}, \textit{embB}, \textit{rpsL}, \textit{rrs}, and \textit{gyrA} genes were detected in 751 of 900 TB patients by PCR-fluorescent probe method, and the rate of drug resistance was 751/900 (83.44%). No mutant genes were detected in the other 149 patients. Compared with DST, the mutant rates of \textit{rpoB}, \textit{katG}/\textit{inhA}, \textit{embB}, \textit{rpsL}, \textit{rrs}, \textit{gyrA} of six drugs were higher than 88%; five of six drugs were higher than 90% except for SM (88.11%). The MDR and XDR mutant gene types were found in 398 (42.22%) and 137 (15.22%) samples. PCR-DS was also employed and confirmed the PCR-fluorescent probe method with the accordance rate of 100%. The PCR-fluorescent probe method is rapid and straightforward in detecting XDR-TB genotypes and is worthy of being applied in hospitals.

Keywords PCR-fluorescent probe · Extensive drug resistance · \textit{Mycobacterium tuberculosis} · Drug susceptibility testing
Background

Tuberculosis (TB) is a chronic infectious disease caused by Mycobacterium tuberculosis. According to the Global Tuberculosis Report 2019, in 2018, there were 10 million new TB cases [1]. It was reported that 3.4% of new patients and 18% of retreat-patients were multidrug-resistant tuberculosis (MDR-TB) and rifampicin-resistant TB (RR-TB), among which extensively drug-resistant tuberculosis (XDR-TB) accounted for 6.2%, and 1.45 million cases died. Nearly 210,000 patients died due to MDR/RR-TB [1]. There were 866,000 new TB cases in China, 66,000 rifampicin resistance cases, and 38,800 deaths, ranking the second in the world for many years. However, only one-third of the patients had access to treatment, far from achieving the goal of eliminating TB. MDR-TB is defined as infected M. tuberculosis resistant to at least both isoniazid (INH) and rifampicin (RFP) in vitro. XDR-TB refers to the infected M. tuberculosis that is not only resistant to INH and RFP but also resistant to at least one kind of fluoroquinolones and second-line anti-TB drug injection (kanamycin, amikacin, capreomycin). Once healthy people inhaled resistant M. tuberculosis, which was exhaled into the air by tuberculosis patients through cough, sneeze, or spit, it will develop as resistant tuberculosis in a certain period of their lives [2].

With the slow growth of M. tuberculosis, diagnosis and drug resistance detection become a challenging problem in clinical treatment [3]. The delayed diagnosis resulted in improper treatment of TB patients and increasing the rate of drug resistance of M. tuberculosis which enormously influenced the treatment’s effect. The emergence and transmission of MDR-TB and XDR-TB hindered TB infection control, thereby developing a stubborn disease. The MDR/XDR-TB is the critical reason for the high lethality of TB [4].

Generally, there are three types of drug resistance mechanisms in M. tuberculosis: (1) reducing cell membrane permeability and efflux pump mechanism; (2) generating catabolic and inactivated enzymes; and (3) altering drug target locus. Chromosome-mediated drug resistance is the primary basis of M. tuberculosis drug resistance [5]. At present, M. tuberculosis drug resistance mechanism research is mainly focused on the drug-targeted locus and the mutation-related genes. The current first-line drugs for TB treatment include isoniazid, streptomycin, rifampicin, pyrazinamide, and ethambutol. The second-line drugs are consisting of fluoroquinolones (levofloxacin, moxifloxacin), ethionamide and protonamide, and injectable drugs such as aminoglycoside (kanamycin and amikacin) and polypeptide antibiotic (capreomycin). Currently, significant drug-resistant genes of M. tuberculosis have been analyzed and identified. The drug-resistant mechanism of isoniazid, an anti-TB chemotherapy drug, is rather complicated, with 92% of the INH-resistant isolates associated with gene mutations from katG, inhA, and ahpC [6–9]. Rifampicin (RFP) targets a DNA-dependent RNA polymerase subunit β (rpoB) in M. tuberculosis, with 95% of the RFP-resistant isolates associated with rpoB gene mutations. The detection of rifampin resistance is a paramount indication of the MDR-TB [10, 11]. EmbB gene mutation interpreted the major molecular mechanism of the 50–60% of ethambutol (EMB) resistance [12, 13]. Moreover, 80% of clinical streptomycin (SM)–resistant isolates of M. tuberculosis were detected mutations in the rpsL or rrs gene [14]. Fluoroquinolones (FQs) included moxifloxacin (Mfx), levofloxacin (Lfx), and others. Mutations of the gyrA gene are related to drug concentration and structure, causing medium and high drug resistance of FQs. In contrast, gyrB gene mutation might result in low drug resistance of FQs by altering drug accumulation in the cell [15, 16]. Mutations associated with resistance to amikacin (AM) are located within rrs, which encodes the ribosomal 16S rRNA. The 60.5% of mutation was a single base substitution of 1401 (A→G). A minority of isolates were 1402 (C→T or A), 1484 (G→T), which occurred mainly on the high resistance strains [17]. The mutation of pncA accompanied by a decrease or loss of PZase activity is the main reason for pyrazinamide (PZA) resistance [18].

Currently, the routine clinical bacteriological laboratory examinations on TB are microscopic smear and culture methods. Conventional mycobacterium culture and drug sensitivity detection method, BactecMGIT960, has been used as the gold standard for drug resistance diagnosis in TB laboratories. Still, its detection cycle is too long to provide timely detection results for clinical practice [19]. GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA) is a new technology that can detect M. tuberculosis and rifampicin resistance. However, its biggest drawback is that it can only detect rifampicin resistance, not other first-line and second-line drugs [20–22]. In this study, we developed a mutation detection system of resistance with isoniazid (INH), rifampicin (RFP), streptomycin (SM), ethambutol (EMB), amikacin (AM), and fluoroquinolones (FQs) including moxifloxacin and/or levofloxacin by rapid detecting clinical sputum specimens with PCR fluorescence probe method, providing guidance to both establish the suitable MDR/XDR mutations detection system and carry out the practical and individualized treatment in the early stage.

Materials and methods

Collection of sputum specimens

We collected 900 cases of morning sputum specimens from 900 TB patients (214 cases were initially treated, 686 cases were retreated) in the TB department of the 8th Medical Center of PLA General Hospital and Heilongjiang Chest
Hospital from January to December 2018. The study protocol was approved by the Research Ethics Committee of the 8th Medical Center of Chinese PLA General Hospital. The informed consent was obtained from all adult participants and parents of the participant under 16 years old. All the 900 sputum samples were positive for acid-fast staining with Ziehl-Neelsen method [23].

**Instruments and reagents**

Mycobacterium nucleic acid detection reagent, fluorescence detection reagent of *M. tuberculosis* nucleic acid amplification, and real-time fluorescence quantitative PCR instrument (ABI7700) were provided by Capital Bio Corporation, Tsinghua University, Beijing. The flow chart of phenotypic drug susceptibility testing (DST), PCR-fluorescent probe method, and PCR-directed sequencing (PCR-DS) is shown in Fig. 1.

*Fig. 1* The flow chart of phenotypic drug susceptibility testing (DST), PCR-fluorescent probe method, and PCR-directed sequencing (PCR-DS)

**Phenotypic drug susceptibility testing**

The rapid culture and drug sensitivity tests of the BACTEC MGIT 960 System (BD Diagnostic, USA) were conducted following the protocol described in the “TB Laboratory Standardization Operation and Network Establishment” [24].

**Primers and probes for fluorescent probe method**

The genome sequences of *rpoB* (GenBank Accession Number: NC_000962, Nucleotide 759807-763325), *katG* (NC_000962, 2153889-2156111), *inhA* (NC_000962, 1674202-1675011), *rpsL* (NC_000962, 781560-781934), *embB* (NC_000962, 4246514-4249810), *gyrA* (NC_000962, 7302-9818), and *rrs* (NC_000962, 1471846-1473382) genes were downloaded from the NCBI database. The forward and reverse primers were designed and synthesized following our previous study [25]. The detailed information of the forward
and reverse primers can be found in Table 1. Probes of these genes were designed based on the resistance-related high-frequency mutation sites. The 5′ ends of these probes were labelled with different fluorescent reporter groups according to the type of drug resistance. The 3′ ends were sequentially labelled with a non-fluorescent quencher (NFQ) and minor groove binder (MGB). The probe primers are listed in Table 2.

**Detection of multiple mutation sites of drug resistance genes in *M. tuberculosis* by fluorescent probe method**

The RT-PCR amplification template was genomic DNA containing mutation sites of drug resistance genes or H37Rv wild-strain DNA. The mutant and wild-type genomes were sequenced. The PCR system consists of 5 sub-reaction systems, and the 5 sub-reaction systems are the same except for the different templates, primers, and probes (Table 1 and Table 2).

1. Sub-reaction system 1 detects mutations of *rpoB* and *katG* genes. The forward and reverse primers involved are respectively: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, and SEQ ID No. 4, and the probe primers involved are as follows respectively: SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 20, SEQ ID No. 21, and SEQ ID No. 22. The probes of SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, and SEQ ID No. 20 have a fluorescent reporter group FAM at the 5′ end, and NFQ and MGB at the 3′ end. The probes with sequences of SEQ ID No. 21 and SEQ ID No. 22 have a fluorescent reporter group VIC at the 5′ end, and NFQ and MGB at the 3′ end. The templates to be added in sub-reaction system 1 are mutant genomic DNA containing *rpoB* 526CAC→TAC, *rpoB* 516GAC→GTC, *rpoB* 526CAC→CGC, *rpoB* 531TCG→TGG, *katG* 315AGC→AAC, or *katG* 315AGC→ACC.

2. Sub-reaction system 2 detects mutations of *rpoB* and *inhA* genes. The forward and reverse primers involved are as follows respectively: SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 5, and SEQ ID No. 6, and the probe primers involved are respectively: SEQ ID No. 13, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, and SEQ ID No. 23. The probes of SEQ ID No. 13, SEQ ID No. 17, SEQ ID No. 18, and SEQ ID No. 19 have a fluorescent reporter group FAM at the 5′ end, and NFQ and MGB at the 3′ end. The probes with sequences of SEQ ID No. 23 have a fluorescent reporter group VIC at the 5′ end, and NFQ and MGB at the 3′ end. The templates to be added in sub-reaction system 2 are mutant genomic DNA containing *rpoB* 511CTG→CCG, *rpoB* 526CAC→CTC, *rpoB* 533CTG→CCG, or *inhA* 15C→A.

3. Sub-reaction system 3 detects mutations of *embB* and *gyrA* genes. The forward and reverse primers involved are as follows respectively: SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, and SEQ ID No. 12, and the probe primers involved are as follows respectively: SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, and SEQ ID No. 29.

### Table 1

| Sequence ID | Gene name | Primer type | Sequence (5′-3′) |
|-------------|-----------|-------------|-----------------|
| No 1        | rpoB      | Forward primer (F) | GAGGCGATCACACCGCAGAC |
| No 2        | rpoB      | Reverse primer (R) | GCCGATCAGACCGATGTTGG |
| No 3        | katG      | F            | CTCTTCGTCAGCTCCCACTCG |
| No 4        | katG      | R            | GTCGGCGGTCACACTTTCG |
| No 5        | inhA      | F            | GGGTTTGGCCCCTTCAGTG |
| No 6        | inhA      | R            | GCCTCGCTGCCCAGAAAG |
| No 7        | rpsL      | F            | CTGGTCCGCAAGGTCGTC |
| No 8        | rpsL      | R            | CCCCAGGTATCCAGCGAACC |
| No 9        | embB      | F            | CTGGTGATATTCCGCTTCTG |
| No 10       | embB      | R            | TGGCCGACCCAGCGAATAG |
| No 11       | gyrA      | F            | AGCATCTCATGCGCAACGG |
| No 12       | gyrA      | R            | ACCGCAGCCACGCAAAGTC |
| No 33       | rrs       | F            | AGAACCTCTACGCGCTACG |
| No 34       | rrs       | R            | GCACACGTCGGTGAAATCG |

8 *rpoB*, gene encoding the beta subunit of RNA polymerase; *katG*, gene encoding the catalase-peroxidase-peroxynitrite T; *inhA*, gene encoding the NADH-dependent enoyl-[acyl-carrier-protein] reductase; *rpsL*, gene encoding the 30S ribosomal protein S12; *embB*, gene encoding the integral membrane indolylacetylinositol arabinosyltransferase; *gyrA*, gene encoding the DNA gyrase (subunit A); *rrs*, gene encoding the 16S ribosomal RNA
The probes of SEQ ID No. 28 and SEQ ID No. 29 have a fluorescent reporter group FAM at the 5' end, and MGB and NFQ at the 3’ end. The probes with sequences of SEQ ID No. 27 have a fluorescent reporter group VIC at the 5' end, and MGB and NFQ at the 3’ end. The templates to be added in sub-reaction system 3 are mutant genomic DNA containing embB 306ATG→ATA, embB 306ATG→GTG, gyrA 90GCG→GTG, or gyrA 90GCG→GAC.

4. Sub-reaction system 4 detects mutations of the rpsL gene. The forward and reverse primers involved are SEQ ID No. 7 and SEQ ID No. 8, and the probe primers involved are as follows respectively: SEQ ID No. 24 and SEQ ID No. 25. The probes of SEQ ID No. 24 and SEQ ID No. 25 have a fluorescent reporter group FAM at the 5’ end, and NFQ and MGB at the 3’ end. The templates added in the sub-reaction system 4 are mutant genomic DNA containing rpsL 88AAG→AGG or rpsL 43AAG→AGG.

5. Sub-reaction system 5 detects mutations of rrs genes. The forward and reverse primers involved are SEQ ID No. 33 and SEQ ID No. 34, and the probe primers involved are as follows respectively: SEQ ID No. 30, SEQ ID No. 31, and SEQ ID No. 32. The probes of SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No. 32 have a fluorescent reporter group FAM at the 5’ end, and NFQ and MGB at the 3’ end. The templates added in sub-reaction system 5 are mutant genomic DNA containing rrs 1401A→G, rrs 1402C→T, or rrs 1484G→T.

The final concentration of each primer and probe in the PCR system in each of the above systems is 0.1 μM, and the concentration of mutant genomic DNA is 100 copies/μl. The PCR system is briefly described as follows: 6 μl of 5× Colorless GoTaq® Flexi Buffer (Promega, Madison, Wisconsin, USA), 0.3 μl of Rox reference dye (50×, Invitrogen, Shanghai, China), 0.2 μM of dNTP/dUTP mixture (Sangon, Shanghai, China), 0.05 U/μl of GoTaq® Hot Start.
Polymerase (Promega, Madison, WI, USA), 0.2 μM of magnesium chloride solution (Promega, Madison, WI, USA), 0.0025 U/μl of uracil-DNA glycosylase (ThermoFisher Scientific, Beijing, China), and 5 μl the template. Finally, the reaction system volume was made up of 30 μl by using primers, probes, and ddH2O.

The five sub-reaction systems were all performed on the ABI7500 fluorescent quantitative PCR instrument. The PCR program is as follows: 50 °C 2 min, 95 °C 10 min, 95 °C 15 s, 63 °C 1 min, and 40 cycles. Among them, 63 °C 1 min is the fluorescence collection step. PCR negative control was 1× TE buffer.

Criteria for determining the results of the fluorescence probe method

The cycle threshold (Ct) value of the positive control should be lower than 40, and the Ct value of the negative control should more than 40. If any of the control is false, all the samples’ results in one experiment are defined as invalid and need to be redetected. In the sub-reaction system 1, if the FAM or VIC amplification curve appears and the Ct value is less than 40, it indicates a point mutation in the rpoB gene or katG gene. In the sub-reaction system 2, if the FAM or VIC amplification curve appears and the Ct value is less than 40, it indicates that there is a point mutation in the rpoB gene or inhA gene. In the sub-reaction system 3, if the FAM or VIC amplification curve appears and the Ct value is less than 40, it means that there is a point mutation in the gyrA gene or embB gene. If the FAM amplification curve appears in the sub-reaction system 4 and the Ct value is less than 40, it indicates a point mutation in the rpsL gene. If the FAM amplification curve appears in the sub-reaction system 5, and if the Ct value is less than 40, it indicates a point mutation in the rrs gene.

PCR-directed sequencing

A total of 20 μl PCR products will be sent to the Invitrogen (Shanghai) Trading Co., Ltd. to verify sequencing further.

Evaluation method

With the BACTEC MGIT 960 System (BD, USA) drug sensitivity results as the standard, the sensitivity and specificity of the PCR fluorescence probe method and the detection coincidence rate of the two methods were evaluated. PCR-DS was used to verify the accuracy of the PCR fluorescence probe method and compare the consistency rate of drug resistance detection between the PCR fluorescence probe method and PCR-DS.

Results

Molecular species identification

Nine hundred specimens of acid-fast staining–positive sputum were identified by PCR fluorescence probe method following a previous study [26]. The results indicated that all the 900 specimens belong to M. tuberculosis complexes.

Phenotypic drug susceptibility testing

Nine hundred clinical culture isolates were analyzed by conventional drug susceptibility testing (Table 3). 501 were RFP-resistant strains (55.67%) and 399 were RFP-susceptible strains (44.33); 451 were INH-resistant strains (50.11%) and 449 were INH-susceptible strains (49.89%); 235 were EMB-resistant strains (26.11%) and 665 were EMB-susceptible strains (73.89%); 293 were FQ-resistant strains (32.56%) and 607 were FQ-susceptible strains (67.44%); 425 were SM-resistant strains (47.22%) and 475 were SM-susceptible strains (52.78%); and 204 were AM-resistant strains (22.67%) and 696 were AM-susceptible strains (77.33%). Furthermore, 427 (47.44%) of strains were resistant to both RFP and INH and were MDR-TB cases, and 146 (16.22%) strains were resistant to all six drugs and were XDR-TB cases.

PCR fluorescence probe method

We analyzed 900 clinical sputum specimens by PCR fluorescence probe method; our results indicated that (Table 3): (1) 480 specimens were rpoB gene mutant type as RFP-resistant (53.33%), and 420 specimens were rpoB gene wild-type as RFP-susceptible (46.67%); (2) 241 specimens were katG gene mutant type (26.78%), and 145 specimens were inhA gene mutant type (16.11%) as INH-resistant; 514 specimens were katG/inhA wild-type as INH-susceptible (57.11%); (3) 190 specimens were embB gene mutant type as EMB-resistant (21.11%), and 710 specimens were embB gene wild-type as EMB-susceptible (78.89%); (4) 239 specimens were gyrA gene mutant type as Lfx-resistant (26.56%), and 661 specimens were gyrA gene wild-type as Lfx-susceptible (73.44%); (5) 342 specimens were rpsL gene mutant type as SM-resistant (38.00%), and 558 specimens were rpsL gene wild-type as SM-susceptible (62.00%); and (6) 163 specimens were rrs gene mutant type as AM-resistant (18.11%), and 737 specimens were rrs gene wild-type as AM-susceptible (81.89%).

The mutations of the rpoB, katG, inhA, rpsL, rrs, embB, and gyrA genes were detected in 751 of 900 specimens by PCR-fluorescent probe method. No mutant genes were detected in the other 149 samples as wild-types. The rpoB, katG, or inhA mutant types were found in 398 specimens (44.22%), MDR gene mutant types, and the related cases were MDR-TB. The rpoB, katG/inhA, gyrA, rpsL, and rrs mutant types

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were simultaneously found in 137 specimens (15.22%), and the related cases were XDR-TB.

DNA sequencing

Among the 900 clinical sputum specimens, 480 (53.33%) were found with eight mutations (T511C, A526G, C526G, C526T, A526T, C531T, and T533C) in five loci associated with the \( rpoB \) gene, 420 were found with no mutation within the \( rpoB \) gene; 241 (26.78%) were detected with two mutations (G315C, G315A) associated with the \( katG \) gene, 145 (16.11%) harbored one mutation (C-15T) in the \( inhA \) gene, and 514 were detected with no mutation in both \( katG \) and \( inhA \) genes; 239 specimens (26.56%) were found with two mutations (C90T, A94G) associated with the \( gyrA \) gene, 661 were found with no mutation in the \( gyrA \) gene; 342 (38.00%) harbored two mutations (A43G, A88G) in the \( rpsL \) gene; 558 were found with no mutation in the \( rpsL \) gene. One hundred sixty-three specimens (18.11%) were found with three mutations (A1401G, C1402T, G1484T) in the \( rrs \) gene, and 737 were found with no mutation in the \( rrs \) gene (Table 3).

Comparison of the coincidence rate

According to Table 4, we can conclude that the lowest positive coincidence rate of the PCR-fluorescent probe method comparing with DST for the detection of amikacin was 75%, and that of the other drugs were higher than 75%, which showed that these two methods have good consistency in positive coincidence rate. At a negative coincidence rate, all six drugs were larger than 97%, indicating that those methods
have a good consistency. All six drugs were larger than 88% in total coincidence rate, five of which were larger than 90%.

Above all, the results revealed that both the PCR-fluorescent probe method and DST have good consistency in drug resistance’s total coincidence rate.

As Table 5 showed, both the positive coincidence rate and the negative coincidence rate of the PCR-fluorescent probe method compared with PCR-DS were 100%. The total coincidence rate was 100%. Additionally, the statistical results illustrated that all the seven drug-resistant genes’ detection results by PCR-fluorescent probe method have good consistency with PCR-DS.

**Table 4** Statistical analysis of detecting clinical sputum specimens by PCR-fluorescent probe method and phenotypic DST

| PCR-fluorescent probe | Type       | DST | Accuracy values |
|-----------------------|------------|-----|-----------------|
|                       |            | Resist | Susceptible     | Sensitivity (%) | Specificity (%) | Total coincidence (%) |
| RFP (rpoB)            | Mutant type | 472   | 8               | 94.21%         | 97.99%          | 95.89%               |
|                       | Wild-type  | 29    | 391             |                |                |                     |
| INH (katG/inhA)       | Mutant type | 378   | 8               | 83.81%         | 98.22%          | 91.00%               |
|                       | Wild-type  | 73    | 441             |                |                |                     |
| EMB (embB)            | Mutant type | 177   | 13              | 75.32%         | 98.05%          | 92.11%               |
|                       | Wild-type  | 58    | 652             |                |                |                     |
| Lfx (gyrA)            | Mutant type | 225   | 14              | 76.79%         | 97.69%          | 90.89%               |
|                       | Wild-type  | 68    | 593             |                |                |                     |
| SM (rpsL)             | Mutant type | 330   | 12              | 77.65%         | 97.47%          | 88.11%               |
|                       | Wild-type  | 95    | 463             |                |                |                     |
| AM (rrs)              | Mutant type | 153   | 10              | 75.00%         | 98.56%          | 93.22%               |
|                       | Wild-type  | 51    | 686             |                |                |                     |
| MDR (rpoB, katG/inhA) | Mutant type | 385   | 13              | 90.16%         | 97.25%          | 93.89%               |
|                       | Wild-type  | 42    | 460             |                |                |                     |
| XDR (rpoB, katG/inhA, gyrA, embB, rpsL, and rrs) | Mutant type | 128   | 9               | 87.67%         | 98.81%          | 97.00%               |
|                       | Wild-type  | 18    | 745             |                |                |                     |

**AM**, amikacin; **DST**, drug susceptibility testing; **EMB**, ethambutol; **FQs**, fluoroquinolones; **INH**, isoniazid; **Lfx**, levofloxacin; **MDR**, multidrug-resistant; **Mfx**, moxifloxacin; **PZA**, pyrazinamide; **RFP**, rifampicin; **SM**, streptomycin; **XDR-TB**, extensively drug-resistant

**Table 5** Statistical analysis of detecting clinical sputum specimens by PCR-fluorescent probe method and PCR-DS

| PCR-fluorescent probe | PCR-DS      | Accuracy values |
|-----------------------|-------------|-----------------|
|                       | Mutant type | Wild-type       | Sensitivity (%) | Specificity (%) | Total coincidence (%) |
| rpoB                  | 480         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 420             |                |                |                      |
| katG                  | 241         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 659             |                |                |                      |
| inhA                  | 145         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 755             |                |                |                      |
| embB                  | 190         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 710             |                |                |                      |
| gyrA                  | 239         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 661             |                |                |                      |
| rpsL                  | 342         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 558             |                |                |                      |
| rrs                   | 163         | 0               | 100%           | 100%            | 100%                  |
|                       | 0           | 737             |                |                |                      |

**Discussion**

The diagnosis and treatment of drug-resistant TB, especially MDR/XDR-TB, are critical and challenging factors in preventing and controlling TB. Currently, conventional culture-based techniques have long turnaround times. We cannot offer timely and effective treatment programs for TB patients, especially those combined with HIV patients [27]. In 2010, WHO endorsed the Gene Xpert MTB/RIF assay [28] that used real-time PCR to identify *M. tuberculosis* complex DNA and the mutations associated with RFP resistance directly from sputum specimens. Nevertheless, Gene Xpert MTB/
RIF assay cannot detect mutations related to other anti-TB drugs such as INH, EMB, SM, AM, and FQs. With the in-depth research on the molecular mechanism of drug resistance, the establishment of a simple, fast, and accurate method for the detection of drug-resistant mutations becomes more and more significant to improve the cure rate, reduce the occurrence of drug resistance, and decrease the risk of recurrence and death rate.

PCR fluorescence probe technology uses double PCR technology and the Taqman probe technique to detect M. tuberculosis and drug resistance by monitoring different fluorescent channels’ fluorescence signal. This technique has strong specificity and sensitivity, and is easy to operate. Our previous study [26] showed that PCR fluorescence probe technology is an essential clinical value in the rapid diagnosis of TB in sputum specimens. However, few studies on the exploratory development of PCR-fluorescent probes in the rapid detection of mutations are associated with XDR-TB.

The conventional DST method takes 3 to 4 weeks. This study is based on the PCR-fluorescent probe method, which has a low cost, simple operation, and only 1.5 h to detect the nucleic acid in specimens. Besides, this method can significantly shorten the detection cycle comparing with phenotypic DST. In this study, we established and evaluated the detection system of multidrug resistance and extensively drug resistance in M. tuberculosis, reflecting that (1) the drug resistance of TB was detected by fluorescence PCR detection technology covered six anti-TB drugs with seven drug resistance genes. Additionally, a multidrug-resistant and extensively drug-resistant mutation detection system was established, and the results showed that the system had high specificity and sensitivity values; (2) The clinical diagnostic performance of PCR detection system was evaluated by testing 900 clinical specimens of M. tuberculosis, and the results were compared with the absolute concentration method of DST. In total coincidence rate, all of the six drugs were larger than 88%, five of which were larger than 90%.

In this study, compared with phenotypic DST, the coincidence rates of rpoB (RFP), katG/inhA (INH), embB (EMB), gyrA (Lfx), rpsL (SM), and rrs (AM) detected by fluorescent probe method were 95.89%, 91%, 92.11%, 90.89%, 88.11%, and 93.22%, respectively. The coincidence rate of two methods of RFP, INH, EMB, FQs, and AM resistance testing is higher than 90%, only SM coincidence rate was 88.11%. The possible reason may be that rpoB, katG/inhA, embB, gyrA, and rrs gene mutations of M. tuberculosis were the main resistance mechanism of RFP, INH, EMB, FQs, and AM. In addition to rpsL gene mutation (50–78%), rrs gene mutation (20–30%) is also the main molecular mechanism of drug-resistant to SM [29, 30]. However, this study had not detected the rrs gene mutation locus of drug resistance to SM of M. tuberculosis, which may be one reason for the low consistency of SM drug resistance detected by PCR-fluorescent probe method compared with phenotypic DST.

In this study, phenotypic DST was used as the standard, the detection rate of MDR-TB by phenotypic DST was 47.44% (427/900), and the detection rate of rpoB and katG/inhA was 42.22% (398/900) by PCR fluorescence probe method. The sensitivity, specificity, and coincidence rate of the PCR-fluorescence probe method for detecting MDR-TB were 90.16%, 97.25%, and 93.89%, respectively. The detection rate of XDR-TB by phenotypic DST was 16.22% (146/900). The detection rate of rpoB, katG/inhA, rpsL, rrs, embB, and gyrA by PCR fluorescence probe method was 15.22% (137/900), the sensitivity and specificity of PCR-fluorescent probe method for detection of XDR-TB were 87.67%, 98.81%, and the coincidence rate was 97.00%.

It is well-known that drug-resistant gene mutations are an effective form of TB resistance. As for the PCR fluorescence probe method, the positive detection rate was lower than DST because drug-resistant gene mutation was just a form of drug resistance. Reducing cell membrane vulnerability and efflux pumps and inactivated enzyme changes were also the causes of TB resistance. On the other hand, only a few loci of 7 common drug-resistant genes of 6 drugs were detected, while other drug-resistant genes or other drug-resistant loci were not developed in this study, such as ndh, efpA, kasA, iniABC operon (for INH resistance) [31], rpoC (for RFP resistance) [32], embA, embC, ubiA (for EMB resistance) [33], and gyrB (for FQs resistance) [34]. These may be reasons for the lower detection rate of the PCR-fluorescent probe method than phenotypic DST.

Conclusion

In summary, with the advantages of simplicity, accuracy, specificity, and high throughput, the PCR-fluorescent probe method gives impetus to the widespread clinical application of molecular diagnostic technology. Our results revealed the association between mutation types, drug-resistant type and dosage, clinical treatment, and prognosis by distinguishing and investigating different mutation types of anti-TB drugs. These findings provided a novel perspective on anti-TB drug development to achieve TB prevention and control truly. Herein, we established a comprehensive detecting system of XDR-TB, including both first-line and second-line anti-TB drugs. Additionally, developed genetic tests will inevitably produce more rapid results for drug-resistant isolates, which will lead to faster identification of MDR and XDR strains, more tailored treatment regimens, and a reduction in the transmission of TB.
Abbreviations  AM, amikacin; DST, drug susceptibility testing; EMB, ethambutol; FQs, fluoroquinolones; INH, isoniazid; Lfx, levofloxacin; MDR-TB, multidrug-resistant tuberculosis; Mfx, moxifloxacin; PCR-DS, PCR-directed sequencing; PZA, pyrazinamide; MTB, *Mycobacterium tuberculosis*; RFP, rifampicin; RR-TB, rifampicin-resistant tuberculosis; SM, streptomycin; TB, tuberculosis; XDR-TB, extensively drug-resistant tuberculosis

Author contribution  Conceptualization: JQL, HRA, and WPG; investigation: JQL, HRA, JZ, YYL, and YQL; formal analysis: WLX; project administration: JQL and HRA; methodology: JQL, HRA, YYL, and WLX; writing - original draft: JQL; and writing - review & editing: WPG, JQL, GXX, and WLX. All authors have read and approved the final manuscript.

Data availability  All data and materials generated or analyzed during this study are included in this published article.

Declarations

Ethical approval and consent to participate  The study protocol was approved by the Research Ethics Committee of the 8th Medical Center of Chinese PLA General Hospital. Written informed consent was obtained from all adult participants.

Consent to participate  The study protocol was approved by the Research Ethics Committee of the 8th Medical Center of Chinese PLA General Hospital, and the informed consent was obtained from all adult participants and parents of the participant under 16 years old.

Consent for publication  Not available.

Conflict of interest  The authors declare no competing interests.

References

1. WHO (2019) Global tuberculosis report 2019. World Health Organization, Geneva, pp 1–297
2. Akshaya KM, Shewade HD, Aslesh OP, Nagaraja SB, Nirgude AS, Singarajipura A, Jacob AG (2017) “Who has to do it at the end of the day? Programme officials or hospital authorities?” Airborne infection control at drug resistant tuberculosis (DR-TB) centres of Kamataka, India: a mixed-methods study. Antimicrob Resist Infect Control 6:111
3. Schön T, Miotta P, Köser CU, Viveiros M, Böttger E, Cambau E (2017) *Mycobacterium tuberculosis* drug-resistance testing: challenges, recent developments and perspectives. Clin Microbiol Infect: Off Publ Eur Soc Clin Microbiol Infect Dis 23(3):154–160
4. Migliori GB, Tiberi S, Zumla A, Palomino JC, Centis R, Gler MT, Crudu V, Alcaide F (2014) Characterization of the embB gene in *Mycobacterium tuberculosis* isolates from Barcelona and rapid detection of main mutations related to ethambutol resistance using a low-density DNA array. J Antimicrob Chemother 69(4):947–953
5. Evans J, Segal H (2010) Novel multiplex allele-specific PCR assays for the detection of resistance to second-line drugs in *Mycobacterium tuberculosis*. J Antimicrob Chemother 65(5):1385–1392
6. Zhang Y, Yew WW (2009) Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberculosis Lung Dis 13(11):1210–1216
7. Li Q, Dong HY, Yang Y, Xia H, Ou XC, Zhang ZY, Li JC, Zhang JK, Huan ST, Chin DP, Kam KM, Zhao YL (2015) Multicenter evaluation of the molecular line probe assay for multidrug resistant *mycobacterium tuberculosis* detection in China. Biomedical and environmental sciences : BES 28(6):464–467
8. Torres JN, Paul LV, Rodwell TC, Victor TC, Amallrajha AM, Elghraoui A, Goodmanson AP, Ramirez-Busby SM, Chawla A, Zadorozhny V, Streicher EM, Sirgel FA, Catanzaro D, Rodrigues C, Gler MT, Crudu V, Catanzaro A, Valafar F (2015) Novel katG mutations causing isoniazid resistance in clinical M. tuberculosis isolates. Emerging Microbes Infect 4(7):e42
9. Liu J, Jiang F, Chen L, Zhao B, Dong J, Sun L, Zhu Y, Liu B, Zhou Y, Yang J, Zhao Y, Jin Q, Zhang X (2018) The impact of combined gene mutations in inhA and ahpC genes on high levels of isoniazid resistance amongst katG non-315 in multidrug-resistant tuberculosis isolates from China. Emerging Microbes Infect 7(1):183
10. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, Hooks DP, Cowan LS, Plkaytis BB, Posey JE (2011) Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 55(5):2032–2041
11. Maningi NE, Daum LT, Rodríguez JD, Sia MD, Peters RPH, Sekyere JO, Fischer GW, Chambers JP, Fourie PB (2018) Multi- and extensively drug resistant mycobacterium tuberculosis in South Africa: a molecular analysis of historical isolates. J Clin Microbiol 56(5)
12. Srivastava S, Ayyagari A, Dhole TN, Nyati KK, Dwivedi SK (2009) emb nucleotide polymorphisms and the role of embB306 mutations in *Mycobacterium tuberculosis* resistance to ethambutol. Int J Med Microbiol : IJMM 299(4):269–280
13. He J, Zhu B, Yang Z, Hu B, Lin L, Zhang Q (2014) Molecular analysis of the rpsl gene for rapid detection of streptomycin-resistant *Mycobacterium tuberculosis*: a meta-analysis. Scand J Infect Dis 46(6):585–592
14. Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC (2004) Multiplex PCR amplifier conformation analysis for rapid detection of gyrA mutations in *Mycobacterium tuberculosis* clinical isolates. Antimicrob Agents Chemother 48(2):596–601
15. Evans J, Segal H (2010) Novel multiplex allele-specific PCR assays for the detection of resistance to second-line drugs in *Mycobacterium tuberculosis*. J Antimicrob Chemother 65(5):897–900
16. Almeida Da Silva PE, Palomino JC (2011) Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. J Antimicrob Chemother 66(7):1417–1430
17. Morlock GP, Crawford JT, Butler WR, Brim SE, Sikes D, Mazurek MH, Woodley CL, Cocksley RC (2000) Phenotypic characterization of pncA mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 44(5):2291–2295
18. Denkinger CM, Kik SV, Cicillo DM, Casenghi M, Shinmack T, Weyer K, Gilpin C, Boehme CC, Schito M, Kimerling M, Pai M (2015) Defining the needs for next generation assays for tuberculosis. J Infect Dis 211 Suppl 2(Suppl 2):S29–S38
19. Martanashidze NM, Soedarsono KT, Koendhori EB, Kusumaningrum D, Koesprjani S, Nuha Z, Chanifah H (2020) Difficulties with the

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implemented xpert MTB/RIF for determining diagnosis of pulmonary and extrapulmonary tuberculosis in adults and children. J Clin Tuberculosis Mycobact Dis 19:100159

21. Kaur R, Kachroo K, Sharma JK, Vatturi SM, Dang A (2016) Diagnostic accuracy of Xpert test in tuberculosis detection: a systematic review and meta-analysis. J Global Infect Dis 8(1):32–40

22. Meyer AJ, Atuheire C, Worodria W, Kizito S, Katamba A, Sanyu I, Andama A, Ayakaka I, Cattamanchi A, Bwanga F, Huang L, Davis JL (2017) Sputum quality and diagnostic performance of GeneXpert MTB/RIF among smear-negative adults with presumed tuberculosis in Uganda. PLoS One 12(7):e0180572

23. Vilchêze C, Kremer L (2017) Acid-fast positive and acid-fast negative Mycobacterium tuberculosis: The Koch Paradox. Microbiol Spectr 5(2)

24. Y Z, Z L (2013) TB laboratory standardization operation and network establishment. People’s Medical Publishing House, Beijing

25. Gong W, Xiong X, Qi Y, Jiao J, Duan C, Wen B (2014) Identification of novel surface-exposed proteins of Rickettsia rickettsii by affinity purification and proteomics. PLoS One 9(6):e100253

26. JianQin L, HuaFang G, HongMin L, ZhiXian Z, GuangYu Z, JinHe W, SuMin W (2012) Study on the reliability of detecting Mycobacteria in sputum specimens by PCR-fluorescent probe. Chin J Antituberculosis 34(5):271–274

27. An P, Nelson GW, Wang L, Donfield S, Goedert JJ, Phair J, Vlahov D, Buchbinder S, Farrar WL, Modi W, O’Brien SJ, Winkler CA (2002) Modulating influence on HIV/AIDS by interacting RANTES gene variants. Proc Natl Acad Sci U S A 99(15):10002–10007

28. WHO (2014) WHO Guidelines Approved by the Guidelines Review Committee. Xpert MTB/RIF implementation manual: technical and operational ‘how-to’; practical considerations. World Health Organization, Geneva

29. Tudó G, Rey E, Borrell S, Alcaide F, Codina G, Coll P, Martín-Casabona N, Montemayor M, More R, Ocaña A, Salvadó M, Vicente E, González-Martín J (2010) Characterization of mutations in streptomycin-resistant Mycobacterium tuberculosis clinical isolates in the area of Barcelona. J Antimicrob Chemother 65(11):2341–2346

30. Sun YJ, Luo JT, Wong SY, Lee AS (2010) Analysis of rpsL and rrs mutations in Beijing and non-Beijing streptomycin-resistant Mycobacterium tuberculosis isolates from Singapore. Clin Microbiol Infect : Off Publ Eur Soc Clin Microbiol Infect Dis 16(3):287–289

31. Nguyen L (2016) Antibiotic resistance mechanisms in M. tuberculosis: an update. Arch Toxicol 90(7):1585–1604

32. Perdigão J, Gomes P, Miranda A, Maltez F, Machado D, Silva C, Phelan JE, Brum L, Campino S, Couto I, Viveiros M, Clark TG, Portugal I (2020) Using genomics to understand the origin and dispersion of multidrug and extensively drug resistant tuberculosis in Portugal. Sci Rep 10(1):2600

33. Farhat MR, Sultana R, Iartchouk O, Bozeman S, Galagan J, Sisk P, Stolte C, Nebenzahl-Guimaraes H, Jacobson K, Sloutsky A, Kaur D, Posey J, Kreisswirth BN, Kurepina N, Rigouts L, Streicher EM, Victor TC, Warren RM, van Soolingen D, Murray M (2016) Genetic determinants of drug resistance in Mycobacterium tuberculosis and their diagnostic value. Am J Respir Crit Care Med 194(5):621–630

34. Farhat MR, Jacobson KR, Franke MF, Kaur D, Sloutsky A, Mitnick CD, Murray M (2016) Gyrase mutations are associated with variable levels of fluoroquinolone resistance in Mycobacterium tuberculosis. J Clin Microbiol 54(3):727–733

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