Rapid screening mutations of first-line-drug-resistant genes in *Mycobacterium tuberculosis* strains by allele-specific real-time quantitative PCR

Pengpeng Yang ¹, Yuzhu Song ¹, Xueshan Xia ¹, A-Mei Zhang ¹

¹ Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China

Corresponding Author: A-Mei Zhang
Email address: zam1980@yeah.net

Tuberculosis (TB) is a worldwide health, economic, and social burden, especially in developing countries. Drug-resistant TB (DR-TB) is the most serious type of this burden. Thus, it is necessary to screen drug-resistant mutations by using a simple and rapid detection method. A total of 32 pairs of Allele-specific PCR (AS-PCR) primers were designed to screen mutation and/or wild-type alleles of 16 variations in four first-line drug-resistant genes (*katG*, *rpoB*, *rpsL*, and *embB*) of TB strains. A pair of primers was designed to amplify 16S rRNA gene and to verify successful amplification. Subsequently, we tested the specificity and sensitivity of these AS-PCR primers. The optimized condition of these AS-PCR primers was first confirmed. All mutations could be screened in general AS-PCR, but only 13 of 16 variations were intuitively investigated by using real-time quantitative PCR and AS-PCR primers. The results of specificity assay suggested that the AS-PCR primers with mutation and/or wildtype alleles could successfully amplify the corresponding allele under optimized PCR conditions. The sensitivity of nine pairs of primers was 500 copy numbers, and the other seven pairs of primers could successfully amplify correct fragments with a template comprising 10³ or 10⁴ copy numbers template. An optimized AS-qPCR was established to screen drug-resistant mutations in TB strains with high specificity and sensitivity.
Rapid screening mutations of first-line-drug-resistant genes in *Mycobacterium tuberculosis* strains by allele-specific real-time quantitative PCR

Pengpeng Yang¹, Yuzhu Song¹, Xueshan Xia¹, A-Mei Zhang¹,*

¹ Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, Yunnan, China

*Corresponding author*

Dr. A-Mei Zhang, Molecular virus Units, Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, Yunnan 650500, China. Tel/Fax: 86-871-65920756; E-mail: zam1980@yeah.net
Abstract

Tuberculosis (TB) is a worldwide health, economic, and social burden, especially in developing countries. Drug-resistant TB (DR-TB) is the most serious type of this burden. Thus, it is necessary to screen drug-resistant mutations by using a simple and rapid detection method. A total of 32 pairs of Allele-specific PCR (AS-PCR) primers were designed to screen mutation and/or wild-type alleles of 16 variations in four first-line drug-resistant genes (katG, rpoB, rpsL, and embB) of TB strains. A pair of primers was designed to amplify 16S rRNA gene and to verify successful amplification. Subsequently, we tested the specificity and sensitivity of these AS-PCR primers. The optimized condition of these AS-PCR primers was first confirmed. All mutations could be screened in general AS-PCR, but only 13 of 16 variations were intuitively investigated by using real-time quantitative PCR and AS-PCR primers. The results of specificity assay suggested that the AS-PCR primers with mutation and/or wildtype alleles could successfully amplify the corresponding allele under optimized PCR conditions. The sensitivity of nine pairs of primers was 500 copy numbers, and the other seven pairs of primers could successfully amplify correct fragments with a template comprising $10^3$ or $10^4$ copy numbers template. An optimized AS-qPCR was established to screen drug-resistant mutations in TB strains with high specificity and sensitivity.

Keywords: AS-qPCR; drug-resistant mutations; TB; specificity and sensitivity
1. **Introduction**

Tuberculosis (TB) is a disease with high prevalence and mortality rate. It is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) infection. One-third of the world population is infected with *M. tuberculosis*, and 5% of infected people developed into TB in their lifetime (Comstock et al. 1974; Koul et al. 2011). In 2017, 10.1 million people suffered from TB, among which 1.6 million people died. Though morbidity and mortality of TB gradually decreased with the appearance of anti-TB drugs, the mutation rate of drug-resistant genes in *M. tuberculosis* seemed to increase recently. The cost of TB treatment and research reached 10.4 billion $ in 2018. Half of this amount was used to treat drug-resistant TB (DR-TB) patients (WHO 2018).

In the middle of 20th century, DR-TB strains were reported for the first time (Crofton & Mitchison 1948). Unfortunately, the researchers did not focus on this phenomena at that time (Zignol et al. 2016). Recently, the number of patients with DR-TB, especially those with multiple drug-resistant TB (MDR-TB), seriously increased. The numbers of patients with MDR-TB reached 160,684 in 2017. A total of 10,800 cases of extensive drug-resistant TB (XDR-TB) were reported by 77 countries, and 88% XDR-TB cases were from European and South-East Asia regions (WHO 2018). Due to abuse of antibiotics and environmental disruption, drug-resistant TB strains have turned out to be barriers to tuberculosis treatment.

First-line anti-TB drugs, including isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide, are still widely used in clinic. Inevitably, drug-resistant genes exist in *M. tuberculosis*, thereby allowing it to resist first-line anti-TB drugs. *katG* and *inhA* genes are two
common candidate genes in isoniazid (INH) -resistant TB strains. Mutations in other genes, such as \textit{sigI}, \textit{ndh}, and others, also reportedly to lead to INH-resistance in TB (Guo et al. 2006).

Similarly, \textit{rpoB}, \textit{embB}, \textit{rpsL}, and \textit{pncA} were major drug-resistant genes for Rifampicin (RIF)-, Ethambutol (EMB)-, Streptomycin (SM)-, and Pyrazinamide (PZA)- resistant TB strains (Lee et al. 2012; Sandy et al. 2002; Zhang & Yew 2009). Although the mutant spectra showed distinction in different countries, there were some hotspot mutations in these candidate genes.

The mutation at codon 315 of the \textit{katG} gene was the most popular INH-resistant mutation, and more than 50% of INH-resistant mutations located in this codon (Afanas'ev et al. 2007; Yuan et al. 2012). Most of RIF-resistant mutations located in hotspot region of the \textit{rpoB} gene, but the mutant frequency varied from 75% to 90% in different countries (Franco-Sotomayor et al. 2018; Thirumurugan et al. 2015). Mutations at codon 43 and 88 were two common SM-resistant mutations in the \textit{rpsL} gene, and over 65% SM-resistant TB strains were caused by these two mutations (Tudo et al. 2010; Zhao et al. 2015). About 70% of EMB-resistant TB strains had mutations which located at codon 306, 406, or 497 in the \textit{embB} gene (Brossier et al. 2015). Thus, rapid screening these mutations was necessary.

Long-time and inappropriate drug usage could lead to drug-resistance. Thus, rapid and convenient diagnosis of drug-resistant TB patients is necessary for further effective treatment.

Based on the drug-resistant mutations (including hotspot and rare mutations) in our previous study (Li et al. 2017), we established an optimized allele-specific real-time quantitative PCR (AS-qPCR) method to screen mutations in drug-resistant TB strains rapidly and with high
sensitivity and specificity.

2. Materials and Methods

2.1 M. tuberculosis strains collection and DNA extraction

Drug-resistant M. tuberculosis strains were collected and cultured in Lowenstein-Jensen (LJ) medium by doctors in Kunming Third People’s Hospital. Drug susceptibility testing (DST) was performed by using the following drugs: INH, 0.2 mg/L; RIF, 40 mg/L; SM, 4.0 mg/L; EMB, 2.0 mg/L; and PZA, 100 mg/L. G+ Bacteria Genomic DNA Kit (ZOMANBIO, China) was used to extract genomic DNA from M. tuberculosis strains according to the manufacturer's instructions. This study was approved by the institutional review board of Kunming University of Science and Technology (Approval No. 2014SK027).

2.2 Primer design and AS-PCR optimization

A total of 32 pairs of primers for AS-PCR were designed to screen the 16 variations (including mutation and wild-type alleles), which were located in four genes (katG, embB, rpsL, and pncA), by using Oligo Primer Analysis Software v.7 (Table S1). To strengthen the specificity of primers, a non-complementary nucleotide in 3’ end of the allele-specific primer was factitiously changed and marked in red in Table S1. One pair of inner control primers (16S 915-F/16S 1018-R) was used to control and identify PCR quantification (Table S1).

PCR was performed in 20 µL reaction volume and involved 30 ng of genomic DNA, 10 µL
2×TSINGKE™ Master Mix (including 1 U DNA polymerase, 1.5 mM MgCl₂, 50 mM KCl, and 100 mM dNTP) (TSINGKE, China) or ChamQ™ SYBR qPCR Master Mix (Vazyme, China), and 0.5 µM each primer (including AS-PCR primers and internal control primers). After optimization, we used the following PCR condition: one cycle of 95 °C for 3 min; 35 cycles of 95 °C for 30 s, optimized temperature for 30 s (Table S1), 72 °C for 10 s; and one extension cycle of 72 °C for 5 min.

2.3 Plasmids construction

Five pairs of primers (Table S2) were designed to amplify fragments containing all mutations. PCR amplification products were ligated into the pClone 007 Blunt Simple Vector by using pClone 007 Vector Kit (TSINGKE, China). All constructed plasmids were identified to carry the specific mutation or wildtype allele by sequencing. Plasmid extraction small Kit (TIANGEN, China) was used to purify the plasmids.

2.4 Specificity and sensitivity tests

We amplified 14 wildtype TB samples (identified by sequencing) and all templates of each mutation to test the specificity of AS-PCR by using both wildtype and mutation primers. If there was only one sample with a certain mutation, we duplicated the AS-PCR by using the same mutation sample. The sensitivity results were determined based on the appearance and intensity of the products on the agarose gel. Moreover, wildtype AS-PCR primers were also used to
amplify plasmids with corresponding mutations. After qualifying the plasmids, we diluted plasmids to $10^4$, $10^3$, and $5 \times 10^2$ copy numbers to achieve a sensitive assay.

2.5 AS real-time quantitative PCR

According to the results of optimized AS-PCR, we performed AS real-time quantitative PCR (AS-qPCR) assays, in order to directly detect the products and determine the mutation or wild type allele. PCR was performed in 20 µL reaction volume and involved 30 ng of genomic DNA, ChamQTM SYBR qPCR Master Mix (Vazyme, China), and 0.3 µM each primer (including mutation and wild type AS-PCR primers of each allele) on Takara Thermal Cycler Dice Real Time System TP800 (TaKaRa, Japan).

3. Results

A total of 16 point mutations in four first-line drug-resistant genes were used to establish AS-PCR detecting method, which were identified in 57 drug-resistant *M. tuberculosis* strains by sequencing in our previous study (Li et al. 2017). After optimizing the conditions of PCR with these primers, we obtained concordant results by sequencing, i.e. the correct bands were successfully amplified by using mutant AS-PCR primers and mutation template but not wild-type samples, and vice versa (Fig. 1). The specificity test results suggested all primers in this study could identify the mutation or wildtype alleles with high fidelity (Fig. S1). As shown in Figure 2, a 206 bp fragment could be amplified by using samples with wild-type *rpsL* gene and
primers $RpsL$ 128A-F/$RpsL$ 128-R (the primer for wild-type allele A at amino acid codon 128). However, this amplicon did not exist when samples with $rpsL$ 128G were used. On the contrary, the primers $RpsL$ 128G-F/$RpsL$ 128-R (for mutant allele G) could amplify a 206 bp fragment when the samples carried the mutation allele 128G in the $rpsL$ gene. The presentation of the 104 bp inner control fragment suggested a successful amplification.

Plasmids with mutation and wildtype alleles were constructed to evaluate the sensitivity of AS-PCR primers. As shown in Figure 3, the sensitivity of primers for amino acid codon 128 in the $rpsL$ gene was estimated by using a plasmid with $10^4$, $10^3$, and $5 \times 10^2$ copy numbers. When the copy number of the plasmids was $5 \times 10^2$, we could visualize a faint band. However, the bands were obvious and clear when we increased the plasmids to $10^3$ or $10^4$ copy numbers. The sensitivity of all 32 pairs of primers were tested, and half of these primers could amplify an observable band when the plasmid copy number was $5 \times 10^2$ (Fig. S2). However, the others needed more copy numbers ($10^3$ or $10^4$, Fig. S2 and Table S1).

To directly and rapidly investigate the testing results of AS-PCR, we combined AS-PCR primers and real-time quantitative PCR for subsequent observation. By observing the melt-curve of these products, we determined whether mutations existed. We firstly defined the baseline at 100 relative fluorescence units (RFU)/min as the detecting level. According to this standard, we tested all 16 mutations by using AS-qPCR. However, only 13 mutations were rapidly and directly genotyped (Fig. S3). As shown in Figure 4, we could rapidly identify the allele at 128 nucleotide in the $rpsL$ gene. By using the optimized AS-qPCR, we rapidly distinguished the
mutation and/or wild-type allele in one PCR reaction with two pairs of primers.

4. Discussion

China is among the top 22 countries with the highest burden of TB and with the second highest burden of DR-TB. About 120,000 persons developed into TB in China each year (Du et al. 2017; Zhao et al. 2012). Many factors could lead to DR-TB, such as contaminative environment, drug abuse, long-time therapy, and host genetic factors. Some TB strains might change to drug-resistance or solo DR-TB might develop to XDR-TB after the long-term treatment of TB patients. Thus, it was important and necessary to rapidly diagnose DR-TB, especially for first-line DR-TB. Point mutations of drug-resistant genes were common reasons for the development of first-line DR-TB strains. Mutations in the katG and/or inhA gene were the two main causes of INH-resistant TB. About 51% and 10% isoniazid-resistant TB strains were caused by mutations in the katG and inhA genes, respectively (Guo et al. 2006). Mutations in the rpoB gene, rpsL gene, embB gene, and pncA gene were the main factors for RIF-resistant, SM-resistant, EMB-resistant, and PZA-resistant TB strains, respectively (Brossier et al. 2015; Scorpio & Zhang 1996; Stoffels et al. 2012; Villellas et al. 2013). Furthermore, some hotspot mutations still exist in these drug-resistant genes (Banerjee et al. 1994; Dalla Costa et al. 2009; Lee et al. 2012; Seifert et al. 2015; Waagmeester et al. 2005). Most commercialized detecting kits for DR-TB strains only contained hotspot mutations. Hence, some rare mutations might be missed. In our previous study, we identified some mutations in these genes of Yunnan DR-TB
strains, and some of them were hotspot mutations and others were rare mutations (Li et al. 2017).

Because no hotspot mutation has been found in the *pncA* gene, eighteen mutations of the other four genes (including *katG*, *rpoB*, *rpsL*, and *embB* genes) were used as candidate mutations in this study. Unfortunately, two mutations, including G1388T (at codon 463) in the *katG* gene and A1490G (at codon 497) in the *embB* gene, could not genotyped by using AS-PCR. Thus, it seemed that not all drug-resistant mutations could be well detected by using AS-PCR.

DST is the classic method and the “gold standard” for the evaluation of drug-resistant TB strains (Ahmad & Mokaddas 2009). Until now, DST is still widely used in laboratory and hospital, but methods to screen mutations of drug-resistant genes were speedily developed after invention of PCR. Multi-fluorescence real-time quantitative PCR is one of the most common methods to detect RIF- and INH-resistant *M. tuberculosis* (Peng et al. 2016). Other technologies, such as whole-genome sequencing (WGS) (Pankhurst et al. 2016), high-resolution melt (HRM), PCR-single strand conformation polymorphism (PCR-SSCP), and oligonucleotide microarrays (Caoili et al. 2006; Herrmann et al. 2006; Pietzka et al. 2009; Traore et al. 2006), have also been widely used to screen the mutations of candidate genes in DR-TB strains. However, these technologies have their advantages and shortcomings. For example, high quality and various mutation type could be detected by using WGS, but the expensive equipment and reagent limited its usage.

Since AS-PCR was first reported in 1989 (Newton et al. 1989), it has been widely used to screen single nucleotide polymorphisms (SNPs) and mutations. Although AS-PCR is considered
as low specificity and sensitivity (Sharma et al. 2016), Onseedaeng et al. identified mutations of
the *gyrA* and *parC* gene in *Escherichia coli* (*E. coli*) with high sensitivity and specificity by
using AS-PCR (Onseedaeng & Ratthawongjirakul 2016). Due to its simple operation, low cost,
and relatively high specificity and sensitivity, we successfully used AS-PCR to screen 16
mutations in four first-line DR-genes. After optimizing reaction conditions, we successfully
amplified the corresponding bands by using AS-PCR primers with high specificity. Most of these
primers detected the corresponding mutations when the DNA template reached 500 copy
numbers. All these results suggested that optimized AS-qPCR could be used to screen mutations
of drug-resistant genes in TB strains with higher specificity and sensitivity. A limitation of
current study was the number of drug-resistant mutations in this study was small size. One
reason was that no more TB-strains with other drug-resistant mutations were obtained in this
experiment; another reason was several drug-resistant mutations could not be well genotyped by
using AS-PCR or AS-qPCR method. In further study, we should collect more drug-resistant TB
strains from various regions and further optimize AS-PCR condition for rapid screening.

5. **Conclusion**

In summary, we established an optimized AS-qPCR method to screen mutations in four
first-line drug-resistant genes of *M. tuberculosis* with relatively high specificity and sensitivity.
This AS-qPCR could be widely used in the future to rapidly screen mutations in drug-resistant
TB strains.
Supplementary Files

Table S1. Information of AS-PCR primers.

Table S2. Information for primers used to construct plasmids.

Figure S1. Electrophoresis map of specific tests.

Figure S2. Electrophoresis map of sensitivity tests.

Figure S3. Melt curves map of real-time quantitative PCR.

Acknowledgments

We thank Mr. Daoqun Li for helping extract DNA from TB strains.

References

Afanas'ev MV, Ikryannikova LN, Il'ina EN, Sidorenko SV, Kuz'min AV, Larionova EE, Smirnova TG, Chernousova LN, Kamaev EY, Skorniakov SN, Kinsht VN, Cherednichenko AG, and Govorun VM. 2007. Molecular characteristics of rifampicin- and isoniazid-resistant Mycobacterium tuberculosis isolates from the Russian Federation. *J Antimicrob Chemother* 59:1057-1064. 10.1093/jac/dkm086

Ahmad S, and Mokaddas E. 2009. Recent advances in the diagnosis and treatment of multidrug-resistant tuberculosis. *Respir Med* 103:1777-1790. 10.1016/j.rmed.2009.07.010

Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle
G, and Jacobs WR, Jr. 1994. inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. *Science* 263:227-230.

Brossier F, Sougakoff W, Bernard C, Petrou M, Adeyema K, Pham A, Amy de la Breteque D, Vallet M, Jarlier V, Sola C, and Veziris N. 2015. Molecular Analysis of the embCAB Locus and embR Gene Involved in Ethambutol Resistance in Clinical Isolates of Mycobacterium tuberculosis in France. *Antimicrob Agents Chemother* 59:4800-4808. 10.1128/AAC.00150-15

Caoili JC, Mayorova A, Sikes D, Hickman L, Plikaytis BB, and Shinnick TM. 2006. Evaluation of the TB-Biochip oligonucleotide microarray system for rapid detection of rifampin resistance in Mycobacterium tuberculosis. *J Clin Microbiol* 44:2378-2381. 10.1128/JCM.00439-06

Comstock GW, Livesay VT, and Woolpert SF. 1974. The prognosis of a positive tuberculin reaction in childhood and adolescence. *Am J Epidemiol* 99:131-138.

Crofton J, and Mitchison DA. 1948. Streptomycin resistance in pulmonary tuberculosis. *Br Med J* 2:1009-1015.

Dalla Costa ER, Ribeiro MO, Silva MS, Arnold LS, Rostirolla DC, Cafrune PI, Espinoza RC, Palaci M, Telles MA, Ritacco V, Suffys PN, Lopes ML, Campelo CL, Miranda SS, Kremer K, da Silva PE, Fonseca Lde S, Ho JL, Kritski AL, and Rossetti ML. 2009. Correlations of mutations in katG, oxyR-ahpC and inhA genes and in vitro susceptibility in Mycobacterium tuberculosis clinical strains segregated by spoligotype families from
tuberculosis prevalent countries in South America. *BMC Microbiol* 9:39. 10.1186/1471-2180-9-39

Du J, Pang Y, Ma Y, Mi F, Liu Y, and Li L. 2017. Prevalence of tuberculosis among health care workers in tuberculosis specialized hospitals in China. *J Occup Health*. 10.1539/joh.16-0251-BR

Franco-Sotomayor G, Garzon-Chavez D, Leon-Benitez M, de Waard JH, and Garcia-Bereguaiin MA. 2018. A First Insight into the katG and rpoB Gene Mutations of Multidrug-Resistant *Mycobacterium tuberculosis* Strains from Ecuador. *Microb Drug Resist*. 10.1089/mdr.2018.0203

Guo H, Seet Q, Denkin S, Parsons L, and Zhang Y. 2006. Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. *J Med Microbiol* 55:1527-1531. 10.1099/jmm.0.46718-0

Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, and Voelkerding KV. 2006. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin Chem* 52:494-503. 10.1373/clinchem.2005.063438

Koul A, Arnoult E, Lounis N, Guillemont J, and Andries K. 2011. The challenge of new drug discovery for tuberculosis. *Nature* 469:483-490. 10.1038/nature09657

Lee JH, Ammerman NC, Nolan S, Geiman DE, Lun S, Guo H, and Bishai WR. 2012. Isoniazid resistance without a loss of fitness in *Mycobacterium tuberculosis*. *Nat Commun* 3:753. 10.1038/ncomms1724
Li D, Song Y, Zhang CL, Li X, Xia X, and Zhang AM. 2017. Screening mutations in drug-resistant Mycobacterium tuberculosis strains in Yunnan, China. *J Infect Public Health* 10:630-636. 10.1016/j.jiph.2017.04.008

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, and Markham AF. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* 17:2503-2516.

Onseedaeng S, and Ratthawongjirakul P. 2016. Rapid Detection of Genomic Mutations in gyrA and parC Genes of Escherichia coli by Multiplex Allele Specific Polymerase Chain Reaction. *J Clin Lab Anal* 30:947-955. 10.1002/jcla.21961

Pankhurst LJ, Del Ojo Elias C, Votintseva AA, Walker TM, Cole K, Davies J, Fermont JM, Gascoyne-Binzi DM, Kohl TA, Kong C, Lemaitre N, Niemann S, Paul J, Rogers TR, Roycroft E, Smith EG, Supply P, Tang P, Wilcox MH, Wordsworth S, Wyllie D, Xu L, Crook DW, and Group C-TS. 2016. Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. *Lancet Respir Med* 4:49-58. 10.1016/S2213-2600(15)00466-X

Peng J, Yu X, Cui Z, Xue W, Luo Z, Wen Z, Liu M, Jiang D, Zheng H, Wu H, Zhang S, and Li Y. 2016. Multi-Fluorescence Real-Time PCR Assay for Detection of RIF and INH Resistance of M. tuberculosis. *Front Microbiol* 7:618. 10.3389/fmicb.2016.00618

Pietzka AT, Indra A, Stoger A, Zeinzinger J, Konrad M, Hasenberger P, Allerberger F, and Ruppitsch W. 2009. Rapid identification of multidrug-resistant Mycobacterium
tuberculosis isolates by rpoB gene scanning using high-resolution melting curve PCR analysis. *J Antimicrob Chemother* 63:1121-1127. 10.1093/jac/dkp124

Sandy J, Mushtaq A, Kawamura A, Sinclair J, Sim E, and Noble M. 2002. The structure of arylamine N-acetyltransferase from *Mycobacterium smegmatis*--an enzyme which inactivates the anti-tubercular drug, isoniazid. *J Mol Biol* 318:1071-1083. 10.1016/S0022-2836(02)00141-9

Scorpio A, and Zhang Y. 1996. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat Med* 2:662-667.

Seifert M, Catanzaro D, Catanzaro A, and Rodwell TC. 2015. Genetic mutations associated with isoniazid resistance in *Mycobacterium tuberculosis*: a systematic review. *PLoS One* 10:e0119628. 10.1371/journal.pone.0119628

Sharma D, Lather M, Dykes CL, Dang AS, Adak T, and Singh OP. 2016. Disagreement in genotyping results of drug resistance alleles of the *Plasmodium falciparum* dihydrofolate reductase (Pfdhfr) gene by allele-specific PCR (ASPCR) assays and Sanger sequencing. *Parasitol Res* 115:323-328. 10.1007/s00436-015-4750-2

Stoffels K, Mathys V, Fauville-Dufaux M, Wintjens R, and Bifani P. 2012. Systematic analysis of pyrazinamide-resistant spontaneous mutants and clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 56:5186-5193. 10.1128/AAC.05385-11

Thirumurugan R, Kathirvel M, Vallayyachari K, Surendar K, Samrot AV, and Muthaiah M.
2015. Molecular analysis of rpoB gene mutations in rifampicin resistant Mycobacterium tuberculosis isolates by multiple allele specific polymerase chain reaction in Puducherry, South India. *J Infect Public Health* 8:619-625. 10.1016/j.jiph.2015.05.003

Traore H, van Deun A, Shamputa IC, Rigouts L, and Portaels F. 2006. Direct detection of Mycobacterium tuberculosis complex DNA and rifampin resistance in clinical specimens from tuberculosis patients by line probe assay. *J Clin Microbiol* 44:4384-4388. 10.1128/JCM.01332-06

Tudo G, Rey E, Borrell S, Alcaide F, Codina G, Coll P, Martin-Casabona N, Montemayor M, Moure R, Orcau A, Salvado M, Vicente E, and Gonzalez-Martin J. 2010. Characterization of mutations in streptomycin-resistant Mycobacterium tuberculosis clinical isolates in the area of Barcelona. *J Antimicrob Chemother* 65:2341-2346. 10.1093/jac/dkq322

Villellas C, Aristimuno L, Vitoria MA, Prat C, Blanco S, Garcia de Viedma D, Dominguez J, Samper S, and Ainsa JA. 2013. Analysis of mutations in streptomycin-resistant strains reveals a simple and reliable genetic marker for identification of the Mycobacterium tuberculosis Beijing genotype. *J Clin Microbiol* 51:2124-2130. 10.1128/JCM.01944-12

Waagmeester A, Thompson J, and Reyrat JM. 2005. Identifying sigma factors in Mycobacterium smegmatis by comparative genomic analysis. *Trends Microbiol* 13:505-509. 10.1016/j.tim.2005.08.009

WHO. 2018. Global Tuberculosis Report. *Available at*
http://www.who.int/tb/publications/factsheet_global.pdf?ua=1.

Yuan X, Zhang T, Kawakami K, Zhu J, Li H, Lei J, and Tu S. 2012. Molecular characterization of multidrug- and extensively drug-resistant Mycobacterium tuberculosis strains in Jiangxi, China. *J Clin Microbiol* 50:2404-2413. 10.1128/JCM.06860-11

Zhang Y, and Yew WW. 2009. Mechanisms of drug resistance in Mycobacterium tuberculosis. *Int J Tuberc Lung Dis* 13:1320-1330.

Zhao LL, Liu HC, Sun Q, Xiao TY, Zhao XQ, Li GL, Zeng CY, and Wan KL. 2015. Identification of mutations conferring streptomycin resistance in multidrug-resistant tuberculosis of China. *Diagn Microbiol Infect Dis* 83:150-153. 10.1016/j.diagmicrobio.2015.06.020

Zhao Y, Xu S, Wang L, Chin DP, Wang S, Jiang G, Xia H, Zhou Y, Li Q, Ou X, Pang Y, Song Y, Zhao B, Zhang H, He G, Guo J, and Wang Y. 2012. National survey of drug-resistant tuberculosis in China. *N Engl J Med* 366:2161-2170. 10.1056/NEJMoa1108789

Zignol M, Dean AS, Falzon D, van Gemert W, Wright A, van Deun A, Portaels F, Laszlo A, Espinal MA, Pablos-Mendez A, Bloom A, Aziz MA, Weyer K, Jaramillo E, Nunn P, Floyd K, and Raviglione MC. 2016. Twenty Years of Global Surveillance of Antituberculosis-Drug Resistance. *N Engl J Med* 375:1081-1089. 10.1056/NEJMsr1512438
Figure 1

Figure 1. Detection of 16 mutations in four first-line drug-resistance genes by using AS-PCR.

M means DNA marker DL2000; m and w mean the AS-PCR primers were used to screen mutation and wildtype alleles, respectively. NC means negative control.
Figure 2

Figure 2. Specificity of AS-PCR primers for A128G in the *rpsL* gene.

**A** PCR were performed by using fourteen DNA templates with allele A and wildtype AS-PCR primers. **B** PCR were performed by using fourteen DNA templates with allele A and mutation AS-PCR primers. **C** PCR were performed by using eight DNA templates with allele G and mutation AS-PCR primers. **D** PCR were performed by using eight DNA templates with allele G and wildtype AS-PCR primers. Fragments at 206 bp mean the specific product by AS-PCR primers; fragments at 104 bp mean the inner control product by inner primers; NC means negative control.
Manuscript to be reviewed

A

250 bp
100 bp

B

250 bp
100 bp

C

250 bp
100 bp

D

250 bp
100 bp

Manuscript to be reviewed
Figure 3

Figure 3. Sensitivity of AS-PCR primers for A128G in the *rpsL* gene.

M means DNA marker DL2000; m and w mean the AS-PCR primers were used to screen mutation and wildtype alleles, respectively.
Figure 4

Figure 4. Detecting allele type of A128G in the \textit{rpsL} genes by using AS-PCR primers and real-time quantitative PCR.

(A) Melt curves of product by using DNA template with allele A. (B) Melt curves of product by using DNA template with allele G.