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

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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 detecting method. A total of 32 pairs of AS-PCR primers were designed to screen mutation and/or wild-type alleles of 16 variations in four firstline 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 Allele-specific PCR (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. Specific assay suggested that mutation and wildtype AS-PCR primers could amplify the corresponding allele under optimized PCR conditions. The sensitivity of third-quarters primers was 500 copy numbers, and the other 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.

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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 detecting method. A total of 32 pairs of AS-PCR primers were designed to screen mutation and/or wild-type alleles of 16 variations in four firstline 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 Allele-specific PCR (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. Specific assay suggested that mutation and wildtype AS-PCR primers could amplify the corresponding allele under optimized PCR conditions. The sensitivity of third-quarters primers was 500 copy numbers, and the other 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 2014, 9.6 million people suffered from TB, among which 1.5 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 8, 000, 000 dollars in 2015. Half of this amount was used to treat drug-resistant TB (DR-TB) patients (WHO 2015).

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 111, 000 in 2014. About 9.7% of MDR-TB were extensively drug-resistant TB (XDR-TB). The MDR-TB persons in 2014 had a 14% growth ratio compared with that in 2013 (WHO 2015). The average cure rate of MDR-TB patients was about 50%, whereas the cure rate of XDR-TB patients was significantly lower (WHO 2015). 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 *sigI*, *ndh*, and others, also reportedly to lead to INH-resistance in TB (Guo et al. 2006). Similarly, *rpoB*, *embB*, *rpsL*, and *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).

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. In this study, 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

74 instructions. A totally of 16 point mutations in 4 genes were identified in 57 drug-resistant *M.*
75 *tuberculosis* strains (Li et al. 2017). This study was approved by the institutional review board of
76 Kunming University of Science and Technology (Approval No. 2014SK027).
77
78 **2.2 Primer design and AS-PCR optimization**
79 A total of 32 pairs of primers for AS-PCR were designed to screen the 16 variations
80 (including mutation and wild-type alleles), which were located in four genes (*katG*, *embB*, *rpsL*,
81 and *pncA*), by using Oligo Primer Analysis Software v.7 (Table S1). To strengthen the
82 specificity of primers, a random nucleotide in 3' end of the allele-specific primer was factitiously
83 changed and marked in red in Table S1. One pair of inner control primers (16S 915-F/16S 1018-
84 R) was used to control and identify PCR quantification (Table S1).
85 PCR was performed at 20 μL reaction volume and involved 30 ng of genomic DNA, 10 μL
86 2×TSINGKE™ Master Mix (including DNA polymerase 1 U， $MgCl_2$ 1.5 mM, KCl 50 mM, and
87 dNTP 100 mM) (TSINGKE, China) or ChamQ™ SYBR qPCR Master Mix (Vazyme, China),
88 and 0.5 μM each primer (including AS-PCR primers and internal control primers). After
89 optimization, we used the following PCR condition: one cycle of 95 °C for 3 min; 35 cycles of
90 95 °C for 30 s, optimized temperature for 30 s (Table S1), 72 °C for 10 s; and one extension
91 cycle of 72 °C for 5min.
92
93 **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 randomly amplified 14 samples by using wildtype strains and AS-PCR primers for mutations to test their specificity. 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 5×10^2 copy numbers to achieve a sensitive assay.

3. Results

Sixteen mutations in four first-line drug-resistant genes were identified by sequencing in our previous study . After optimizing the conditions of 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 wild-type alleles with high quality (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 5×10^2 copy numbers. When the copy number of the plasmids was 5×10^2, 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 5×10^2. However, the others needed more copy numbers (10^3 or 10^4, 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 defined the baseline at 100 relative fluorescence units (RFU)/min, and 13 mutations were genotyped by the AS-real-time quantitative PCR (AS-qPCR) (Fig. S2). 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 (Fig. 4).

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 isoniazid-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, and *pncA* gene were the main factors for RIF-resistant, SM-resistant, and PZA-resistant TB strains, respectively (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.

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 used AS-qPCR to screen 16 mutations in four first-line DR-genes. After optimizing reaction conditions, we successfully amplified the corresponding bands and melt curves 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.

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 sensitivity tests.

Figure S2. Melt curves map of real-time quantitative PCR.

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Figure 1(on next page)

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

M means DNA marker DL2000; 1 and 2 mean the AS-PCR primers were used to screen mutation and wildtype alleles, respectively.

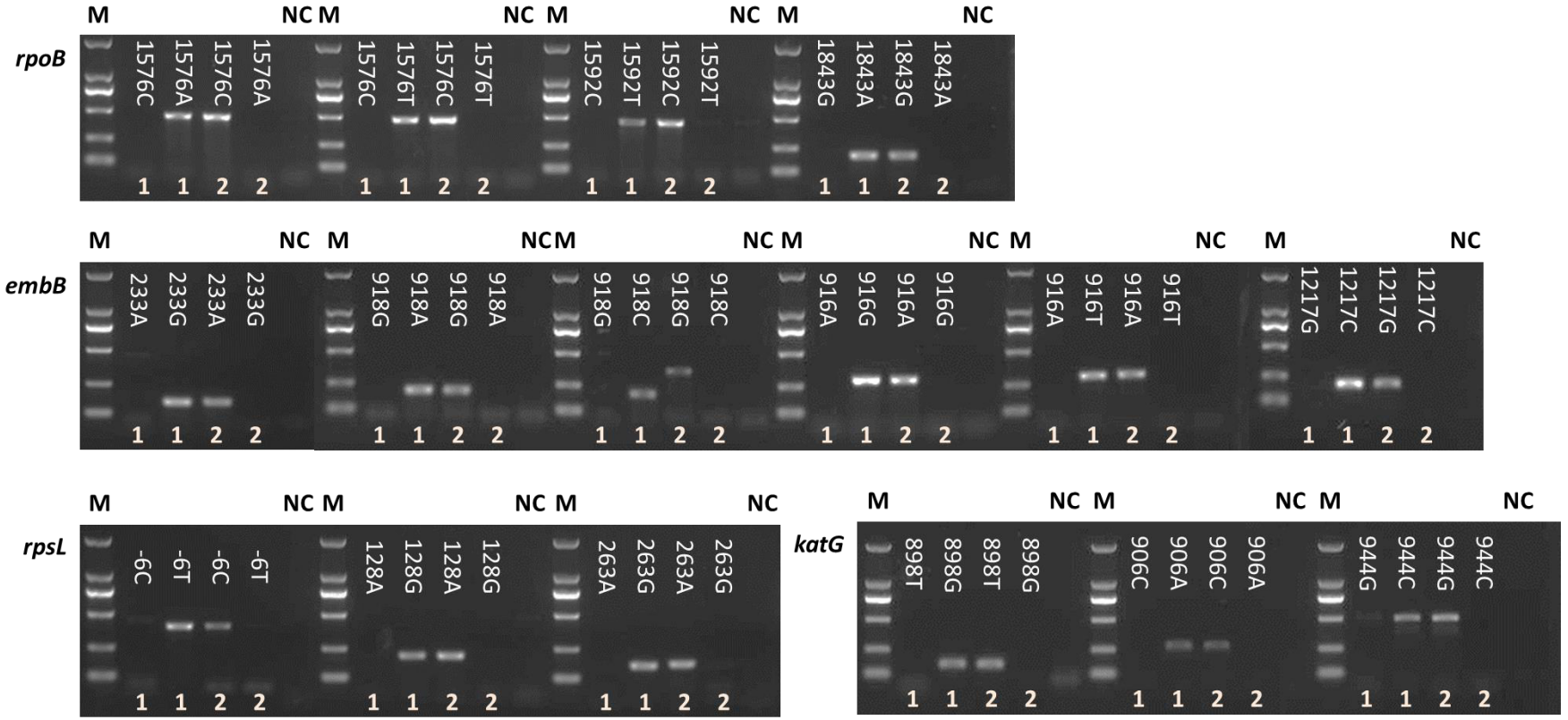

rpoB
M NC M NC M NC M NC
1576C 1576A 1576C 1576A
1576C 1576T 1576C 1576T
1592C 1592T 1592C 1592T
1843G 1843A 1843G 1843A
1 1 2 2
embB
M NC M NC M NC M NC M NC M NC
233A 233G 233A 233G
918G 918A 918G 918A
918G 918C 918G 918C
916A 916G 916A 916G
916A 916T 916A 916T
1217G 1217C 1217G 1217C
rpsL
M NC M NC M NC
-6C -6T -6C -6T
128A 128G 128A 128G
263A 263G 263A 263G
katG
M NC M NC M NC
898T 898G 898T 898G
906C 906A 906C 906A
944G 944C 944G 944C

Figure 2(on next page)

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

(A) PCR were performed by using DNA template with allele A and wildtype AS-PCR primers. (**B)** PCR were performed by using DNA template with allele A and mutation AS-PCR primers. (**C)** PCR were performed by using DNA template with allele G and mutation AS-PCR primers. (**D)** PCR were performed by using DNA template 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; M means DNA marker DL2000; NC means negative control.

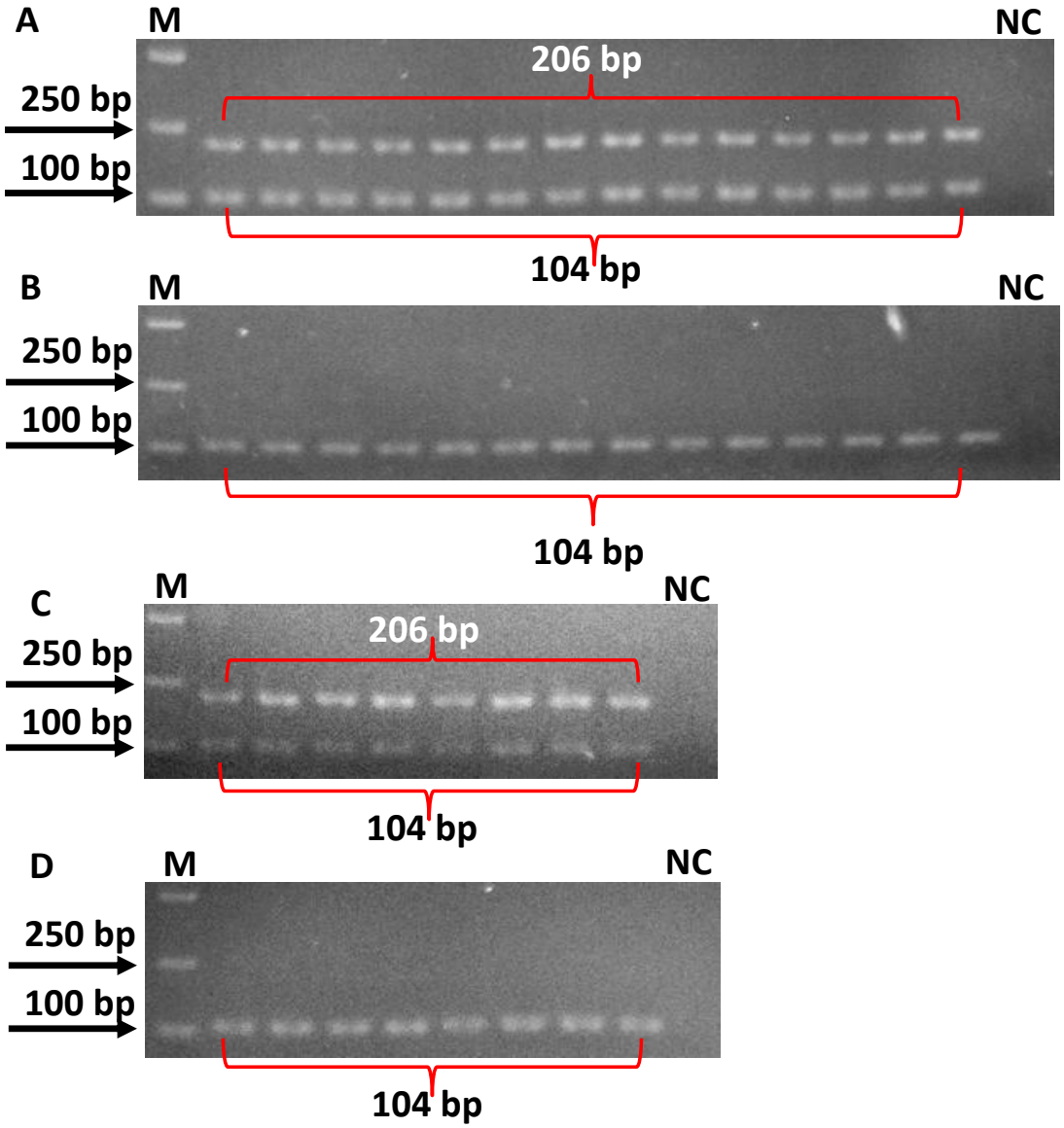

A
M
NC
206 bp
250 bp
100 bp
104 bp
B
M
NC
250 bp
100 bp
104 bp
C
M
NC
206 bp
250 bp
100 bp
104 bp
D
M
NC
250 bp
100 bp
104 bp

Figure 3(on next page)

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

M means DNA marker DL2000; 1 and 2 mean the AS-PCR primers were used to screen mutation and wildtype alleles, respectively.

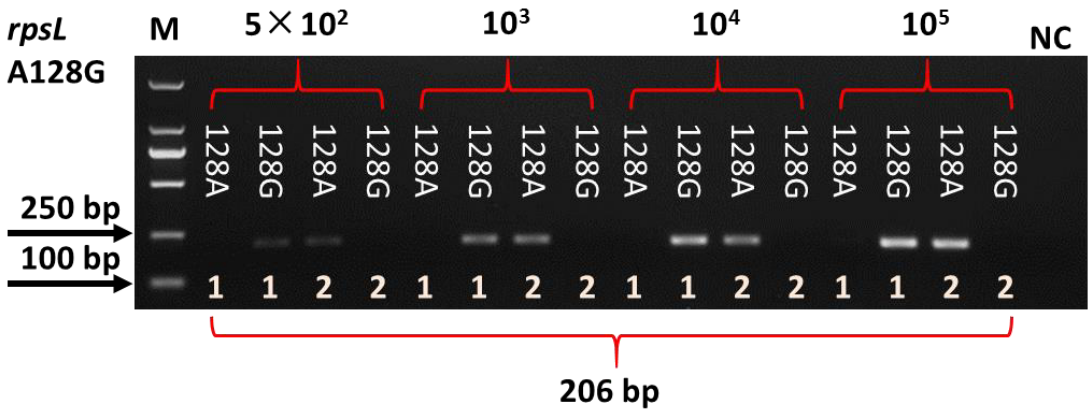
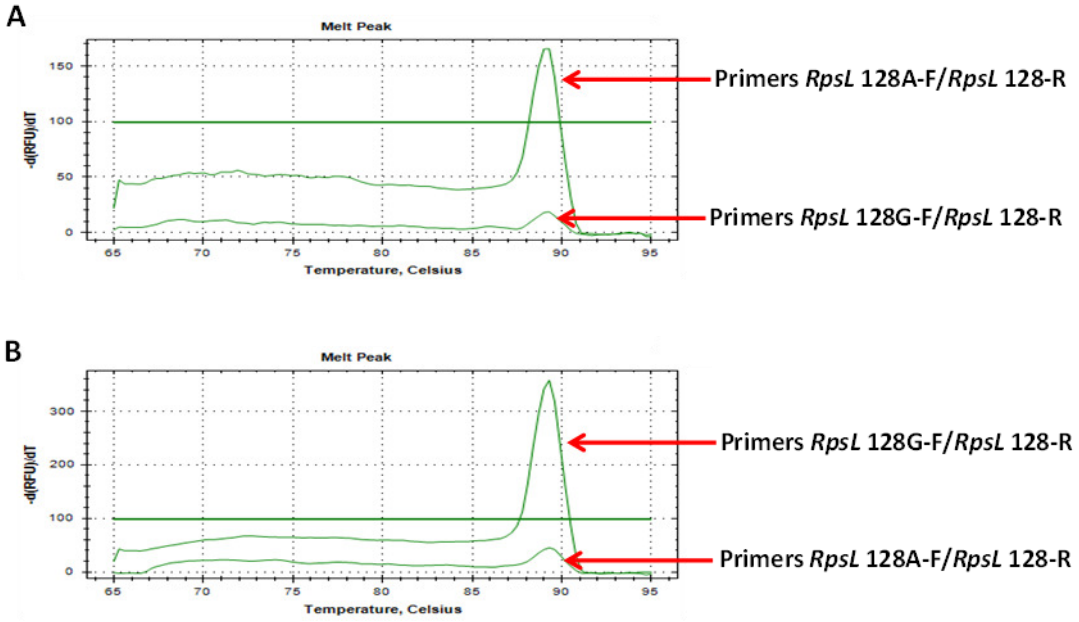

rpsL
A128G
M
5×10²
10³
10⁴
10⁵
NC
250 bp
100 bp
128A 128G 128A 128G 128A 128G 128A 128G 128A 128G 128A 128G 128A 128G 128A 128G
1 1 2 2 1 1 2 2 1 1 2 2 1 1 2 2
206 bp

Figure 4(on next page)

Detecting allele type of A128G in the *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.


A
Melt Peak
-d(RFU)/dT
Temperature, Celsius
Primers RpsL 128A-F/RpsL 128-R
Primers RpsL 128G-F/RpsL 128-R
B
Melt Peak
-d(RFU)/dT
Temperature, Celsius
Primers RpsL 128G-F/RpsL 128-R
Primers RpsL 128A-F/RpsL 128-R