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Detection of resistance to fluoroquinolones and injectable drugs among antituberculosis drugs by allele-specific primer extension on a microsphere-based platform

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ABSTRACT

Molecular drug susceptibility testing (DST) for antituberculosis drugs is important for improving the efficacy of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) treatment. In this study, we developed a molecular high-throughput assay system based on allele-specific primer extension (ASPE) and MagPlex-TAG microspheres, referred to here as TAG-ASPE, which can detect mutations related to resistance to injectable second-line drugs and fluoroquinolones. Target genes were amplified by multiplex PCR using DNA from H37Rv and 190 clinical *Mycobacterium tuberculosis* strains and extended by ASPE using 22 ASPE primers. ASPE products were then sorted on the TAG-ASPE array and detected using a Luminex 200 system. The performance of the TAG-ASPE method was compared with that of sequencing and phenotypic DST. Comparison of the TAG-ASPE method with sequencing showed that the sensitivity and specificity of the TAG-ASPE method were 100% [95% confidence interval (CI), 96.38–100%] and 100% (95% CI, 95.70–100%) for the *rrs* gene and 100% (95% CI, 96.90–100%) and 100% (95% CI, 95.07–100%) for the *gyrA* gene, respectively. Compared with phenotypic DST, the sensitivity and specificity of the TAG-ASPE method for detecting drug-resistance mutations against injectable second-line drugs were 92.52% (95% CI, 85.8–96.72%) and 98.7% (95% CI, 92.98–99.97%), respectively. Additionally, the sensitivity and specificity for fluoroquinolone-resistance detection were 85.4% (95% CI, 78.36–90.85%) and 100% (95% CI, 92.38–100%), respectively. The results of this study demonstrate that the TAG-ASPE method can effectively detect mutations conferring resistance to second-line antituberculosis drugs in numerous clinical specimens.

1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* and has long been a leading cause of death worldwide (Su et al., 2017). In 2015, the World Health Organization (WHO) estimated that approximately 10.4 million people developed TB, including 580,000 people with multidrug-resistant tuberculosis (MDR-TB) and that 1.4 million patients died from the disease (WHO, 2016). Increasing TB incidence due to MDR-TB [defined as resistance to at least two of the first-line anti-TB drugs, rifampin (RIF) and isoniazid (INH)] interferes with efforts to control and manage TB (Lee et al., 2015). Because MDR-TB treatment is associated with severe side effects, high cost, long treatment period, and low cure rates, it is important to prevent the spread of MDR-TB in public health clinics (WHO, 2016).

MDR-TB with additional resistance to fluoroquinolones [FQs; ofloxacin (OFX), levofloxacin (LVX), and moxifloxacin (MXF)] and second-line injectable drugs [SLIDs; amikacin (AMK), kanamycin (KAN), and capreomycin (CPM)] is also observed. The global success rate for treating MDR-TB is approximately 52% (WHO, 2016), and TB strains resistant to all TB drugs have emerged (Dheda et al., 2017). On average, 9.4% (95% confidence interval (CI), 7.4–11.6%) of MDR-TB strains are extensively drug-resistant TB (XDR-TB) strains resistant to both FQs and SLID (Falzon, et al., 2013).

The drugs OFX, LVX, and MXF are FQs that selectively inhibit the TB DNA enzymes gyrase and topoisomerase IV. Gyrase is encoded by *gyrA* and *gyrB*, and topoisomerase IV is encoded by *parC* and *parE* (Pestova et al., 1999). Because *M. tuberculosis* produces only one DNA gyrase, it can develop resistance to FQs by acquiring mutations in the *gyrA* and

Abbreviations: ASPE, allele-specific primer extension; CI, confidence interval; DST, drug susceptibility testing; FQs, fluoroquinolones; INH, isoniazid; L–J, Lowenstein–Jensen; LVX, levofloxacin; MDR-TB, multidrug-resistant tuberculosis; MXF, moxifloxacin; OFX, ofloxacin; RIF, rifampin; SLIDs, second-line injectable drugs; TB, tuberculosis; WHO, World Health Organization; XDR-TB, extensively drug-resistant tuberculosis

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gyrB genes (Palomino and Martin, 2014), with the most frequent mutations found at positions 90 and 94 of *gyrA*.

AMK and KAN are aminoglycosides, and CPM is a cyclic peptide antibiotic. The aminoglycosides act by binding to the 30S ribosomal subunit and cause misreading of the genetic code, resulting in aberrant protein synthesis. CPM acts by binding across the 23S rRNA and 16S rRNA, resulting in inhibition of protein synthesis. Resistance to AMK, KAN, and CPM is mainly caused by mutations at positions 1401 and 1402 of the bacterial *rrs* gene, with mutations at position 1484 also reported (Palomino and Martin, 2014; Sowajassatakul et al., 2014).

Rapid and reliable drug susceptibility testing (DST) should be established for treating patients with MDR-TB. Conventional DST is performed by using solid media containing anti-TB drugs and is a low-cost method that has been used for decades, although > 8 weeks are required to obtain the results, which substantially delays diagnosis. In recent years, it has become possible to diagnose TB more quickly and simply using various molecular or non-molecular tests (Ignatyeva et al., 2012; Lin et al., 2009; Rufai et al., 2014). The diagnosis of resistant TB, which is currently the most important problem in TB control, can be diagnosed faster than with conventional DST, and quicker methods will contribute positively to TB control. Previously, we developed a microsphere-based test based on the allele-specific primer extension (ASPE) method for detecting 14 different drug-resistance mutations in four target genes responsible for resistance to the first-line anti-TB drugs INH, RIF, and ethambutol (Lee et al., 2015). Here, we modified the microsphere and ASPE-based platform to diagnose mutations conferring resistance to FQs and SLIDs.

2. Materials and methods

2.1. Mycobacterial strains, DST, and DNA extraction

In this study, 190 *M. tuberculosis* strains isolated from clinical isolates and *M. tuberculosis* H37Rv (ATCC 27294) were provided by the Korean Institute of Tuberculosis (Cheongju, Korea). Because strains from clinical isolates were used in this study, ethical approval was exempted by the Institutional Review Board. The reference strain *M. tuberculosis* H37Rv was used as the wild-type control. All clinical *M. tuberculosis* strains and H37Rv were subjected to phenotypic DST using Lowenstein–Jensen (L–J) medium. The critical concentrations of the drugs tested were based on WHO guidelines (WHO, 2012). A loop of fresh bacterial culture grown on L–J media was transferred to a screw-cap microcentrifuge tube containing 400 μL of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. After boiling at 100 °C for 15 min in a water bath, heat-killed bacteria were centrifuged for 1 min at 16,000*g*, and each supernatant was stored at − 20 °C before use (Lee et al., 2015).

2.2. Multiplex PCR

Each multiplex PCR mixture consisted of 50 ng genomic DNA, 0.2 μM each primer, 1 × Qiagen PCR reaction buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, and 2.5 U Qiagen HotStart Taq polymerase (Qiagen, Hilden, Germany). A negative control (without template DNA) was included in each test. The PCR primer sequences are shown in Table 1. Amplification was performed using the following thermocycling protocol: 95 °C for 15 min; 40 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 45 s; followed by a final extension at 72 °C for 7 min (Lee et al., 2015). Multiplex PCR products were stored at 4 °C until use and analyzed by agarose gel electrophoresis.

Table 1
Multiplex PCR primer sequences.

Gene	Size (bp)		Sequence, 5′ → 3′
rrs	308	Forward	GCG AAT CCT TAA AAG CCG GTC T
		Reverse	GCC TAC GCC CCA CCA GTT GGG G
gyrA	160	Forward	ACC GCA GCC ACG CCA AGT C
		Reverse	CCT GGC GAG CCG AAG TTG C

2.3. Exonuclease I-shrimp alkaline phosphatase treatment

To remove unused primers and nucleotides, multiplex PCR products were treated with exonuclease I-shrimp alkaline phosphatase. ExoSAP-IT reagent (3 μL; Affymetrix, Cleveland, OH, USA) was mixed with 7.5 μL of the multiplex PCR product and incubated at 37 °C for 30 min, heated to 80 °C for 15 min to inactivate the enzyme, and stored at 4 °C until use (Lee et al., 2015).

2.4. Multiplex ASPE reaction

Each multiplex ASPE reaction was performed in a final volume of 20 μL that consisted of 5 μL ExoSAP-IT-treated PCR product, 1 × ASPE buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 1.25 mM $MgCl_2$, 0.75 U Tsp DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 μM dATP, dTTP, and dGTP (Invitrogen), 5 μM biotin-dCTP (Invitrogen), and 25 nM each TAG-ASPE primer. The TAG sequence information was provided by Luminex Corporation (Austin, TX, USA), and the TAG-ASPE primer sequences are listed in Table 2. The ASPE reaction was performed at 96 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 74 °C for 2 min, after which ASPE reactions products were stored at 4 °C until use (Lee et al., 2015).

2.5. Hybridization to MagPlex-TAG microspheres

Each hybridization reaction was prepared with 2500 MagPlex-TAG microspheres (of each set) per reaction (Luminex Corporation). The MagPlex-TAG microsphere mixture was diluted to 100 of each microsphere set per μL in 2 × Tm hybridization buffer [0.4 M NaCl, 0.2 M Tris, and 0.16% Triton X-100 (pH 8.0)] and mixed by vortexing. The MagPlex-TAG microsphere mixture (25 μL) was aliquoted into each well, followed by addition of 20 μL of distilled water. Five microliters of each ASPE reaction was then added directly to each well, and the mixture was denatured at 96 °C for 90 s and hybridized at 37 °C for 30 min. Microspheres were pelleted by placing the plate on a magnetic separator, and the supernatant was removed. This step was performed twice with 1 × Tm hybridization buffer [0.2 M NaCl, 0.1 M Tris, and 0.08% Triton X-100 (pH 8.0)]. Tm hybridization buffer (1 ×; 75 μL) containing 5 μg/mL streptavidin-R-phycoerythrin (Invitrogen) was added to each well and incubated for 15 min at 37 °C. The microspheres were analyzed using a Luminex 200 system (Luminex Corporation) (Lee et al., 2015).

2.6. Sequencing

To confirm the performance of the microsphere-based TAG-ASPE method, we performed sequencing commercially (Cosmogenetech, Daejeon, Korea). DNA samples not analyzed for the presence of each mutation were excluded.

2.7. Data analysis

Median fluorescence intensity (MFI) was used to calculate the allelic ratio for each biallelic marker, as follows:

$$\text{Wild} - \text{type allelic ratio} = \frac{(NET\ MFI)\ wild\ type\ allele}{(NET\ MFI)\ wild\ type\ allele + (NET\ MFI)\ mutant\ allele}$$