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# Molecular drug resistance profiles of *Mycobacterium tuberculosis* from sputum specimens using ion semiconductor sequencing

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ABSTRACT

The increasing burden of multidrug resistant (MDR)-TB, defined by resistance to rifampin (RFP) and isoniazid (INH), and extensively drug resistant-TB, defined by MDR-TB with additional resistance to fluoroquinolones (FQs) and more than one second-line injectable drug, is a serious impediment to global TB control. We evaluated the feasibility of full-length gene analysis including *inhA*, *katG*, *rpoB*, *pncA*, *rpsL embB*, *eis*, and *gyrA* using a semiconductor NGS with the Ion AmpliSeq TB panel to directly analyse 34 sputum specimens confirmed by phenotypic DST: INH, RFP, ethambutol (EMB), pyrazinamide (PZA), amikacin, kanamycin, streptomycin (SM), FQs including ofloxacin, moxifloxacin, and levofloxacin. The molecular drug resistance profiles showed "very good" and "substantial" strength of agreement for the phenotypic DST results of RFP and EMB, PZA, SM, FQs resistance with specificities of 96%, and 88%, 97%, 100% and sensitivities of 100%, and 88%, 60%, 67%, respectively. The strength of agreement for the detection of resistance to INH was "substantial", compared between *katG* mutation and phenotypic INH only. Ion semiconductor NGS could make possible detection of several uncommon or novel amino acid changes in the full coding regions of these eight genes. However, molecular drug resistant profile should be complemented and validated by subsequent phenotypic DST studies at the same time.

## 1. Introduction

*Mycobacterium tuberculosis* (MTB), an obligate pathogenic bacterial species for tuberculosis (TB), is a highly transmissible agent with significant morbidity and mortality. Global strategies for TB treatment and control are designed to accurately and rapidly diagnose, treat, and reduce the transmission of drug-susceptible and drug-resistant TB (World Health Organization, 2016). The standard short-course treatment is effective for drug-susceptible TB, with cure rates > 95% (Hopewell et al., 2014). Unfortunately, the increasing burden of multidrug resistant (MDR)-TB, defined by resistance to rifampin (RFP) and isoniazid (INH), and extensively drug resistant (XDR)-TB, defined by MDR-TB with additional resistance to fluoroquinolones (FQs) and more than one second-line injectable drug, is a serious impediment to global TB control (World Health Organization, 2016). The latest outcome data show a treatment success rate of 83% for TB (2014 cohort), 52% for MDR-TB (2013 cohort), and 28% for XDR-TB (2013 cohort) (World Health Organization, 2016). Currently, the reference method for determining drug susceptibility of MTB, using either solid or liquid media, requires weeks to months to yield results due to the pathogen's slow growth. However, genotypic tests such as the commercial GeneXpert MTB/RIF® (Cepheid, Sunnyvale, CA) (Dharan et al., 2016) and GenoType MTBDRplus® (Hain Lifescience, Nehren, Germany) (Lacoma et al., 2008; Meaza et al., 2017) assays offer rapid assessment of drug resistance mutations of the *rpoB*, *inhA*, *katG*, and *aphC* genes against key first-line anti-TB drugs; the GenoType MTBDRsl® (Hain Lifescience) assay (Brossier et al., 2010; Zeng et al., 2016) can also identify the most common mutations of the *rrs*, *gyrA*, and *embB* genes, which result in resistance to second-line agents. However, the number of mutations evaluated by current genotypic tests is limited, and the mechanisms of resistance are not completely understood. Thus, more reliable, informative, and rapid diagnostic tools for identifying MDR/XDR-TB are essential to appropriately treat and control the disease.

In recent years, whole genome sequencing (WGS) has offered novel

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insight into the evolution and spread of MTB, but its application in routine patient care has been limited due to the high cost, questionable clinical relevance, complexity of interpretation, and slow turn-around times (TATs) (Colman et al., 2016; Operario et al., 2017; Pankhurst et al., 2016; Witney et al., 2016). Fortunately, significant and continued advances have made routine use of targeted sequencing feasible as a viable public health and clinical tool. By using drug resistance determining regions of TB genes, medical laboratories can provide results within a clinically relevant time frame. Among the various next-generation sequencing (NGS) platforms, amplicon-based NGS has been promoted as for use in public health surveillance of MDR-TB (Colman et al., 2016; Daum et al., 2014; Daum et al., 2012). Ion semiconductor sequencing utilises a small chip for the detection of released hydrogen ions emitted during DNA polymerisation when deoxynucleotide triphosphates are incorporated into a growing strand of DNA (Rothberg et al., 2011).

In this study, we evaluated the feasibility of full-length gene analysis using a semiconductor NGS with the Ion AmpliSeq TB panel to directly analyse 34 sputum specimens. To determine if ion semiconductor sequencing could predict drug resistant phenotypes, the results were compared with data obtained by the Advansure™ MDR-TB Genoblot assay and the conventional phenotypic drug susceptibility testing (DST).

## 2. Materials and methods

### 2.1. Specimens and DNA preparation

Sputum specimens were liquefied and decontaminated using an equal volume of 4% NaOH and N-Acetyl-L-Cysteine (NALC) at room temperature for 15 min. After decontamination, all specimens were neutralized with phosphate-buffered saline (PBS) (pH 6.8) and centrifuged at 3000*g* for 15 min. The sediment obtained after centrifugation was resuspended in 2 mL of PBS and was used for Auramine-Rhodamine staining, TB/NTM PCR, and inoculation for culture medium. The leftover sediments were frozen at − 20 °C until used.

The presence of MTB was screened by Auramine-Rhodamine staining and AdvanSure TB/NTM real-time PCR (LG Life Sciences, Seoul, Korea) that targets the *IS6110* and the *rpoB* genes specific for the MTB complex. In addition, the MTB isolates form sputum specimens were confirmed by conventional culture methodology: the sediment was cultivated using a BACTEC MGIT 960 liquid culture system (Becton Dickinson, Franklin Lakes, NJ) for 6 weeks and with 3% Ogawa solid media for 8 weeks in 5–10% $CO_2$ incubators.

Of 268 samples requested for phenotypic DST from Daejeon St. Mary's Hospital (Daejeon, Korea) to the Korean Institute of Tuberculosis (Cheongju, Korea) between July 2015 and August 2017, 34 (9.2%) with resistance to ≥ 1 drug resistance were selected. Mycobacterial genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hamburg, Germany) according to manufacturer's protocol from 34 frozen leftover sediments stored at the Department of Laboratory Medicine, Daejeon St. Mary's Hospital. Nucleic acid quality and quantity were assessed by a Nano-Drop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and agarose gel electrophoresis. DNA concentrations were determined using a broad range Qubit DNA Kit and a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA).

### 2.2. Advansure™ MDR-TB Genoblot assay

Species identification of MTB isolates were archived from Ogawa solid media or the BACTEC MGIT 960 system. Genotypic resistance due to common mutations conferring resistance to RFP and INH was determined using the Advansure™ MDR-TB Genoblot assay (LG Life Sciences, Seoul, Korea) which can detect mutations in the 81-bp hotspot region of *rpoB*, the 313-319 codon region of the *katG* gene, and the *inhA* and *ahpC* promoter regions (Kim et al., 2012) according to the manufacturer's instructions at the Korean Institute of Tuberculosis.

### 2.3. Phenotypic drug susceptibility testing

Phenotypic resistance for first- and second-line drugs was determined using the conventional DST at the Korean Institute of Tuberculosis. The methodology used was the absolute concentration method with egg-based Löwenstein-Jensen medium. The critical concentrations of first-line anti-tuberculosis drugs were follows as: INH 0.2 μg/mL, RFP 40 μg/mL, and ethambutol (EMB) 2.0 μg/mL. Pyrazinamide (PZA) susceptibility was determined by the pyrazinamidase activity test. The critical concentrations of second-line drugs were as follows: streptomycin (SM) 10 μg/mL, amikacin (AMK) 30 μg/mL, kanamycin (KM) 30 μg/mL, ofloxacin (OFX) 4.0 μg/mL, moxifloxacin (MXF) 2.0 μg/mL, and levofloxacin (LEV) 2.0 μg/mL.

### 2.4. Molecular drug resistance profiles by ion semiconductor sequencing

To identify gene variants correlating with drug resistance within the genomic DNA of MTB extracted directly from sputum specimens,

**Table 1**
Major *M. tuberculosis* genes associated with acquisition of drug resistance in Ion AmpliSeq TB panel.

| Drug | Gene | Rv No. | Length (bp) | Function |
|---|---|---|---|---|
| | | | | Product |
| First line | | | | |
| Isoniazid | *katG* | Rv1908c | 2223 | Catalase, broad-spectrum peroxidase, and peroxynitritase activities<br>Catalase-peroxidase-peroxynitritase T KatG |
| | *inhA* | Rv1484 | 810 | Involved in mycolic acid biosynthesis<br>NADH-dependent enoyl-[acyl-carrier-protein] reductase InhA |
| Rifampin | *rpoB* | Rv0667 | 3519 | Catalyzes the transcription of DNA into RNA<br>DNA-directed RNA polymerase β chain RpoB |
| Ethambutol | *embB* | Rv3795 | 3297 | Involved in the biosynthesis of the mycobacterial cell wall arabinan<br>Integral membrane indolylacetylinositol arabinosyltransferase EmbB |
| Pyrazinamide | *pncA* | Rv2043c | 561 | Converts amides such as nicotinamide to corresponding acid<br>Pyrazinamidase/nicotinamidase PncA |
| Second line | | | | |
| Streptomycin | *rpsL* | Rv0682 | 375 | Involved in the translation initiation step<br>30S ribosomal protein S12 RpsL |
| Amikacin, Kanamycin | *eis* | Rv2416c | 1209 | Involved in intracellular survival and associated with the cell surface and secretion<br>Enhanced intracellular survival protein Eis, GCN5-related *N*-acetyltransferase |
| Fluoroquinolones | *gyrA* | Rv0006 | 2517 | Catalyzes the interconversion of topological isomers of double-stranded DNA rings<br>DNA gyrase GyrB |