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Short communication

RNA expression analysis of efflux pump genes in clinical isolates of multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* in South Korea

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ABSTRACT

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis*, is an important communicable disease. Various mechanisms of resistance to antituberculosis drugs have been reported; these are principally mutations in target genes. However, not all *M. tuberculosis* resistance can be explained by mutations in such genes. Other resistance mechanisms associated with drug transport, such as efflux pumps, have also been reported. In this study, we investigated the expression levels of three putative efflux pumps and mutations in target genes associated with injectable agents and fluoroquinolones with clinical MDR and XDR-TB isolates. Thirty clinical isolates of *M. tuberculosis* that had been phenotypically characterized were obtained from the Korean Institute of Tuberculosis. Of these, 14 were MDR-TB isolates resistant to at least one injectable aminoglycoside (amikacin; AMK, kanamycin; KAN, and/or capreomycin; CPM) and 16 were XDR-TB isolates. *M. tuberculosis* H37Rv (ATCC 27249) was used as a reference strain. Five putative genes (*Rv*1258*c*, *Rv*2686*c*, *Rv*2687*c*, *Rv*2688*c* and *pstB*) were selected for analysis in this study. Sequencing was performed to detect mutations in *rrs* and *eis* genes. qRT-PCR was performed to investigate expression levels of five efflux pump genes. Of the 30 isolates, 25 strains had mutations in *rrs* associated with resistance to KAN, CPM and AMK and two strains had *eis* mutations, as well as mutations in *rrs*. *pstB* (*Rv*0933) exhibited increased expression and *Rv*2687*c* and *Rv*2688*c* exhibited decreased expression compared to the reference strain. Increased expression of *pstB* in clinical drug-resistant tuberculosis isolates may contribute to drug resistance in *M. tuberculosis*. In our case, overexpression of *Rv*1258*c* may have been associated with resistance to kanamycin. No correlation was evident between *Rv*2686*c*, *Rv*2687*c* or *Rv*2688*c* expression and fluoroquinolone resistance. To explore the details of efflux pump drug-resistance mechanisms, further studies on efflux pump inhibitors, transcriptional regulators, such as *whiB7*, and additional efflux pump genes are needed.

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis*, is an important communicable disease. Although the global prevalence of the disease is declining slowly, ~9.6 million new cases of TB and 1.5 million deaths caused by TB were recorded in 2014 (World Health Organization, 2015). Treatment of TB is difficult because of the need to take multiple drugs for several months. Furthermore, multidrug-resistant tuberculosis (MDR-TB), resistant to at least isoniazid (INH) and rifampicin (RIF), and extensively drug-resistant tuberculosis (XDR-TB; additionally resistant to fluoroquinolones and at least one injectable agent including amikacin; AMK, kanamycin; KAN, and/or capreomycin; CPM), render treatment problematic. Globally, ~3.3% of new patients had MDR-TB and 9.7% of them had XDR-TB in 2014 (World Health Organization, 2015).

Various mechanisms of resistance to antituberculosis drugs have been reported; these are principally mutations in target genes (Zhang & Yew, 2009). However, not all *M. tuberculosis* resistance can be explained by mutations in such genes. Other resistance mechanisms associated with drug transport, such as efflux pumps, have also been reported (Ball et al., 1980).

Five families of efflux pumps have been associated with drug resistance: three superfamilies including the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS) and the resistance nodulation division (RND) superfamily; and two other families including the multidrug and toxic-compound extrusion (MATE) family and the small multidrug resistance (SMR) family (De Rossi et al., 2006; Piddock, 2006; Sarathy et al., 2012; Stavri et al., 2007). These five

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Table 1
Primers used for this study.

Gene	Product size (bp)	Primer (5′-3′)	Reference
rrs	1524	F-AGAGTTTGATCCTGGCTCAG R-AAGGAGGTGATCCAGCCGCA	(Loffler et al., 2000)
eis	257	F-ATCGGTGAAACTGGCCGCGG R-CGGGGTATGCGTCGACGTGG	This study
*Rv*1258*c*	110	F-CCTCAACCTGGCCTTTATTG R-GGATGGACAACCCGAATG	This study
*Rv*2686*c*	137	F-CGGTGGCGAACAACAAG R-AGCCCAGTACGGTGGTAAGA	This study
*Rv*2687*c*	128	F-ATCATCGGGTTCTTCTTCGT R-GCCAGCAGCACAGTTAGTTT	This study
*Rv*2688*c*	142	F-CAGTCCCACCGAACTGAA R-GTTTCGCAGCACGGAGT	This study
pstB	94	F-CTGGACCCGACTACCACCGAGAA R-GCCTGGGCAAGGTTATGGGTC	(Lu et al., 2014)
polA	180	F-GTCGTGGTTGGACCTTGGAGGG R-GCGTCCGTATCGTCGTCATCG	(Lu et al., 2014)

bp, base pairs.

families are classified on the basis of energy source, size, and the number and substrates of transporters (De Rossi et al., 2006; Piddock, 2006; Sarathy et al., 2012; Stavri et al., 2007). Since the efflux mechanism of drug resistance was first reported in the 1980s (Ball et al., 1980; McMurry et al., 1980), a number of studies have focused on efflux-mediated antimicrobial resistance and efflux determinants (Poole, 2000a; Poole, 2000b). Rv1258c is reported to be a target of multiple drugs (Sarathy et al., 2012; Ainsa et al., 1998; Sharma et al., 2010; Siddiqi et al., 2004). *Rv*2686*c*-*Rv*2687*c*-*Rv*2688*c* is a target of fluoroquinolones (Sarathy et al., 2012; Pasca et al., 2004). *PstB* (*Rv*0933) is also a target of fluoroquinolones (Banerjee et al., 1998; Bhatt et al., 2000). In this study, we investigated the expression levels of five putative efflux pump genes and mutations in target genes (*rrs*, *eis*, *gyrA* and *gyrB*) with clinical MDR and XDR-TB isolates.

Thirty clinical isolates of *M. tuberculosis* that had been phenotypically characterized were obtained from the Korean Institute of Tuberculosis. Of these, 14 were MDR-TB isolates resistant to at least one injectable aminoglycoside (amikacin, kanamycin and/or capreomycin) and 16 were XDR-TB isolates. *M. tuberculosis* H37Rv (ATCC 27249) was used as a reference strain.

Drug susceptibility testing was performed using the Lowenstein-Jensen media absolute concentration method described previously (Kim et al., 2014); critical concentrations of DST were INH, 0.2 μg/mL; RIF, 40 μg/mL; streptomycin (SM), 10 μg/mL; KAN, 40 μg/mL; CPM, 40 μg/mL; AMK, 40 μg/mL; ofloxacin (OFX), 2.0 μg/mL; levofloxacin (LEV), 2.0 μg/mL; and moxifloxacin (MXF), 2.0 μg/mL.

Putative efflux pump genes were selected by searching articles in PubMed using combinations of the following keywords: “efflux pump”, “tuberculosis”, “quinolone” and “aminoglycoside.” After review of previous articles, five putative genes (*Rv*1258*c*, *Rv*2686*c*, *Rv*2687*c*, *Rv*2688*c* and *pstB*) were selected for analysis in this study (Sarathy et al., 2012; Ainsa et al., 1998; Sharma et al., 2010; Siddiqi et al., 2004; Pasca et al., 2004; Banerjee et al., 1998; Bhatt et al., 2000).

To detect mutations in *rrs* and *eis*, genomic DNA was extracted from bacteria cultured on 2% Ogawa slants using a heat extraction method. PCR amplification was performed with the aid of AccuPower HF PCR PreMix (Bioneer); 45 μL distilled water, 2 μL primer mix, and 3 μL template DNA were mixed. Table 1 lists the primers that were used. The PCR conditions for *rrs* and *eis* were listed in supplementary data (Table S1). Sequencing was performed with the aid of a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems).

To extract total bacterial RNA, *M. tuberculosis* clinical isolates were cultured in 30-mL of Middlebrook 7H9 broth (BD) supplemented with

Table 2
Results of drug susceptibility testing and detection of mutations in target genes.

Strain	INH	RIF	SM	KAN	CPM	AMK	OFX	LEV	MXF	*rrs*	*eis*	*gyrA*[b]	*gyrB*[b]
M1	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M2	R	R	R	R	R	R	S	S	S	A1401G	None	None	None
M3	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M4	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M5	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M6	R	R	S	R	S	S	S	S	S	None	None	None	None
M7	R	R	S	R	R	R	S	S	S	None	None	None	None
M8	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M9	R	R	R	R	R	R	S	S	S	A1401G	None	None	None
M10	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M11	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
M12	R	R	R	R	S	S	S	S	S	C517T	G-37 T	None	None
M13	R	R	R	R	R	R	S	S	S	A1401G	None	None	None
M14	R	R	S	R	R	R	S	S	S	A1401G	None	None	None
X1	R	R	S	R	R	R	R	R	S	A1401G	None	A90V	None
X2	R	R	R	R	R	R	R	R	R	A1401G	None	D94A	L479F
X3	R	R	S	R	R	R	R	R	R	A1401G	None	None	None
X4	R	R	R	R	R	R	R	R	R	A1401G	None	D94G	None
X5	R	R	R	R	R	R	R	R	R	A1401G	None	D94G	None
X6	R	R	S	R	S	R	R	R	R	A1401G	None	D94G	None
X7	R	R	S	R	R	R	R	R	R	A1401G	None	D94G	None
X8	R	R	R	R	R	R	R	R	R	A1401G	None	S91P	None
X9	R	R	S	R	R	R	R	R	R	A1401G	None	D94G	None
X10	R	R	S	R	R	R	R	R	R	A1401G	None	D94N	None
X11	R	R	S	R	R	R	R	R	R	A1401G	None	A90V	None
X12	R	R	S	R	R	R	R	R	R	A1401G	None	[a]D94G, Y, C	None
X13	R	R	R	R	R	R	R	R	S	A1401G	None	None	None
X14	R	R	R	R	R	R	R	S	S	A1401G	None	None	None
X15	R	R	S	R	R	R	R	R	R	C517T	C-14 T	A90V	None
X16	R	R	S	S	R	S	R	R	R	None	None	A90V, D94G	None

INH, isoniazid; RIF, rifampicin; SM, streptomycin; KAN, kanamicin; CPM, capreomycin; AMK, amikacin; OFX, ofloxacin; LEV, levofloxacin; MXF, moxifloxacin.
[a] Multi-peaks on sequencing.
[b] Results of a previous study using the same isolates (Kim et al., 2014).