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Expression analysis of 10 efflux pump genes in multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates

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ABSTRACT

Objectives: Active extrusion of antituberculosis drugs via efflux pumps (EPs) has been suggested as contributing to drug resistance in *Mycobacterium tuberculosis*. This study was conducted to determine the role of 10 drug efflux transporters in the development of drug resistance in a series of clinical *M. tuberculosis* isolates.

Methods: A total of 31 clinical *M. tuberculosis* isolates without drug exposure [21 multi/extensively drug-resistant (M/XDR-TB) and 10 drug-susceptible isolates] were studied. The expression profile of 10 EP genes, including *efpA*, *mmr*, *stp*, *drrA*, *drrB*, *mmpL7*, Rv1250, Rv1634, Rv2994 and Rv1258c, was investigated against the H37Rv standard strain by quantitative reverse transcription PCR (RT-qPCR).

Results: Among the 21 M/XDR-TB isolates, 10 showed significantly increased levels of gene expression (>4-fold) for at least one of the studied EPs. Moreover, of the isolates with overexpressed genes, three and seven lacked genetic alterations in the surveyed regions of the *rpoB* + *katG* + *inhA* and *katG* + *inhA* genes, respectively. Whilst no elevation was observed in the expression of *mmr*, Rv1250, Rv1634 and Rv1258c genes in any of the isolates, *drrA*, *stp* and *drrB* were found to be the most commonly overexpressed, being overexpressed in seven, five and three isolates, respectively. Decreased minimum inhibitory concentrations (MICs) of rifampicin, but not isoniazid, were observed in the presence of the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP).

Conclusion: Overexpression of EP genes can contribute to the emergence of a MDR phenotype in *M. tuberculosis*. Inhibition of EPs may provide a promising strategy for improving tuberculosis treatment outcomes in patients infected with M/XDR-TB isolates.

1. Introduction

Tuberculosis (TB) is still one of the biggest threats to public health worldwide. Despite a declining trend in TB incidence worldwide, rising cases of multidrug-resistant (MDR)-TB represent a notable concern for TB control programmes. The global burden of multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB) was 3.5% of new cases and 18% of previously treated patients in 2017. Among the MDR-TB cases, 8.5% were estimated to be extensively drug-resistant TB (XDR-TB) [1]. The intrinsic resistance of *Mycobacterium tuberculosis* to most of the anti-TB drugs is primarily attributed to the low permeability of its cell wall [2]. However, mutations in genes encoding drug targets are considered to be the main cause of acquired resistance to isoniazid (INH) and rifampicin (RIF), which are the most powerful anti-TB agents, resulting in the emergence of MDR-TB. Molecular studies have demonstrated that resistance to RIF is mainly caused by mutations in the RNA polymerase β subunit gene *rpoB* [3]. Resistance to INH is predominantly linked to mutations in *katG* and *inhA* genes, accounting for approximately 80% of INH-resistant *M. tuberculosis* isolates [4–6]. Acquisition of mutations in drug target genes is known to be the principal mechanism of drug resistance in *M.*

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tuberculosis, however such mutations are not found in some clinical drug-resistant *M. tuberculosis* isolates, suggesting the contribution of other resistance mechanisms such as active efflux anti-TB drugs via efflux pumps (EPs) [7]. Previous studies have reported overexpression of EPs in various drug-resistant *M. tuberculosis* isolates [8,9], and in some studies it has been suggested that the emergence of MDR-TB is attributed to combinational or inducible expression of EPs [10]. Moreover, inhibition of EPs by EP inhibitors and generation of knockout mutants of EPs were associated with reduced bacterial drug tolerance caused by EP activity [11]. The increasing activity of EPs is due to long-term exposure to low concentration of drugs (insufficient treatment) or to overexpression of EP genes [12]. The *M. tuberculosis* genome encodes many known drug EPs and some studies have demonstrated a correlation of these EPs and resistance to tetracycline, aminoglycosides, fluoroquinolones, RIF, INH and chloramphenicol in mycobacteria [12–18]. Some of these EPs have narrow substrate specificity, however many extrude different classes of structurally unrelated drugs and are found to contribute to the emergence of MDR-TB [19,20]. Although *M. tuberculosis* has genes encoding putative drug efflux transporters, little information is available on the expression profile of genes encoding drug EPs in *M. tuberculosis* clinical isolates. We have previously characterised the contribution of four EPs (Rv1410c, Rv2459, Rv1218c and Rv1273c) to drug resistance in several M/XDR-TB clinical isolates [21]. Overall, we found that 57% (12/21) of M/XDR-TB isolates were characterised by increased EP activity. We also found that 71% of MDR-TB isolates with no mutation in *rpoB*, *inhA* or *katG* genes revealed overexpressed EP genes. In the current study, the role of another 10 putative EPs, including *efpA* (Rv2846c), *mmr* (Rv3065), *stp* (Rv2333c), *drrA* (Rv2936), *drrB* (Rv2937), *mmpL7* (Rv2942), Rv1250, Rv1634, Rv2994 and Rv1258c, in developing resistance among *M. tuberculosis* clinical isolates was examined.

2. Materials and methods

2.1. Bacterial isolates, RNA extraction and cDNA synthesis

A total of 31 *M. tuberculosis* clinical isolates, including 10 drug-susceptible isolates, 20 MDR-TB and 1 XDR-TB as determined by the proportion method, were studied. Total RNA isolation (GeneJET RNA Purification Kit; Thermo Fisher Scientific GmbH, Dreieich, Germany) and cDNA synthesis (RevertAid^{TM} First Strand cDNA Synthesis Kit; Thermo Fisher Scientific GmbH) were performed according to the manufacturer's instructions. DNA was also extracted from drug-resistant isolates [22] for PCR sequencing and detection of mutations in the *rpoB*, *katG*, and *inhA* genes as described previously [21].

2.2. Primers

All of the primers used for evaluating the expression of EP-related genes were specifically designed for this assay, and the primer for the *hsp65* gene was designed during our previous work [21] (Table 1). Gene sequences were obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). After designing the required primers, OligoCalc: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) and Vector NTI software were used to analyse the characteristics of the selected primer sequences. Finally, the BLAST tool in NCBI was used to check primer specificity.

2.3. Quantitative reverse transcription PCR (RT-qPCR)

A fluorescence quantitative detection system (Bioer, Hanqzhou, China) was used to perform relative RT-qPCR using SYBR® Green Low ROX Master Mix (Ampliqon, Brighton, UK). Each reaction had the following components in a 0.2-mL PCR microtube: 0.4 pM of each primer; 1 μL of template cDNA (50 ng/μL); 12.5 μL of 2× SYBR® Green Master Mix; and 9.5 μL of PCR-grade water (Metabion, Steinkirchen, Germany) in a final volume of 25 μL. The PCR reactions were run under the following conditions: 1 cycle of 95 °C for 5 min; and 50 cycles of 95 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s. Melt curve analyses were performed after each run to ensure single amplicon production. The heat shock protein 65 gene (*hsp65*) and *M. tuberculosis* H37Rv reference strain were used as an internal control and a reference strain, respectively. All of the reactions were run in duplicate and the mean value was considered as an expression level of each tested gene against the reference strain after normalisation to the *hsp65* housekeeping gene.

Table 1
Nucleotide sequences of primers used in quantitative reverse transcription PCR (RT-qPCR) analysis.

Gene/Rv number	Primer sequence	T_m (°C)	Amplicon size (bp)	Product
efpA (Rv2846c)	F, 5′-CTGTAGCCCAAGATGTCCTG-3′	60.5	124	Possible integral membrane efflux protein
	R, 5′-CGTTCCACTTGTTCCGCGA-3′	59.5		
mmr (Rv3065)	F, 5′-AGCACGGAAGGGTTCACTC-3′	59.5	207	Multidrug-transport integral membrane protein
	R, 5′-GCCAACCACCTTCATCACAG-3′	60.5		
stp (Rv2333c)	F, 5′-ACCTATCAAGTACCAGCGGC-3′	60.5	141	Integral membrane drug efflux protein
	R, 5′-GACATCCAGCGCAGTTTCG-3′	59.5		
drrA (Rv2936)	F, 5′-CTGGTGTTGTTTGGTCGTCTG-3′	61.2	94	Daunorubicin-dim-transport ATP-binding protein ABC transporter
	R, 5′-CGGCATGTACGAGGCTGAAT-3′	60.5		
drrB (Rv2937)	F, 5′-GGCCCTCTATATCGTCGGTT-3′	60.5	102	Daunorubicin-dim-transport integral membrane protein ABC transporter
	R, 5′-GCGTCTGGGTTCCTGGTAA-3′	59.5		
mmpL7 (Rv2942)	F, 5′-CTGCCACAAGATGCCAAGAC-3′	60.5	96	Conserved transmembrane transport protein
	R, 5′-TTCCACGACGAGATAGGCGA-3′	60.5		
Rv1250	F, 5′-GGGCGATGATGGTTGGCTT-3′	59.5	99	Probable drug-transport integral membrane protein
	R, 5′-TGGCGTAACCGATGCGTAG-3′	59.5		
Rv1634	F, 5′-GCTATGGATTCTGTCGCCG-3′	59.5	158	Possible drug efflux membrane protein
	R, 5′-ATGTATAGCCCACGAGCCG-3′	59.5		
Rv2994	F, 5′-AATCCCGCACGAAAGCCTC-3′	59.5	98	Probable conserved integral membrane protein
	R, 5′-CATCATCAGCAACGCCGAC-3′	59.5		
Rv1258c	F, 5′-GAACAACAGCGGCAGCATG-3′	59.5	108	Probable conserved integral membrane transport protein
	R, 5′-GGTGATGGCGTCTCGATAG-3′	59.5		
hsp65	F, 5′-AAGTCGGTGGCGGTCAAG-3′	58.4	122	60-kDa chaperonin 2 GroEL2
	R, 5′-GCGTTCTCCAGCGTCAGG-3′	60.8		

T_m, melting temperature.

2.4. Analysis of gene expression

RT-qPCR was performed to investigate the expression level of 10 genes coding for EPs in 31 clinical *M. tuberculosis* isolates as well as the H37Rv reference strain. Line-Gene K software of the Bioer detection system was used to analyse the collected data. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method compared with those of H37Rv strain [23]. A result equal to 1 indicates that the expression level of the gene is the same as the reference strain, and an overexpression level of >4-fold is considered as the cut-off for significantly different gene expression.

2.5. Determination of the effect of an efflux pump inhibitor on the minimum inhibitory concentrations (MICs) of rifampicin and isoniazid

MICs of RIF and INH were tested in the presence and absence of the EP inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in 96-well plates. First, all of the wells were inoculated with 50 μL of 7H9 Middlebrook broth medium. Then, 50 μL of medium containing RIF or INH (256 μg/mL) was added into only the first well and then two-fold serial dilutions were performed horizontally. Mycobacterial suspensions were then diluted (1:50) in the same medium and 50 μL of each suspension was added to the wells (except for the control column). The final concentration of CCCP was 2 μg/mL in the desired wells. All of the plates were sealed and were incubated at 37 °C for 2 weeks. H37Rv was used as a control strain in the experiment.

2.6. Statistical analysis

The one-sample *t*-test was used for statistical analysis of the results obtained from drug-susceptible and drug-resistant groups for all genes against the standard strain, which was equal to 1. The *t*-test was also used to compare the mean values of drug-susceptible and drug-resistant isolates with each other. Statistical analysis was performed using IBM SPSS Statistics v.21 (IBM Corp., Armonk, NY).

3. Results

3.1. Genetic background of isolates

Based on sequencing findings, all of the tested isolates could be divided in four groups as follows: (i) isolates without any mutations in *rpoB*, *katG* or *inhA* genes; (ii) isolates with *rpoB* mutations only; (iii) isolates with mutations in *rpoB* and *katG*; and (iv) isolates with mutations in *rpoB* and *inhA* (Supplementary Table S1) [21]. All of the MDR-TB isolates were also subjected to spoligotyping [24], restriction fragment length polymorphism

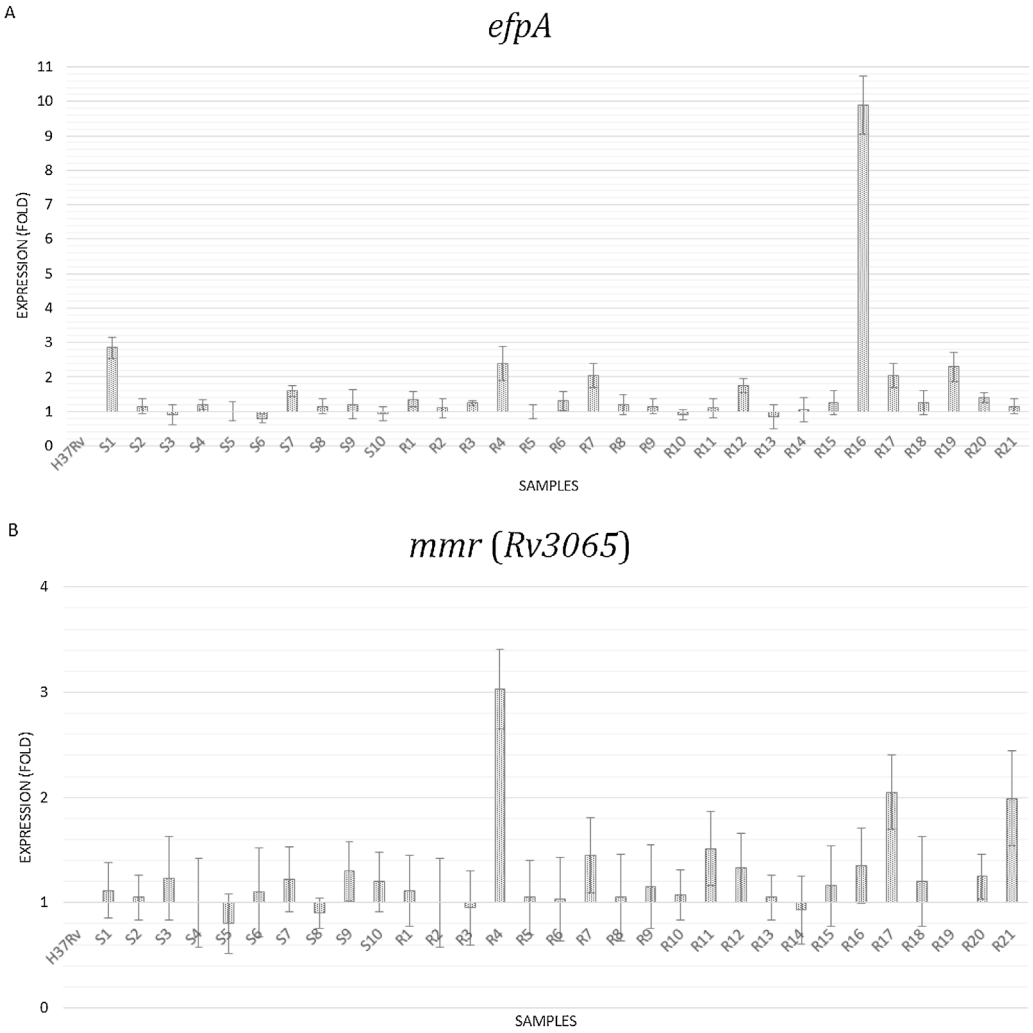

Fig. 1. Expression profile of ten efflux pump genes among 10 drug-susceptible (S1–S10) and 21 drug-resistant (R1–R21) *Mycobacterium tuberculosis* clinical isolates compared with the reference strain H37Rv.

using the polymorphic guanine–cytosine-rich sequence (RFLP-PGRS) typing (data not shown) and mycobacterial interspersed repetitive unit variable-number tandem-repeat (MIRU-VNTR) analysis [25] according to methods described previously. The isolates were genetically characterised as belonging to different clusters and/or spoligotypes (Supplementary Table S1).

3.2. Transcriptional analysis of efflux pump genes

Fig. 1 shows the expression levels of the 10 target genes in the clinical isolates compared with the reference strain H37Rv. Differences in expression levels between the drug-susceptible group and the standard strain were not statistically significant for

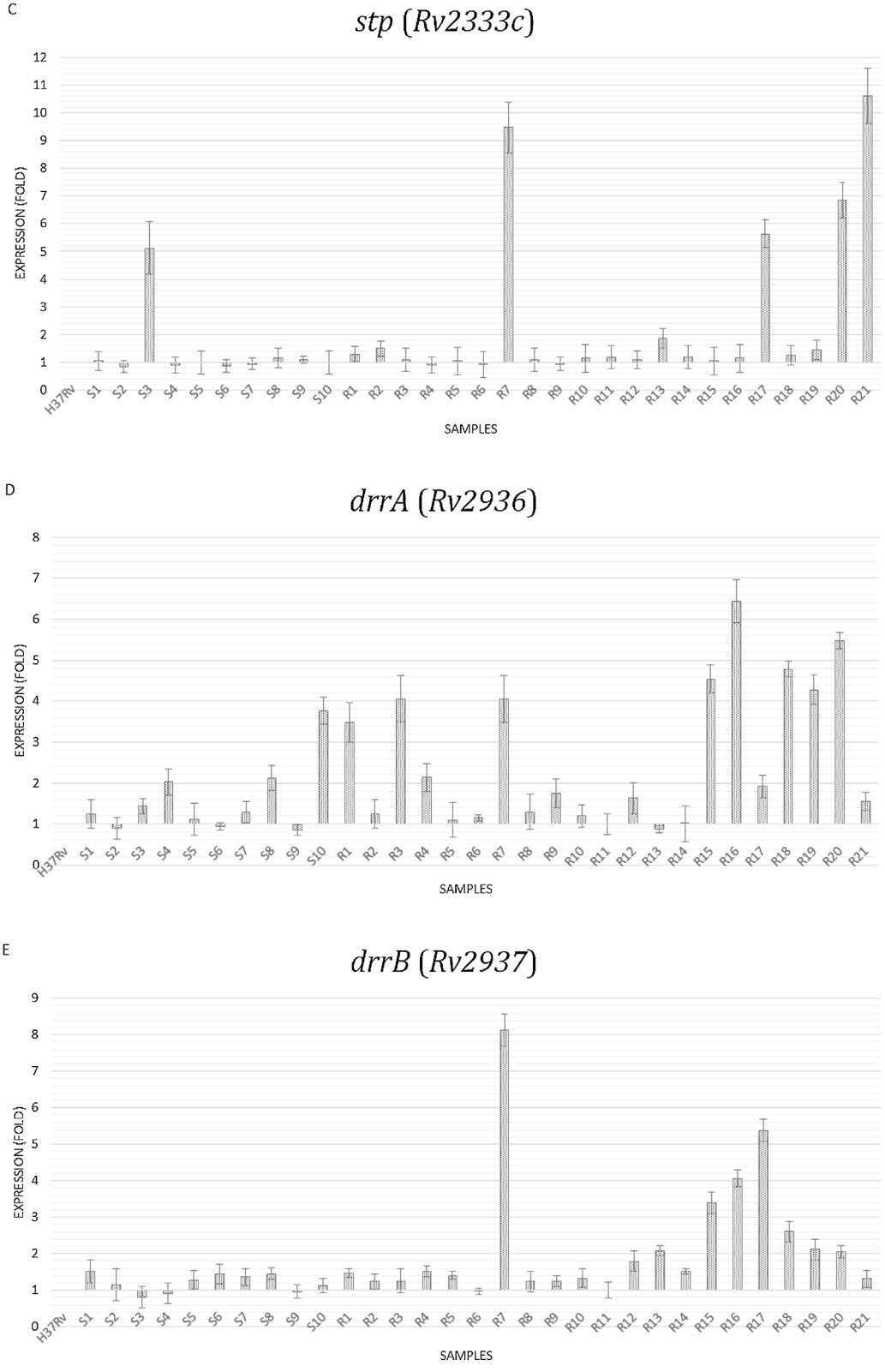

Fig. 1. (Continued)

all of the studied genes ($P > 0.05$), except for *drrB*, Rv1634 and Rv2994. However, for drug-resistant isolates the levels were significantly different from those of the H37Rv standard strain ($P < 0.05$), except for the *efpA* gene. On the other hand, statistical analysis of the mean expression levels between the susceptible and resistant groups demonstrated a significant discrepancy for all genes ($P \leq 0.05$), with the exception of the Rv1258c gene. Unlike most drug-susceptible strains that showed a similar expression pattern to that of the H37Rv reference strain, one drug-susceptible isolate (strain S3) was characterised by overexpression of some genes, notably *stp*, for which a >4-fold expression level was observed. Among the 21 drug-resistant isolates, 10 had a higher expression level (>4-fold) for at least one of the studied EP genes. Whilst no elevation was observed in the expression of *mmr*, Rv1250, Rv1634 and Rv1258c genes in any of the isolates, *drrA* and *stp* were found to be the most commonly overexpressed genes, being overexpressed in seven and five isolates, respectively. This was followed by the *drrB* gene, which was found to be

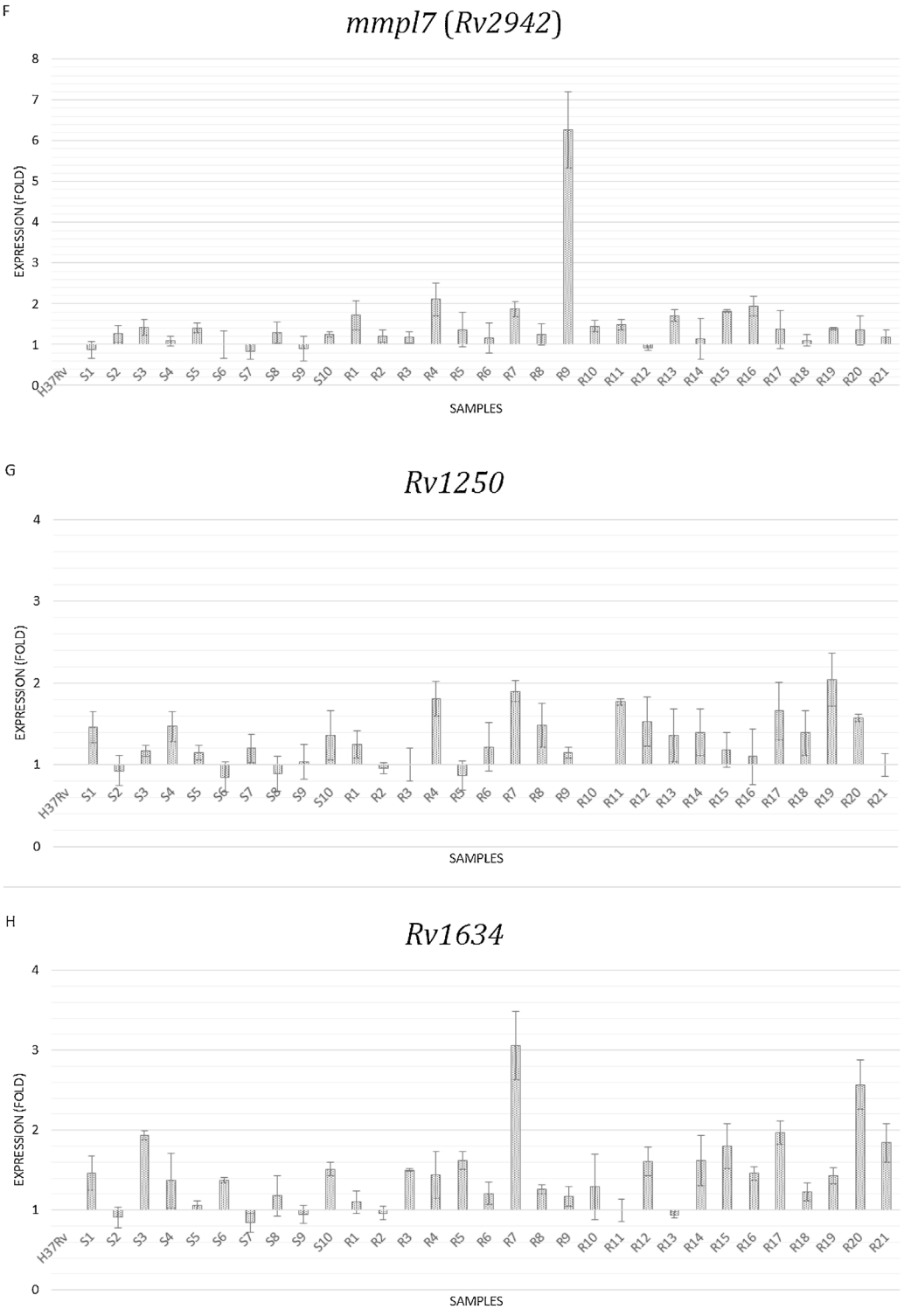

Fig. 1. (Continued)

overexpressed in three isolates, whilst the remaining three genes showed elevated expression in only one isolate. Among the M/XDR-TB isolates with elevated levels of EP gene expression, three lacked any genetic alteration in the surveyed regions of the *rpoB*, *katG* and *inhA* genes (Supplementary Table S1) and seven did not harbour any mutation in the common INH resistance genes.

3.3. Minimum inhibitory concentration changes with and without carbonyl cyanide 3-chlorophenylhydrazone

MICs of the different isolates ranged between 8 μg/mL and >64 μg/mL for RIF and between 4 μg/mL and >64 μg/mL for INH (Table 2). Fold changes in the MICs of RIF and INH in the presence of the EP inhibitor CCCP are given in Table 2. Several fold decreases were seen in the MICs of RIF with CCCP (related to EP gene expression), but no change in the MIC of INH with CCCP was observed in this assay.

4. Discussion and conclusion

McMurry et al. were the first to report that resistance to antibiotics involves active EPs [26]. Since then, active EPs have been recognised to have a major role in bacterial drug resistance [27,28]. Current TB research has shown an increasing focus on drug efflux mechanisms to efficiently treat TB patients [29]. In this study, the role of EPs along with mutations in drug target genes in the development of drug resistance in *M. tuberculosis* isolates was investigated. It was found that overexpression of EP genes can contribute to the emergence of MDR-TB. According to our results, expression levels of EP genes in clinical drug-susceptible isolates were approximately similar to those of the reference strain H37Rv and the observed differences were not statistically significant between these two groups. On the other hand, significantly different rates of expression were found between the drug-susceptible and M/XDR *M. tuberculosis* isolates. We had also previously found that most of the susceptible clinical isolates had expression values of approximately 1, like H37Rv, and drug-resistant isolates showed a significantly different pattern of expression compared with susceptible isolates and the H37Rv reference strain. However, in contrast to our previous results on the expression profile of four EPs, in the current study there was one drug-susceptible isolate (S3) that showed high expression levels for the *stp* and Rv1258c genes (5.1- and 3.4-fold, respectively). Also, an expression level of 3.8-fold for the *drrA* gene was recorded for the drug-susceptible strain S10. Other studies have also reported overexpression of some EPs in susceptible *M. tuberculosis* isolates [8]. Among the 21 drug-resistant isolates, 10 had a >4-fold increase in expression level for at least one of the ten EP genes studied, with

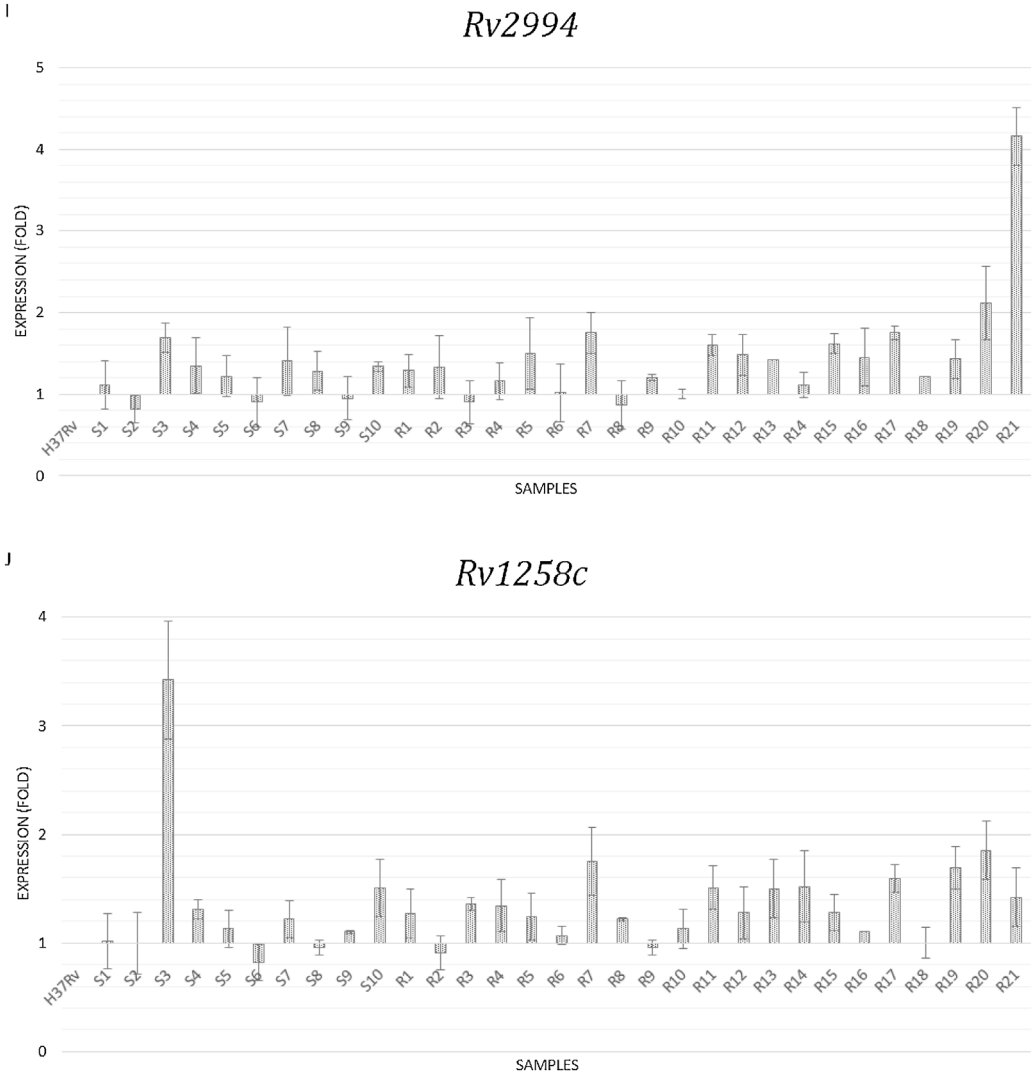

Fig. 1. (Continued)

the *drrA* and *stp* genes being the most commonly overexpressed genes. Moreover, in our previous work Rv1218c and Rv1410c showed overexpression in 7 of 21 M/XDR-TB isolates, followed by 4 and 3, respectively, for Rv1273c and Rv2459 [21]. By referring to RT-qPCR analysis and *rpoB*, *katG*, and *inhA* sequencing data, M/XDR-TB strains with overexpressed EP genes were classified into three groups: (i) isolates carrying mutations in both RIF and INH resistance-related genes (three isolates); (ii) isolates harbouring mutations in the *rpoB* gene (four isolates); and (iii) isolates lacking mutation in the studied INH/RIF resistance-related genes (three isolates). Among the ten MDR-TB isolates with overexpressed EP genes, three isolates lacked genetic alteration in the studied genes, reinforcing the hypothesis that EPs might be mediating drug resistance in these isolates. However, absence of mutation in the studied regions of these three genes does not necessarily exclude the role of resistance-conferring mutations. Acquisition of mutations in other regions of the studied genes or even other genes may be linked to resistance in these isolates. EPs can also be involved in drug resistance in isolates harbouring mutations in the drug target-encoding genes. Indeed, reduction in intracellular levels of antibiotics caused by increased activity of efflux systems may facilitate acquisition of chromosomal mutations that provide higher levels of resistance [30,31]. Therefore, a combination of EP activity and resistance-conferring mutations can direct drug resistance in *M. tuberculosis* isolates with chromosomal mutations and overexpressed EPs. Furthermore, determination of the MICs of RIF and INH demonstrated some MIC alterations in the presence of CCCP. The effect of the EP inhibitor CCCP on the MICs of RIF ranged from no fold changes to an eight-fold decrease. However, INH was not affected by CCCP in the isolates tested in the current experiment.

In general, the data obtained from the studied clinical *M. tuberculosis* isolates are consistent with those reported by Calgin et al. who compared the expression level of 15 EPs in 10 drug-susceptible and 10 MDR-TB clinical isolates without drug exposure [32]. They showed that the expression levels of all EP genes in clinical isolates were higher than those of the same genes in the reference strain. However, in contrast to the current results, they found no significant difference between the two groups of drug-susceptible and MDR-TB isolates in the expression of EP genes [32]. Increased expression of some EP genes even in the absence of antibiotic stress was also reported by Kanji et al. who declared that increased levels of *drrB* and Rv2688 efflux mediator genes were involved in XDR-TB strains [33]. In agreement with their findings, *drrB* was also overexpressed in three of the isolates in the current study. In a study performed by Li et al., the *drrA*, *drrB*, *efpA* and *jefA* (Rv2459) genes were found to be the most commonly overexpressed EP genes, which showed expression levels >4-fold in nine, four, five and six of nine MDR-TB isolates under INH pressure, respectively [8]. However, the expression level of *stp*, which was the most frequently overexpressed gene in the current study, was not elevated [8]. Decreased MICs of RIF in two MDR-TB isolates without mutation (DP13 and DP19) and some isolates harbouring *rpoB* mutations revealed possible involvement of EPs in these isolates. In accordance with our results, lack of a synergistic effect of INH and CCCP in some INH-resistant *M. tuberculosis* isolates with no mutations in *katG* or *inhA* genes was also reported by Jaiswal et al. [34]. However, recent studies with different EP inhibitors showed weaker inhibitory effects of CCCP on mycobacterial isolates compared with verapamil [34,35]. Hence, the synergistic effect of CCCP and RIF combinations along with elevated expression of some EP genes is corelated and may support the idea that EPs contribute to drug resistance development [36].

Machado et al. [35,37] and Gupta et al. [38] have also demonstrated the role of EPs in the development of MDR and XDR phenotypes in *M. tuberculosis* strains. EPs have been shown in several other studies to contribute to drug resistance and there is no doubt that EPs are alternative mechanisms potentiating the development of M/XDR phenotypes [9,37,38]. On the other hand, some in vitro studies with EP inhibitors have shown that inhibition of EPs would render MDR-TB susceptible to anti-TB drugs and suggested addition of these compounds to TB treatment regimens as therapy adjuvants [35,39,40].

In summary, in this study 48% (10/21) of M/XDR-TB isolates studied in this work had overexpressed EP genes, mediating drug resistance alone in strains without mutations or in combination with resistance-conferring chromosomal mutations in drug target genes. Therefore, these putative efflux transporters can provide a promising target for drug discovery campaigns for the development of novel classes of anti-TB compounds. Administration of EP inhibitors along with available anti-TB drugs can efficiently improve the outcome of TB treatment in patients infected with MDR- or XDR-TB strains and reduce the cost and duration of treatment. Conducting further studies with larger groups of bacteria is required to better understand the exact molecular mechanisms of non-susceptibility of *M. tuberculosis* isolates to anti-TB drugs and to identify factors that potentiate the development of M/XDR phenotypes.

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Competing interest

None declared.

Ethical approval

Not required.

Table 2
Fold changes in the minimum inhibitory concentrations (MICs) of rifampicin and isoniazid in the presence and absence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP).

Isolate[a]	Rifampicin		Isoniazid	
	MIC (μg/mL)	Fold decrease in presence of CCCP	MIC (μg/mL)	Fold decrease in presence of CCCP
DR1	64	2	8	–
DR5	64	2	4	–
DR6	>64	–	8	–
DR10	>64	–	>64	–
DR13	32	2	32	–
DR16	8	8	64	–
DR19	64	4	64	–

[a] Because of some missing isolates during the years, only seven different isolates were tested in this experiment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2019.01.003.

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