The effect of anti-tuberculosis drugs therapy on mRNA efflux pump gene expression of *Rv1250* in *Mycobacterium tuberculosis* collected from tuberculosis patients

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Abstract

Efflux pumps are transmembrane proteins that vigorously participate in extruding a wide range of substrates, including drugs, outside the bacterial cell. We aimed to investigate the mRNA expression level of the *Rv1250* efflux pump gene in *Mycobacterium tuberculosis* isolated from individuals with tuberculosis who received drug therapy, at the 1st, 3rd and 5th months, and newly diagnosed patients with tuberculosis who will receive drug therapy (0 month). The study was a multiple cross-sectional longitudinal design—50 different *M. tuberculosis* isolates and a reference strain H37Rv were subcultured in LJ medium and confirmed by multiplex PCR for identification of *M. tuberculosis* and collected for RNA extraction. Total bacterial mRNA was analysed using real-time quantitative PCR to evaluate mRNA quantification gene expression. There were differences in the level of *Rv1*250 mRNA expression between sensitive (n = 11) and resistant (n = 40) groups of 5.961 ± 0.414 and 10.192 ± 1.978, respectively (fold changes; $p < 0.05$). There were significant differences of expression level among *M. tuberculosis*-resistant groups ($p < 0.05$) specifically 7.573 ± 0.424 for 0-month drug therapy (n = 10), 9.438 ± 0.644 for 1st month drug therapy (n = 10), 11.057 ± 0.262 for 3rd month drug therapy (n = 10) and 12.701 ± 0.460 for 5th month drug therapy (n = 10). We assume that the extent of *Rv1250* gene expression in *M. tuberculosis* clinical isolates is a result of the exposure to antimicrobials during treatment, which affect the basic expression of the efflux pump *Rv1250* gene.

Keywords: Drug-resistant, efflux pump, mRNA expression level, *Mycobacterium tuberculosis*, *Rv1250*gene

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Introduction

The advancement of antibiotic usage was one of the most important stages in controlling infectious diseases in the twentieth century. However, various forms and processes of antibiotic resistance dissemination in pathogenic organisms have caused most common antibiotics to become ineffective [1]. As a result, the re-emergence of tuberculosis (TB) infection is generating severe public health problems and is a major cause of global mortality [2]. The main short-term anti-TB drug therapy uses streptomycin, isoniazid, rifampicin and ethambutol, which form a potent drug combination for infections involving susceptible strains of *M. tuberculosis* [3]. The *M. tuberculosis* strains are changing from being susceptible to become resistant towards all first- and second-line drugs.

Mycobacterium tuberculosis has the potential to alter cell metabolic pathways into other pathways, including aerobic and anaerobic cell respiration (e.g. oxidative phosphorylation and nitrate reduction). This flexibility is one of the abilities of *M. tuberculosis* that allows it to adapt and survive inside the human body, where conditions can change from aerobic (in the alveolar lung region) to microaerophilic/anaerobic (in the tuberculous granuloma region) [4].

The intrinsic resistance of *M. tuberculosis* to various antimicrobials can be caused by the presence of a permeable barrier in the cell wall against the compounds that are toxic to cells [5]. The peptidoglycan and arabinogalactan layers limit the transport

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of hydrophobic molecules into the cell, and mycolic acid limits the movement of hydrophilic and hydrophobic molecules into the cell. Hydrophobic antibiotics such as rifampicin and fluoroquinolone can enter bacterial cells through diffusion into the bilayer hydrophobic layer. However, hydrophilic antibiotics and nutrients that cannot diffuse through the cell wall enter the cell through porin. Porin is a non-specific protein canal in the outer membrane of bacteria that allows the transport of hydrophilic solutions [6]. Because of the unique structure of bacterial cells, mutations of the antibiotic target genes are known to be the main reason for resistance to drug therapies. Not all drug target gene mutations in various first-line drug resistances can be explained in clinical cases [3].

The efflux pumps system can cause resistance by extruding the antibiotic molecules that enter the cell, where the intracellular concentration of the antibiotics that are given depends on the balance of influx and efflux of the cell. It is very important to understand the mechanism of drug influx through porin and of drug efflux through efflux pumps to prevent antibiotic resistances [6].

Efflux pumps (transport membrane proteins) are encoded by genes that are responsible for several physiological processes in eubacterial cells (including mycobacteria), such as cell division mechanisms, pH homeostasis and secretion of intracellular metabolites. Based on bioenergetic criteria, the efflux pump system can be divided into two parts, primary and secondary transporters. Primary transporters obtain energy sources from ATP hydrolysis and ATP formation. Secondary transporters gain energy sources from electrochemical gradients that are bound to protons being transported and distributed on the surface of bacterial cells— the proton motive force. Transporters are divided into five families, namely ATP binding cassette; Major Facilitator Superfamily; Multi-Antimicrobial Extrusion Protein; Small Multidrug Resistance; Resistance Nodulation Division [7].

Effective drug therapy for TB infection is needed, especially new drugs, drug targets and factors that influence drug resistance. DNA sequencing predicts that the *M. tuberculosis* genome of H37Rv strain encodes various putative efflux proteins, which have not been generally characterized. A study implicated that mycobacteria extrude various drugs through the active efflux system [8]. Some efflux pumps are specific towards certain antibiotics, whereas other efflux pumps extrude components that are structurally and functionally different, for example in multidrug-resistance efflux pumps [9]. An experimental procedure to identify the overexpression of efflux pumps in mutant bacteria through drug induction is limited on a laboratory scale [10]. Some research on efflux pump overexpression has been carried out using clinical isolates. Generally, most efflux-pump-related research has used experimental design [3,11]. This study aims to investigate the mRNA expression level of the efflux pump *Rv1250* gene in an *M. tuberculosis*-resistant strain isolated from follow up of individuals with multidrug-resistant TB (MDR-TB) in Makassar. We compared the level of *Rv1*250 mRNA gene expression in clinical isolates that were obtained independently from the followed up patients with MDR-TB who received antibiotic therapy in the 1st month, 3rd month and 5th months, and also newly diagnosed TB patients who would receive drug therapy (0 months). The study also aimed to compare the expression level of the *Rv1250* gene in resistant and susceptible groups of clinical isolates obtained from patients diagnosed with TB who had positive lung X-ray results.

Materials and methods

Antibiotics and chemicals

The BBL *Mycobacterium* Preparation Kit, Mycobacterium Growth Indicator Tubes containing 7 mL of Middlebrook 7H9 broth, the Polymyxin-B Amphotericin-B Nalidixic Acid Trimethoprim Azlocillin Mycobacterium Growth Indicator Tubes Growth Supplement Kit and oleic acid albumin dextrose catalase enrichment were purchased from BD (Becton, Dickinson and Company, Sparks, MD USA). Streptomycin, isoniazid, rifampicin, ethambutol, kanamycin, ofloxacin, capreomycin, moxifloxacin, sodium hydroxide and verapamil were purchased from Sigma-Aldrich (St Louis, MO, USA); dimethyl sulphoxide was purchased from Merck (KgaA, Darmstadt, Germany); and SD BIOLINE TB Ag MPT64 was from Standard Diagnostic, Inc. (Abbot, Illinois, USA). Drugs were dissolved in specific diluents according to the manufacturer's instructions. The optimal concentration of verapamil was 60 mg/L for anti-TB drug-resistant isolates [12]. The aminoglycosides (capriomycin, streptomycin, kanamycin), isoniazid, and verapamil were dissolved in sterile distilled water (dH_2O), while the fluoroquinolones (ofloxacin, moxifloxacin) were dissolved in 0.1% sodium hydroxide. Rifampicin and ethambutol were dissolved in 100% dimethyl sulphoxide.

Clinical isolates and drug susceptibility testing

This study was approved by the Institutional Research Board of the Medical Faculty of Hasanuddin University, Makassar, Indonesia; registered approval number 42/H4.8.4.5.31/PP36-KOMETIK/2018, dated 18 January 2018. Written informed consent for this study was obtained from all participants or their parents/guardians accompanied by the authorized nurses who were in charge of managing patients with TB.

Forty clinical strains of *M. tuberculosis* were recovered from different sputum samples of patients with MDR-TB at follow up and ten clinical strains of *M. tuberculosis* from patients

diagnosed with pulmonary TB were sent to the Tuberculosis Laboratory of the Makassar Medical State Laboratory (BBLK Makassar), Indonesian Ministry of Health as reference laboratory for bacterial solid and liquid culture. The isolate profiles of drug susceptibility were evaluated using the proportional method using a Mycobacterium Growth Indicator Tubes 960 System with the following critical drug concentrations: streptomycin 1.00 mg/L, isoniazid 0.10 mg/L, rifampicin 1.00 mg/L, ethambutol 5.00 mg/L, kanamycin 2.5 mg/L, ofloxacin 2.0 mg/L, capreomycin 2.5 mg/L and moxifloxacin 0.25 mg/L. The critical proportion of resistant bacillus necessarily to define a resistant strain is 1% for all tested drugs [13]. All 40 clinical isolates were rifampicin-resistant, MDR and extensively drug-resistant (XDR) phenotypes; and ten clinical isolates were phenotipically susceptible to all drugs that were tested, including reference strain H37RV. All samples were confirmed by multiplex PCR method [14–16].

Nucleic acid extraction

All 51 *M. tuberculosis* strains (including H37RV) were subcultured in LJ medium and collected for quantitative real-time PCR assay. First, a nucleic acid RNA extraction was carried out. Scraped bacterial colonies were put into 900-µL of an 'L6' solution consisting of 120 g guanidium thiocyanate (Fluka Chemie AG, Buchs, Switzerland, cat no. 50990) in 100 mL 0.1 M Tris–HCl, pH 6.4, 22 mL 0.2 M EDTA pH 8.0 and 2.6 g Triton X-100 (Packard Instruments, Fallbrook, CA, USA) with a final concentration of 50 mM Tris–HCl, 5 M guanidium thiocyanate, 20 mM EDTA, 0.1% Triton X-100. Then it was spun at 16,128 g. Sediment was added with a 20-µL diatom suspension consisting of 50 mL H_2O and 500 µL from 32% (w/v) 'Celite' (diatom) (Jansen Chimica, Beerse, Belgium); where 20 µL of diatom suspension can bind 10 µg bacterial RNA. This was then vortexed before being centrifuged in a 1.5-mL Eppendorf tube at 16,128 g for 15 min. The supernatant was removed and the sediment was washed with 'L2' solution comprising 120 g of guanidium thiocyanate in 100 mL of 0.1 M Tris–HCl, pH 6.4 by adding 1 mL of 'L2' solution. It was then vortexed and centrifuged at 16,128 g for 15 min, then the washing was repeated twice using an 'L2' solution, followed by washing with 1 mL of 70% ethanol twice and 1 mL of acetone. The results were then heated in a waterbath at 56°C for 10 min and 60 µL of 'TE' solution consisting of 1 mM EDTA in 10 mM Tris–HCl pH 8.0 were added, then vortexed and centrifuged at 16,128 g for 30 seconds, before being incubated in an oven for 10 minutes at a temperature of 56°C. After being vortexed and re-centrifuged for 30 seconds at a speed of 16,128 g the supernatant was taken. Supernatant from this process will be obtained from nucleotide extraction and stored at −80°C before PCR analysis [14,17].

Quantification of gene expression using quantitative real-time PCR

Quantitative real-time PCR using Brilliant II Sybr® following the manufacturer's instructions was carried out in a CFX connect thermocycler (Bio-Rad laboratories Inc., Hercules, CA, USA). The primer for the *Rv1250* gene and the *polA* gene, used as the housekeeping gene, were from Macrogen® (Seoul, Korea) and are described in Table 1. Briefly, mastermix PCR used 22.5 µL of total volume for each reaction in a sterile microtube containing10 µL of nuclease-free water, 1.25 µL of forward primer (10 pmol), 1.25 µL of reverse primer (10 pmol) and 10 µL 2× Kapa2G fast ready-mix (Sigma-Aldrich); this was spun down and vortexed at low speed. Then, 2.5 µL PCR mix was inserted into each PCR strip tube and 2.5-µL samples were added into each PCR strip tube and slowly flicked to make sure all solutions had dropped down to the bottom of the tube or spun down using a mini centrifuge. The thermal cycling conditions were as follows: initial denaturation 95.0°C for 3 min, denaturation 95°C for 30 sec, then 40 cycles of annealing at 55°C for 30 sec. Profile of *Rv1*250 mRNA gene expression was determined by calculating the slope of the *polA* housekeeping gene dilution multiplied by log template (obtained from the DNA quantity before amplification) plus the starting quantity. Changes in gene expression are given in fold-change units. The standard curve was made and as an indication of good amplification efficiency (90%–-100%).

Data analysis

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used to perform Wilcoxon test on all sample groups to evaluate the effects of drug therapy on *Rv1*250 mRNA gene expression ($p < 0.05$). Paired *t* test or difference test between sample groups were carried out for quantitative data (intervals and ratios) that was normally distributed. The Shapiro–Wilk statistical test shows the p value of each data group is >0.05, which indicates the data is normally distributed, so the paired *t* test can be performed.

Results

Table 2 shows the average mean of *Rv1*250 mRNA gene expression in the susceptible and resistant isolate groups. The results of the independent samples test analysis showed $p < 0.05$, so the data sets were not homogeneous ($p > 0.05$). An analysis of variance test was performed to see whether the difference in *Rv1*250 mRNA gene expression between the two groups was $p < 0.05$. The result shows that mean values of *Rv1*250 mRNA gene expression in sensitive and resistant isolates were 5.961-fold and 10.192-fold change.

TABLE 1. Primers used in this study

Gene	Primers	Sequences	Tm	Length (bp)
Rv1250	*Rv1250*-F	5′-GCAGCCTTGGATTTGGGCGGTGAT-3′	60.5°C	133 bp
	Rv1250-R	5′-GGACAAGCTGAAGTTCCGGTCGTT-3′	62.2°C	
polA	*polA*-F	5′-GTCGTGGTTGGACCTTGGAGGG-3′	64.7°C	181 bp
	polA-R	5′-GCGTCCGTATCGTCGTCATCG-3′	66.4°C	

F forward, R reverse, bp base pair, Tm melting temperature.

TABLE 2. *Rv1250* mRNA gene expression of *Mycobacterium tuberculosis* susceptible and resistant strains

	n	*Rv1250* mRNA gene expression (fold change; mean ± SD)	p
Drug-susceptible clinical isolates (including H37Rv)	11	5.961 ± 0.414	0.000
Drug-resistant clinical isolates	40	10.192 ± 1.978	

Table 3 shows the average level of *Rv1250* mRNA gene expression. Kolmogorov–Smirnov's statistical test results brought out in each group data sets were $p > 0.05$, so a paired *t* test was performed. It shows that mean values of *Rv1250* mRNA gene expression fold changes, subsequently, were 5.960 in the control group; 7.573 in the before drug therapy group; 9.438 in the 1st month of drug therapy group; 11.057 in the 3rd month of drug therapy group; and 12.701 in the 5th month of drug therapy group.

Table 4 shows the significant differences in the effect of anti-TB drugs against *Rv1250* mRNA gene expression with paired *t* test result $p < 0.05$.

The increasing changes of *Rv1250* mRNA gene expression in *M. tuberculosis* H37RV as reference strain, the drug-susceptible group as control, and drug-resistant groups can be seen in Fig. 1. The *Rv1250* mRNA efflux pump gene expression in *M. tuberculosis* H37RV strain was 5.973-fold change, which was almost the same level as the susceptible-strain group (mean 5.960 ± 0.437).

Discussion

Efflux pumps contribute to the resistance mechanism in several ways. Bacterial cells have a basal level of efflux activity that generates in the decreasing of bacterial cell susceptibility to one or several types of antibiotics (intrinsic resistance); increasing of genes expression that encode proteins which regulate efflux

TABLE 3. Description and normality test of *Rv1250* mRNA gene expression of *Mycobacterium tuberculosis* clinical isolates

	n	*Rv1250* mRNA gene expression (fold change; mean ± SD)	p
Drug-susceptible group (Control)	10	5.960 ± 0.437	0.983
Before drug therapy[a] group (0 month)	10	7.573 ± 0.424	0.820
1st month of drug therapy[a] group	10	9.438 ± 0.644	0.975
3rd month of drug therapy[a] group	10	11.057 ± 0.262	0.983
5th month of drug therapy[a] group	10	12.701 ± 0.460	0.998

[a]Drug therapy consists of streptomycin, isoniazid, rifampicin, ethambutol, ofloxacin, amikacin, kanamycin.

TABLE 4. The effect of anti-tuberculosis drug therapy towards *Rv1250* mRNA gene expression in resistant *Mycobacterium tuberculosis* clinical isolates

Paired *t* test	*n*	Difference in mean values	p
Drug-resistant group: 1 month of drug therapy[a] – before drug therapy (0 month)	50	1.86640	0.000
Drug-resistant group: 3 months of drug therapy[a] – before drug therapy (0 month)		3.48330	
Drug-resistant group: 5 months of drug therapy[a] – before drug therapy (0 month)		5.12750	
Drug-resistant group: 3 months of drug therapy[a] – 1 month of drug therapy		1.61890	
Drug-resistant group: 5 months of drug therapy[a] – 3 months of drug therapy		1.64420	
Drug-resistant group: 5 months of drug therapy[a] – 1 month of drug therapy, bulan		3.26310	

[a]Drug therapy consists of streptomycin, isoniazid, rifampicin, ethambutol, ofloxacin, amikacin, kanamycin.

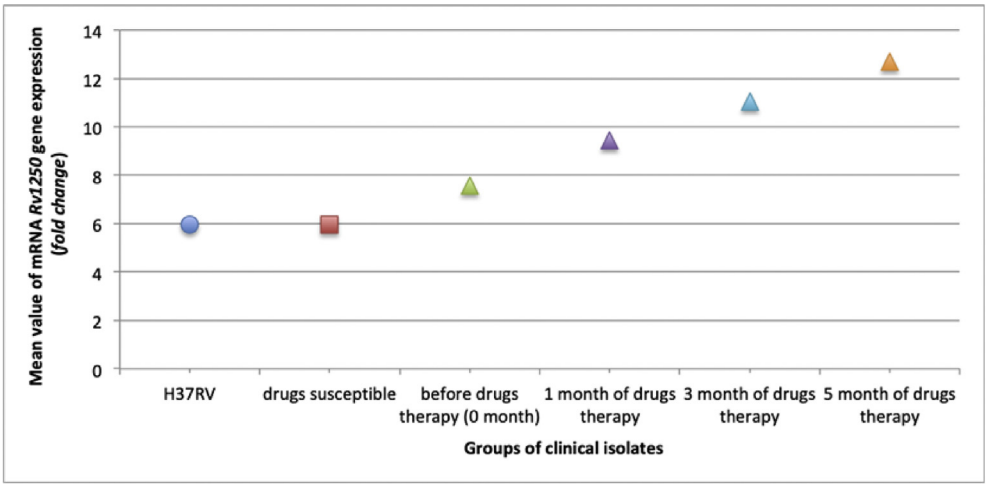

FIG. 1. Effect of drug therapy on *Rv1*250 mRNA gene expression level ($p < 0.05$) of *Mycobacterium tuberculosis* in drug-susceptible clinical isolates ($n = 10$), drug-resistant clinical isolates (before drug therapy (0 month), 1 month of drug therapy, 3 months of drug therapy, 5 months of drug therapy) and reference strain (H37Rv).

pumps mechanism and is the first stage of the drug resistance process; the diversity of components that can be extruded from cells through efflux pumps mechanism, which causes bacterial cells to have resistance phenotype; decreased levels of intracellular antibiotic concentrations through efflux mechanism, which allows bacterial cells to survive for long periods of time until chromosomal mutation occurs against certain antibiotics [18–20].

Machado et al. proved the contribution of efflux pumps in an *M. tuberculosis* strain toward isoniazid resistance through an *in vitro* study [20]. The *M. tuberculosis* isoniazid-resistant strain was obtained from a susceptible strain that was induced through exposure to certain levels of antibiotics for 3 weeks, and then the susceptible trait was restored with the addition of an efflux pump inhibitor. Over-expression of efflux pump genes was compatible with the increased function of the efflux pump. However, in one of the antibiotic induction paired panels, different results were obtained in the same strain where one series of the *M. tuberculosis* strain did not undergo mutation. Hence, bacterial evolution with the same treatment can lead to non-deterministic effects, where each bacterial cell is significantly different in terms of both genotype and phenotype [19].

Meenakshi et al. in his study suggested that efflux pumps play an important role in anti-TB drug activity of *M. tuberculosis* [20]. The study used *M. tuberculosis* wild-type strains and an *M. tuberculosis* knockout mutant, which had mutations in the *Rv1258c*, *Rv0849*, *Rv1218* and *Rv3065* genes. The results showed that wild-type strains were unable to withstand antibiotic stress even with the addition of efflux pump inhibitors, whereas knockout mutant strains showed a susceptible phenotype after the addition of efflux inhibitors [20].

In our study, we aimed to investigate the direct effect of drug therapy against the expression level of the *Rv1*250 mRNA efflux pump gene in *M. tuberculosis*. This study was essentially an *in vivo* study, because the clinical isolates that were obtained from TB patients were not exposed to any drug induction during laboratory experiments. In addition to this, bacterial strains were passaged in growth medium before RNA extraction. Based on the previous research, it is known that efflux pumps will display over-expression after drug induction, so that *M. tuberculosis* bacilli that infect individuals with MDR-TB who are currently receiving drug therapy would show an increasing level of *Rv1*250 mRNA efflux pump gene expression during the 1st, 3rd and 5th month periods of TB treatment. We also engaged a group of *M. tuberculosis* isolates that were obtained from individuals who were newly diagnosed with MDR-TB who had not yet received any drug therapy (0-month), which acted as a comparison to evaluate an increase in *Rv1*250 mRNA gene expression. The group of *M. tuberculosis* susceptible strain and the American Type Culture Collection (H37RV) reference bacteria acted as controls.

Our results indicate a significant difference ($p < 0.05$) in *Rv1*250 mRNA efflux pump gene expression, with two-fold increased expression between the *M. tuberculosis*-resistant isolates ($n = 40$) and susceptible isolates ($n = 11$) groups. Fig. 1 shows a realistic escalation of *Rv1*250 mRNA gene expression in the 0-month, 1-month, 3-month and 5-month groups. The major mean value difference could be seen between the control group with 5.960 ± 0.437 fold change and the 5th month of drug therapy group with 12.701 ± 0.460 fold change. It could be concluded that there were a major effect of prolonged drug therapy duration toward the level expression of efflux pump

Rv1250 gene. Hence, there was a major effect of prolonged drug therapy on the level of expression of efflux pump *Rv1250* gene.

There are various mechanisms that cause bacterial resistance, such as the complexity of cell walls, which prevents antibiotics from reaching the targeted area in bacterial cells, through to changes of membrane cell permeability [21]. Other mechanisms work by modifying and inactivating enzymes that are drug-related targets, so that there is no interaction between drug molecules and enzymes, or drug function cannot recognize and bind with targeted molecules [9]. Spontaneous mutations in the bacterial genome can cause resistance through gene transfer, that harbours mutations from other bacterial cells [22]. However, the most relevant mechanism of MDR and XDR phenotypes is caused by the presence of the efflux system, which actively transports molecules or substrates outside the bacterial cells [23,24]. *Mycobacterium tuberculosis* resistance is pertinent to the induction of the efflux pump system, because the intracellular drug concentration in bacterial cells depends on the balance of influx and efflux, cell wall permeability and the efflux system [11].

It is known that the targeted gene mutation is a fundamental reason for resistance, but it is not possible to study all of the related genes causing resistance to various drugs. McMurry et al. provided the first evidence of the role of efflux pumps in antibiotic resistance [25]; since then, efflux mechanisms have been perceived to be major performers in bacterial drug resistance [25,26]. Research conducted by Mustafa et al. said that there was no significant difference between efflux pump gene expression of *M. tuberculosis* susceptible and resistant isolates, but there were differences in the transcription level of all efflux pump-related genes. The transcription level of an *M. tuberculosis* clinical isolate was much higher than the *M. tuberculosis* reference strain in the same genotype [27]. A number of studies have shown an increased level of efflux pump gene expression due to the presence of drugs [28,29]. In conclusion, the efflux pump gene expression of *M. tuberculosis*, which was isolated directly from patients with TB, had a higher expression level, although it was not an *in vitro* induced drug [27].

The *Rv1250* gene encodes an integral protein drug transporter on the cell membrane of *Mycobacterium tuberculosis*. Rv1250 is included in the Major Facilitator Superfamily, which is an element of 25% transporter proteins in bacterial cells [30]. The Major Facilitator Superfamily transporter is a single polypeptide uniporter, symporter or antiporter carrier that is relevant to clinical and pharmaceutical functions, and has the role of transporting small molecules (inositol, secondary metabolites from the Krebs cycle, sugar, phosphate, drugs, nucleosides, neurotransmitters, amino acids and peptides) caused by the motive force protons process. From this study, we can gather a basic consideration that efflux pump overexpression in clinical isolates is one of the reasons for the emergence of the XDR phenotype. XDR-TB is a phenotype with isoniazid- and rifampicin-resistant (MDR), fluoroquinolone-resistant, and resistant against at least one of the three second-line drugs (i.e. amikacin, kanamycin, capreomycin). Although a multidrug efflux pump recognizes substrate with various chemical structures and components, the types of substrate most widely excreted from cells through efflux mechanisms are quinolone, macrolide and tetracyclines. Hence, the efflux pump gene overexpression is a preliminary to the emergence of the XDR-TB phenotype. Therefore, further research is needed to evaluate the relationship between the substrate specification of efflux pump proteins and the emergence of XDR-TB strain.

Conclusion

Our finding reports that there were differences in the level of *Rv1250* mRNA expression between sensitive and resistant groups of 5.961 ± 0.414 and 10.192 ± 1.978, respectively, $p < 0.05$ and significant differences ($p < 0.05$) among resistant strain group (0-month 5.960 ± 0.437, 1st month 7.573 ± 0.424; 3rd month 11.057 ± 0.262, and 5th month of drug therapy 12.701 ± 0.460) of mRNA expression levels in *M. tuberculosis* isolated from follow-up TB patients.

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Availability of data and materials

All test methods and data analysed in this study are presented in this article. The results of the quantitative real-time PCR in 51 isolates are tabulated in the Supplementary material, Table S1.

Conflict of interests

The authors have declared that no competing interest exists.

Authors' contributions

FU, MH, DRH and RN initiated and designed the study. FU, MH, DRH, RD, ARJ, MRP and RN drafted the manuscript. FU, MH, DRH, RD, MRP and ARJ supervised the field activities and the microbiology work. FU, MH, DRH, RD, ARJ, MRP and RN helped to collect isolates. All authors have read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2019.100609.

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