EFFLUX PUMP GENE EXPRESSION AND EFFLUX PROTEINS IN MULTIDRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS COMPLEX*

Çoklu Ilaç Dirençli Mycobacterium Tuberculosis Kompleks Suşlarında Eflüks Pompasını Oluşturan Genlerın Ekspresyonları Ve Eflüks Proteinleri

Aymen Azeez Khalid AL-BAYATI
Biyoteknoloji Anabilim Dalı

Fatih KÖKSAL
Biyoteknoloji Anabilim Dalı

ÖZET

İzoniyazid (INH) ve rifampisin (RIF) tüberküloz tedavisinde kullanlan en etkili anti tüberküloz ilaçlardır. Bu önemli tüberküloz ilaçları için moleküler direnç mekanizmalarının bilinmesi, çoklu ilaç dirençli (MDR) tüberküloz ve yaygın ilaç dirençli tüberkülozun hızlı teşhisi için çok önemlidir.

Çok sayıda çalışma, dışa atım (eflüks) pompalarının antibiyotik direnci ile ilişkili olduğun ve antitüberküloz ilaçlarla birlikte dışa atım inhibitörlerinin kullanımının başarıyı arttığını göstermektedir.

Çalışmamıza 20 dışa atım pompa (EP) geninin ekspresyonunu ölçmek ve dirence sebep olan rpoB (RIF), katG (INH), inhA promotör (INH) ve oksiR-ahpC (INH) genlerindeki mutasyonlarını değerlendirmek için 40 MDR Mycobacterium tuberculosis izolatı ve 40 klinik duyarlı izolat dahil edildi.

katG, inhA ve oksiR-ahpC mutasyonu olmayan MDR izolatlarında, mutasyonları taşıyanlara kıyasla eflüks pompa genlerinde daha fazla ekspresyon tespit edilmiştir. drrA, drrB, efpA, jefA, mmr, Rv0849, Rv1634 ve Rv1250 INH veya RIF stresi altında aşırı ekspresyon gözlemlenmiştir.

İlaç etkisi olmadan, MDR izolatlarında duyarlı olanlara göre; efpA, Rv0849, Rv1250, Rv1634, Rv2994, stp, Rv2459, pstB, drrA, ve drrB ekspresyon düzeyleri dikkate değer şekilde daha yüksekti. ($P < 0.05$)

Sonuç olarak, eflüks pompa mekanizması özellikle katG ve inhA genlerinde mutasyonu olmayan INH direncinde oldukça önemlinde önemli rol oynayabileceği düşülmektedir. MDR ve hassas suşlar arasındaki EP genleri ekspresyon seviyelerinin anlaşılması, dirençli tüberküloz tanı ve tedavisinde faydalı olacaktır.

Anahtar Kelimeler: tüberküloz, eflüks pompa, çoklu ilaç dirençli, tedavi

ABSTRACT

Isoniazid (INH) and rifampicin (RIF) are the most effective drugs in the treatment of tuberculosis. Known of molecular resistance mechanisms for these important tuberculosis drugs is essential to quick diagnoses of multidrug-resistant (MDR) tuberculosis and extensive drug-resistant tuberculosis.

Numerous studies show efflux pump as an important mechanism for antibiotic resistance and using of efflux inhibitors with antituberculosis drugs during treatment, enhanced success.

40 clinical MDR Mycobacterium tuberculosis isolates and 40 clinical pan-sensitive isolates were included to evaluate the expression of 20 putative drug efflux pump (EP) genes and sequence mutations in rpoB (RIF), katG (INH), the inhA promoter (INH), and oxyR-ahpC (INH). Nine and three MDR isolates were induced to over- express efflux pump genes by INH and RIF, respectively.

* Produced from the Doctoral Thesis of the Same Title.

MDR isolates that carried the wild-type katG, inhA, and oxyR-ahpC efflux pump genes were most overexpressed under INH stress in a than in those that carried mutations. drrA, drrB, efpA, jefA, mmr, Rv0849, Rv1634, and Rv1250 were overexpressed under INH or RIF stress.

Without drug inducement, efpA, Rv0849, Rv1250, Rv1634, Rv2994, stp, Rv2459, pstB, drrA, and drrB expression levels were significantly higher ($P < 0.05$) in 40 MDR isolates than in 40 pan- sensitive isolates.

In conclusion, EP may play an important role in INH acquired resistance in MDR tuberculosis especially wild type katG and INH genes. Understanding of expression levels for EP genes between MDR and sensitive tuberculosis some may be helpful to diagnose and treat resistant tuberculosis.
Key words: Tuberculosis, efflux pump, MDR, Isoniazid, Treatment.

INTRODUCTION

The history of tuberculosis (TB) changed significantly after the discovered of the first drugs with anti-mycobacterial activity. The introduction of Streptomycin in 1943 to treat TB resulted in a decrease in death rates associated with TB disease" Shortly thereafter, especially in 1948, the first antibiotic was given for the treatment of TB. However, shortly after the first antibiotic was introduced in 1943, drug resistance developed mainly due to the use of streptomycin as monotherapy.

The genus is divided into two wide taxonomic groups depend on the growth rates of individual species. The first group which takes more than a week known as slow growers to form colonies includes slow-growing species such as the well-known pathogens *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae*. The other group that give colonies within seven days such as *Mycobacterium smegmatis*, those are termed fast growers or non-pathogenic/ opportunistic bacteria (Ethiological agents of human tuberculosis (TB), bovine tuberculosis (BTB) and leprosy respectively) (Forrellad et al., 2013).

MATERIAL AND METHODS

Ethics approval of research

This study has been performed in accordance with the Declaration of Helsinki. Since the study used only isolates that were routinely collected from Tropical Disease Research and Application Center in Adana /Turkey, The Ethical Review Committee at the cukurova university /college of medicine approved the study procedures.

Bacterial strains and drug susceptibility test using BACTEC MGIT 960 system

M. tuberculosis clinical isolates were selected from the storage collection of the Tropical Disease Research and Application Center,university of cukurova The isolates were from sputum specimens obtained from patients and cultured on BACTEC-MGIT 960, A total of 80 clinical isolates *M. tuberculosis* along with *M. tuberculosis* reference strain H37RV were included in the present study. were selected and divided into two groups the first group includes Forty MDR-TB isolates, which are resistant to INH and RIF, have been selected and Forty sensitive-TB isolates which are sensitive to Antibiotics used .

The isolate profiles of drug susceptibility were evaluated by the proportional method using BACTEC MGIT 960 system with the following: RIF 1.0 µg/mL and INH 0.1 µg/mL.

Genomic DNA Extraction & Amplification

Genomic DNA was extracted from cultures grown on BACTEC-MGIT 960 were harvested and killed by heating at 80°C for 30 min. using the mickle method (Figure 4.2A), as described previously and the extracted DNA was analyzed immediately or was stored at −20°C , PCR amplification assays were carried out for the *rpoB, katG, inhA and oxyR-ahpC* genes. Primers for each of the genes were selected from published literature or designed. Table 1 contains specific annealing temperatures and primer sequences used for amplification.

Each 25-µL PCR mixture contained 12.5 µL of HotStarTaq master mix , primers 0.25 µL of the forward, and 0.25 µL of the reverse ; 6 µL of double-distilled H2O,1 µL of Dimethyl sulfoxide (DMSO) ; and 5 µL of genomic DNA,the reaction was performed in applied biosystem thermal cycler (MJ Research, Inc.) for *rpoB, katG, inhA and oxyR-ahpC* genes under the following conditions, Amplification was carried out for 30 cycles (an initial denaturation step at 94°C for 5 min, followed by denaturation at 94°C for 30 s), annealing temperature for three genes (katG,, rpoB and inhA: 60 °C) for 30 s and elongation at 72 °C for 30 s , with a final elongation step at 72 °C for 5 min, and the oxyR-ahpC gene Amplification was carried out for 35 cycles (an initial denaturation step at 94°C for 5 min, followed by denaturation at 94°C for 45 s, annealing temperature 60 °C for 45 s and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 7 min, The amplification of the gene was detected by 2% agarose gel electrophoresis with ethidium bromide staining and visualized under UV light.

Table 1. Primers used to amplify and sequence

Gene	**Primer**	**Sequence (5′→3′)**	**Amplicon size (bp)**
rpoB	F	ACCGACGACATCGACCACTT	450
	R	GTACGGCGTTTCGATGAACC	
katG	F	AATCGATGGGCTTCAAGACG	500
	R	CTCGTAGCCGTACAGGATCTCG	
inhA	F	CCTCGCTGCCCAGAAAGGGA	248
	R	ATCCCCCGGTTTCCTCCGGT	
oxyR-ahpC	F	GAGACCGGCTTCCGACCACC	293
	R	GCTGGTAGGCGGGGAATTGAT	

DNA Sequencing and mutation detection

The sequences of genes including rpoB for RIF, katG and inhA regulator sequence, and oxyR-ahpC genes for INH (reported to carry major mutations associated with RIF and INH resistance) were analyzed by PCR, positive isolates and DNA sequencing It amplified a product was designed to identify the presence or absence of the gene. The sequences of the specific primers and the sizes of the amplicons are presented in (Table 1). Partial PCR products were characterized by DNA sequencing using the forward primers on an Applied Biosystems 3130xl Genetic Analyzer. The resulting DNA sequences were analyzed using the basic local alignment search tool

(http://www.ncbi.nih.gov/BLAST), and the specific mutations in protein sequences of the individual isolates were identified.

The PCR products were used as templates for targeted DNA sequencing. Sequencing of both strands of the PCR product was performed on an ABI373 sequencing instrument according to the protocol supplied by the manufacturer using the Big Dye™Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Mutations were determined by comparing with *M. tuberculosis* H37Rv sequence of katG, rpoB, oxyR-ahpc, and inhA genes where a PCR product was detected, it was purified using the SeqFinder Sequencing System MEFV Kit Protocol (Talstrasse, Altendorf, Switzerland) following manufacturer's instructions.

Quantification of gene expression using real-time quantitative PCR (qPCR)

The primers of the 21 genes are described in Table 1.2. The assay was performed using a Syber green qPCR kit (Promega Company, UK) in Applied Biosystems 7300 Real-Time PCR System . Briefly, each 0.2 mL tube contained 12.5 µL 2× Syber green master mix, 0.25 µL each primer, 150 ng cDNA, and 9.5 µL RNase-free water. The thermal cycling conditions were as follows: 95°C for 10 min, then 45 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 1min, and the last step consisted of a melting curve analysis (65–95°C). The fold change in the genes expression in eighty (10) MDR-TB isolates was calculated by 2-ΔΔCT method of Livak and Schmittgen (Livak and Schmittgen, 2001). When the expression of genes compared with nonexposed control genes displaying levels more than 1 were found to be increased; equal to or above four were found to be overexpressed (Livak and Schmittgen, 2001; DeMarco et al., 2007; Rodrigues et al., 2012; Bustin et al., 2010). The expression of genes of (10) MDR-TB isolates without drug inducement was calculated by 2-ΔCT method. The delta Ct values were derived by subtracting the CT value of housekeeping gene (*polA*) from the CT value obtained for each gene. The values of ΔΔCT were derived by subtracting the value of ΔCT obtained for each gene without drug stress from the value of ΔCT of genes induced by INH/RIF. polA which is expressed at a stable level in the isolates and can used as an internal invariant control, is described as a housekeeping gene

RESULT AND DISCUSSION

Selection strains and Susceptibility testing

For the study, 80 clinical isolates were used (Table 1.3). Of these, 40 isolates were pan-susceptible, 40 isolates were resistant to the first-line drugs, i.e. isoniazid (INH) and rifampin (RIF). The concentrations of INH were 0.1 µg/ml and RIF were 1.0 µg/ml (Adami et al., 2017). *M. tuberculosis* clinical isolates, which showed resistant to both drugs, including INH and RIF, are considered as MDR-TB (Nathanson et al., 2010; Organization, 2010). Whereas, XDR-TB is caused with additional resistance to a fluoroquinolone such as ciprofloxacin and one second-line injectable agent including amikacin or kanamycin (Kolyva and Karakousis, 2012).

Table 2 Characteristics of sample

Mycobacterial Strains	Number of TB isolates	Notes
Sensitive TB	40 sample	Sensitive to INH and RIF
MDR-TB	40 sample	Resistance to INH and RIF

DNA Amplification

A total of 89 bacterial strains for both strains (sensitive and resistant starins) were isolated from the tuberculosis center as that mentioned in material and method section, Moreover all strains were subjected to the molecular identification procedure by means of PCR amplification of inhA ,katG, oxyR-ahpC and rpoB gene and show in table 1.1. all strains , For this purpose genus specific primers were used in PCR ,all isolated were amplified were used previous genes as described to confiremed the mycobacterium tuberculosis were identified using specific primers sets that mentioned earlier in Table 2 (Materials and Methods section).

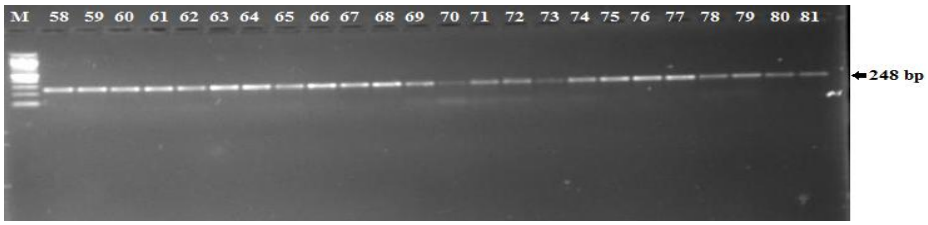

Figure 1. PCR products of amplified inhA gene MDR-TB isolated Lanes: M, 100 bp DNA marker

Correlation between the Phenotype and Mutations Associated with Antibiotic Resistance

First, in order to establish a relationship between the level of phenotypic drug resistance and the presence of resistance-associated mutations, we looked for mutations in genes associated with drug resistance to the antibiotics studied.

All pan-susceptible strains showed no mutation in any of the genes evaluated. Resistance to isoniazid was associated with mutations in the katG and inhA genes according to published data (Cambau et al., 2015; Domínguez et al., 2016). Among the MDR strains, 28 harbored mutations in katG, of which 27 had the S315T mutation and one has a rare mutation at codon 735. The remaining 12 strains harbored double mutations in inhA. The double mutations C-15T/S94A were detected in strains MtbPT7 and MtbPT9 and the pair C-15T/I194T was detected in MtbPT10 and MtbPT11.

As for rifampicin resistance, all MDR strains and two rifampicin monoresistant strains showed S531L mutation in rpoB. It is the most common mutation in rifampicin resistant clinical isolates.

According to the most widely accepted dogma on the mechanisms of drug resistance in *M. tuberculosis*, the only cause of resistance levels recognized in these strains is expected to be derived from the reduced affinity of the mutated genes against the respective antibiotic only. (Zhang and Yew, 2009; Domínguez et al., 2016).

Effect of Antibiotics on Efflux Pump Gene Expression

To further confirm these findings, we analyzed the transcriptional profile of the possible pumps described in the literature that the *M. tuberculosis* strains were associated with antibiotic resistance phenotypes. (Viveiros et al., 2012; Black et al., 2014; Silva da et al., 2016). RT-qPCR was done for efflux genes previously shown to be overexpressed in response to antibiotic exposure.

The cDNA concentrations of the transcripts were calculated by comparing the 2-ΔΔCT values ofthe tested cDNA samples with those of the external controls. The results showed that significant changes in expression levels occurred in all strains after exposure to antibiotics. MDR strains were exposed to rifampicin or isoniazid and similar models were observed(Table 1.3). Three to six genes were overexpressed after exposure to isoniazid or rifampicin. P55 showed the highest level of expression in both strains and for both antibiotics. In general, EfpA was overexpressed in all strains, independent of the antibiotic exposure. For the remaining flow pumps analyzed, we were unable to find any correlation antibiotic to overexpression of a particular efflux pumps. We observed a general pattern of gene expression upon exposure to antibiotics.

Drug resistance in *M. tuberculosis* has long been associated with the development of mutations in genes encoding drug targets, but it has recently been recognized that waste flow pump activity plays an important role in the development of drug-resistant phenotypes in *M. tuberculosis*. Several recent studies have demonstrated the importance of overexpression of overflow pump genes in clinical strains of MDR and XDR *M. tuberculosis*. However, most of these studies are based on a simple assessment and evaluation of the expression levels of the *M. tuberculosis* discharge pump genes, and few have identified the effect of discharge inhibitors on the MICs of antituberculosis drugs and their over-expressed flow systems. Therefore, further studies are needed to investigate the contribution of overactivity of overflow pumps to resistance levels in *M. tuberculosis* strains. (Kanji et al., 2016; Machado et al., 2016; Oh et al., 2017).

Next, we analyzed the expression levels of putative efflux pumps upon exposure to the antibiotics in the H37Rv susceptible strain, and in the monoresistant, MDR *M. tuberculosis* clinical isolates. The efflux pump genes mmr, mmpL7, Rv1258c, p55, and efpA were shown to be overexpressed in presence of antibiotics demonstrating the contribution of these efflux pumps to the resistance phenotype of the strains studied, with the exception of the H37Rv strain. We have noticed a general overexpression of almost all efflux genes studied upon exposure to the antibiotics in the drug resistant strains independently on the genotype of the strains.

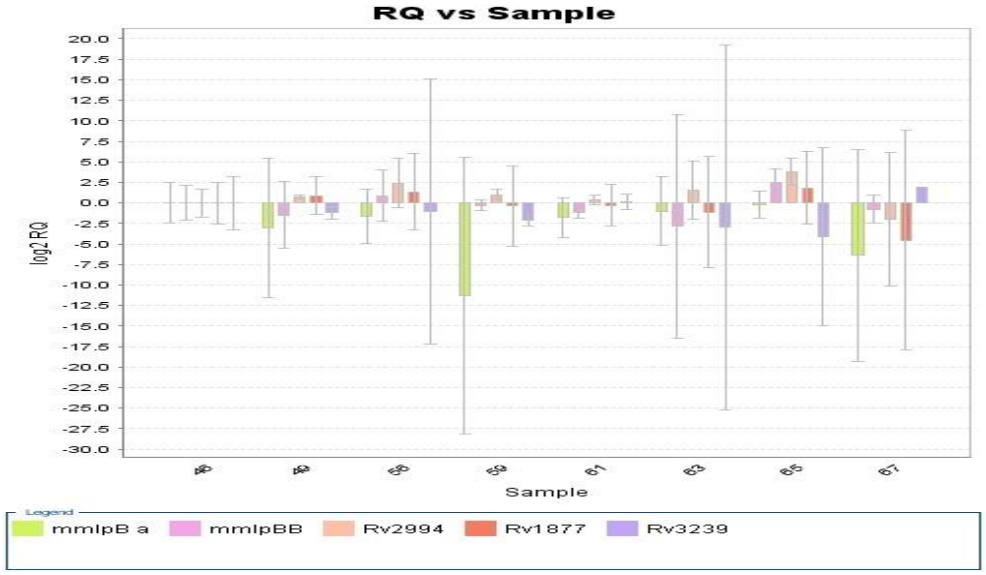

Figure 2. result of quantative Real time PCR for MDR strains (46,49,56,59,61,63,65,67) caluculated by log 2RQ

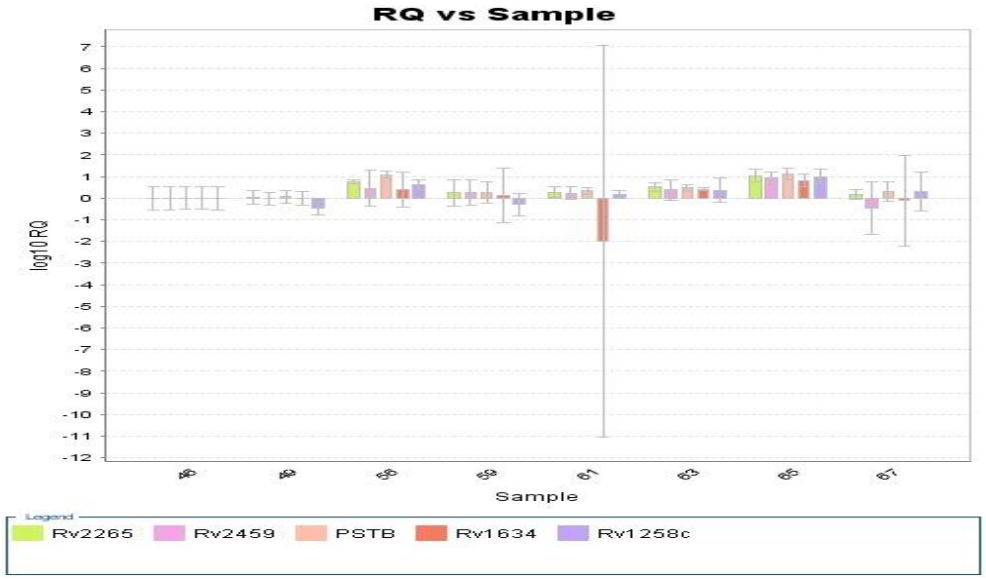

Figure 3. result of quantative Real time PCR for MDR strains (46,49,56,59,61,63,65,67) caluculated by log 2RQ

Gupta et al. showed that the induced efpA expression was 4-and 4.5-fold greater under INH stress, respectively, which was consistent withthe resultsin ourstudy. jefA (Rv2459) has been reported to respond to INHstress, and Gupta et al. found tha tthe increased transcription ofjefA (Rv2459) leads to increased resistance to EMB and INH in *M.tuberculosis*. Mmr has been reported to mediate the efflux of different chemical classes and antibiotics. Gupta et al found thatmmr had two-fold higher expression in two isolates under the INH-stressed condition and three-fold higher expression in three isolates under the levofloxacin-stressed condition. Rv1634 is a member ofthe majorfacilitator superfamily in *M. tuberculosis,* similar to many antibiotic resistance (efflux) proteins, including DTDP-glucose dehydratase fromStreptomyces violaceoruber. Rv1634 has been reported to decrease susceptibility to various fluoroquinolone swhen overexpressed in *M. smegmatis* and involve in norfloxacin and ciprofloxacin efflux. Louwet al. reported that Rv1634was up regulated in strains with a Beijing genotype after exposure to RIF for 24h. Balganesh et al. reported that the efflux pump encoded by Rv0849 mediates the efflux of RIF Rv1250 isaprobable drugtransport integral membrane protein having 579 amino acid (aa) and highl ysimilar to the tetracenomycin Cp rotein from *Streptomyces glaucescen* (32.9% identity in517 aaoverlap). Rv1250is alsosimilartoRv3239C from *M. tuberculosis* (31.9% identity in 423aa overlap). No further information on this protein ha sbeen reported. Together, these results indicate thatmore efflux pumps may respond toINH than RIF in *M. tuberculosis*. A possible explanation for this may be that INH exerts its bactericidal activity byattacking the cellwall mycolic acidafter being converted to arangeof oxygenated andorganic toxic radicals b ybifunctional bacterial enzyme catalase-peroxidase (KatG), whereas RIF acts by arresting DNA-directed RNA synthesis inthe cytoplasm of *M. tuberculosis.*

In conclusion, this study allowed us to show that the main mechanisms associated with drug resistance in *M. tuberculosis* correlates mutations in target genes with increased efflux and that compounds that inhibit efflux activity can significantly reduce the phenotypic level of such resistance. The level of drug resistance in *M. tuberculosis* is a combination between the presence of a mutation in the drug target genes and a general stress response to the presence of noxious compounds that regulates the intracellular level of a drug. The data obtained in the presented study corroborated our previous findings (Machado et al., 2017) now tested on a larger and diverse panel of *M. tuberculosis* clinical strains. The demonstration that the efflux activity modulates the levels of antibiotic resistance by complementing the resistance due to target-gene mutations, is a very relevant finding in the context of the ongoing discussion on the ability and clinical reliability of sole molecular based detection of the target-gene mutations as the future routine DST for *M. tuberculosis* (Böttger, 2011; Domínguez et al., 2016; Pankhurst et al., 2016; Schön et al., 2016). The use of efflux inhibitors as adjuvants of the antituberculosis therapy may be a promise for the development of new and shorter therapeutic strategies as they may potentiate the activity of the current antituberculosis drugs shortening the recommended 6 month treatment for cure, they can increase the activity of drugs that are no longer used due to the emergence of resistance, and they may also be used to protect the activity and usefulness of the new antituberculosis drugs from the development of drug resistance.

CONCLUSION

Efflux pumps may play an important role in INH acquired resistance in MDR M. tuberculosis, especially in strains without mutations in katG, inhA, and oxyR-ahpC, which are associated with INH resistance.

The basal expressional differences of some drug efflux pump genes between MDR and pan-sensitive isolates may be helpful to diagnose and treat resistant tuberculosis. However, we acknowledge that we were unable to demonstrate a direct relationship between the resistance level of INH and/or RIF and the activation of specific genes.

Further efforts are required to elucidate the actual roles of drug efflux pumps in the drug resistance of *M. tuberculosis.*

REFRENCES

Adami, AG., Gallo, JF., Pinhata, J MW., Martins, MC., Giampaglia, CMS., and De Oliveira, R. S., 2017. Modified protocol for drug susceptibility testing of MGIT cultures of Mycobacterium tuberculosis by the MGIT 960. Diagnostic microbiology and infectious disease, 87:108-111.

Black P., Warren R., Louw G., van Helden P., Victor T., Kana B.,2014. Energy metabolism and drug efflux in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 58, 2491–2503. 10.1128/AAC.02293-13.

Böttger E.,2011. The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing. Clin. Microbiol. Infect. 17, 1128–1134. 10.1111/j.1469-0691.2011.03551.x

Bustin, SA., Beaulieu, JF., Huggett, J., Jaggi, R., Kibenge, FS., Olsvik, PA., Penning, LC., and Toegel, S., 2010. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BioMed Central, 11: 1-5.

Cambau E., Viveiros M., Machado D., Raskine L., Ritter C., Tortoli E., et al. , 2015. Revisiting susceptibility testing in MDR-TB by a standardized quantitative phenotypic assessment in a European multicentre study. J. Antimicrob. Chemother. 70, 686–696. 10.1093/jac/dku438.

da Silva P. E. A., Machado D., Ramos D., Couto I., von Groll A., Viveiros M., 2016. Efflux pumps in mycobacteria: antimicrobial resistance, physiological functions, and role in pathogenicity, in Efflux-Mediated Antimicrobial Resistance in Bacteria, eds Li X-Z., Elkins C. A., Zgurskaya H. I., editors. (Cham: Springer International Publishing AG;), 527–559.

Demarco, CE., Cushing, LA., Frempong-Manso, E., Seo, SM., Jaravaza, TA., and Kaatz, GW., 2007. Efflux-related resistance to norfloxacin, dyes, and biocides in bloodstream isolates of *Staphylococcus aureus*. Antimicrobial agents and chemotherapy, 51: 3235-3239.

Domínguez J., Boettger E. C., Cirillo D., Cobelens F., Eisenach K. D., Gagneux S., et al., 2016. Clinical implications of molecular drug resistance testing for *Mycobacterium tuberculosis*: a TBNET/RESIST-TB consensus statement. Int. J. Tuberc. Lung Dis. 20, 24–42. 10.5588/ijtld.15.0221

Forrellad MA, Klepp LI, Gioffré A, Sabio y García J., 2013. Virulence factors of the *Mycobacterium tuberculosis* complex. Virulence; 4(1): 3–66.

Kanji A., Hasan R., Zhang Y., Shi W., Imtiaz K., Iqbal K., et al., 2016 . Increased expression of efflux pump genes in extensively drug-resistant isolates of *Mycobacterium tuberculosis*. Int. J. Mycobacteriol. 5:S150. 10.1016/j.ijmyco.2016.09.067

Kolyva, AS., and Karakousis, PC., 2012. Old and new TB drugs: mechanisms of action and resistance. Understanding Tuberculosis-New Approaches to Fighting Against Drug Resistance. InTech.

Livak, KJ., and Schmittgen, TD., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods, 25: 402-408.

Machado D, Coelho TS, Perdigão J, Pereira C, Couto I, Portugal I, Maschmann RDA, Ramos DF, von Groll A, Rossetti MLR, Silva PA and Viveiros M ., 2017. Interplay between Mutations and Efflux in Drug Resistant Clinical Isolates of Mycobacterium tuberculosis. Front. Microbiol. 8:711. doi: 10.3389/fmicb.2017.00711

machado D., Pires D., Perdigão P., Couto I., Portugal I., Martins M., et al., 2016. Ion channel blockers as antimicrobial agents, efflux inhibitors, and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*. PLoS ONE 11:e0149326. 10.1371/journal.pone.0149326

Nathanson, E., Nunn, P., Uplekar, M., Floyd, K., Jaramillo, E., Lönnroth, K., Weil, D., and Raviglione, M., 2010. MDR tuberculosis—critical steps for prevention and control. New England Journal of Medicine, 363: 1050-1058.

Oh T. S., Kim Y. J., Kang H. Y., Kim C. K., Cho S. Y., Lee H. J., 2017. RNA expression analysis of efflux pump genes in clinical isolates of multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* in South Korea. Infect. Genet. Evol. 49, 111–115. 10.1016/j.meegid.2017.01.002

Organization, WH., and Initiative, ST., 2010. Treatment of tuberculosis: guidelines, World Health Organization.

Pankhurst L. J., del Ojo Elias C., Votintseva A. A., Walker T. M., Cole K., Davies J., et al., 2016. Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. Lancet Respir. Med. 4, 49–58. 10.1016/S2213-2600(15)00466-X