

The Inhibitory Effect of Thioridazine on *adeB* Efflux Pump Gene Expression in Multidrug-Resistant *Acinetobacter baumannii* Isolates Using Real Time PCR

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Abstract

Background: The purpose of the present study was to investigate the antimicrobial effects of berberine and thioridazine, as well as their effect on the gene expression of the AdeABC efflux pump system in Multidrug-Resistant (MDR) *Acinetobacter baumannii* (*A. baumannii*) isolates.

Methods: This study was carried out in five MDR clinical isolates of *A. baumannii* and a sample of standard strain (*A. baumannii* PTCC1797). The effect of Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of berberine, thioridazine, and ciprofloxacin alone and their combination on *A. baumannii* was evaluated by broth microdilution method. Also, their effect on the expression of *adeB* efflux pump gene was evaluated using real time PCR method.

Results: The MIC of thioridazine, berberine, ciprofloxacin+thioridazine, ciprofloxacin+berberine, thioridazine+berberine, and ciprofloxacin+thioridazine+berberine on MDR *A. baumannii* isolates was 64, 256, 128, 256, 128, and 128 $\mu\text{g/ml}$, respectively. The results showed that treatment of strains with thioridazine alone and in combination with berberine and ciprofloxacin significantly ($p < 0.05$) decreased the expression of *adeB* efflux pump gene.

Conclusion: Due to the inhibitory effects of thioridazine on bacterial isolates and *adeB* efflux pump gene, this compound can be used as a potential antimicrobial agent against MDR *A. baumannii*.

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Keywords: *Acinetobacter baumannii*, Ciprofloxacin, Gene expression, Real-time polymerase chain reaction, Thioridazine

Introduction

Acinetobacter baumannii (*A. baumannii*) plays an important role in nosocomial infections¹. This bacterium causes infection and hospitalization, especially in the intensive care unit, burns unit, and surgery wards and increases mortality in people with impaired immune systems². Multidrug Resistant (MDR) *A. baumannii* shows resistance through various mechanisms such as aminoglycoside modifying enzymes, change of outer membrane, biofilm formation, and increased expression of efflux pump³. Efflux pump is one of the main mechanisms in the development of resistance in *A. baumannii*². One of the most important of these

efflux pumps is the AdeABC pump belonging to Resistance-Nodulation-Division (RND) family and one of the main components, called *adeB*, as a transporter protein, causes antibiotics to be expelled from the bacterial cell⁴. Studies have shown that disrupting the efflux pump can increase the antimicrobial properties of antibiotics and biocides against bacteria. In recent decades, the use of herbal medicines and biocides alone or in combination has been widespread to increase the effectiveness of antibiotics⁵. One of these phytochemical compounds is berberine, which has several phenolic rings as an isoquinoline alkaloid. According to studies,

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this substance affects the bacterial cell membrane and disrupts its structure, causing the material to leak out of the cell ⁶. Another antimicrobial compound used is thioridazine from the phenothiazine family. According to studies, thioridazine, by having a synergistic effect with cloxacillin and miconazole, can reduce the expression of the efflux pump gene in methicillin-resistant *Staphylococcus aureus* (*S. aureus*) ⁷. Considering the important role of efflux pumps in antibiotic resistance in *Acinetobacter* isolates and the possible role of berberine and thioridazine in inhibiting some of these pumps, the purpose of this study was to investigate the antimicrobial effects of these two compounds alone and in combination against MDR *A. baumannii* and also investigate the effect of these two substances on the gene expression of AdeABC efflux pump system using real time PCR.

Materials and Methods

Bacterial strains

In this experimental study, 6 strains of *A. baumannii* including five MDR clinical isolates and a standard strain (*A. baumannii* PTCC1797) obtained from Iran Scientific and Industrial Research Center were included in the study. *A. baumannii* isolates were identified using standard biochemical test and finally confirmed by amplification of *gltA* gene using PCR method.

Determination of the MIC

The antimicrobial effects of the compounds were evaluated by broth microdilution method with the following treatments: group 1 received thioridazine; group 2, berberine; group 3, ciprofloxacin; group 4, thioridazine and berberine; group 5, thioridazine and ciprofloxacin; group 6, berberine and ciprofloxacin; and group 7, thioridazine and berberine and ciprofloxacin. MIC determination was done by broth microdilution method, according to CLSI guideline ⁸. Then, serial dilution process was administered and dilutions equivalent to 256 $\mu\text{g/ml}$, 128 $\mu\text{g/ml}$, 64 $\mu\text{g/ml}$, 32 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$, and 4 $\mu\text{g/ml}$ were prepared from thioridazine, berberine, and ciprofloxacin solutions. Also, dilutions in the range of 256 $\mu\text{g/ml}$ to 4 $\mu\text{g/ml}$ were prepared for combinations of each compound. In order to determine the MBC, 10 microliters per well were cultured separately in Mueller–Hinton agar medium in which the bacterium was not grown with 5% sheep blood. Then, incubation at 37°C was done for 18-24 hr, and finally, the lowest concentration of the treatment in which no bacterial growth was observed was reported as MBC concentration ⁹.

Detection of *adeB* gene by PCR

Genomic DNA of bacteria was extracted using boiling method ¹⁰. PCR amplification was performed in a 25 μl total volume containing 12.5 μl master mix (Amplicon, Denmark), 25 pmol of each primer, and 3 μl of DNA template.

Expression of *adeB* efflux pump gene by real time PCR

Sediment preparation: The volume required for sedimentation of seven different treatments was calculated according to the MIC concentration in 1 ml of the microtube. All calculations were based on $C1V1=C2V2$ formula. After calculations, the required volume of each treatment including 100 μl of bacteria grown overnight at a concentration of 10^7 CFU/ml was poured into a 1.5 ml microtube. Then, the remaining volume of 1 cc was brought to final volume with sterile Mueller–Hinton broth culture medium. After 20 hr of incubation, the microtubes were centrifuged at 15,000 rpm for 10 min. The microtube supernatant was removed and the contents were washed three times with distilled water. The final precipitate was stored at -20°C for further analysis. All of these stages were performed on 7 treatment groups and a control group.

RNA extraction and cDNA synthesis

After sedimentation, RNA was extracted from clinical and standard samples using RNA extraction kit (Pars Toos Company, Iran) according to the instructions. Then, cDNA synthesis was performed using a cDNA synthesis kit (Pars Toos Company, Iran) in one cycle with two different temperatures (37°C for 15 min and 85°C for 5 s).

Quantitative real time PCR method

At this stage, BIO RAD CFX96 was used to quantitatively evaluate the gene expression of *A. baumannii*. To quantify the expression of the *adeB* gene, a master mix (containing SYBER Green) was prepared; the volume of the master mix was 20 μl , including 1 μl of each primer, 3 μl of distilled water, 10 μl of buffer, and then 5 μl of cDNA was added to master mix. In this reaction, *adeB* and *rpoB* primers were used according to table 1. The *rpoB* gene was used as an internal control with the ability to be stable in different conditions in order to compare and properly measure gene expression. Finally, the relative expression of *adeB* gene in *A. baumannii* isolates was calculated by $\Delta\Delta\text{CT}$ method. To calculate the expression of the desired gene by real time PCR (due to SYBER Green use), the relative comparison method (Ct: cycle threshold) was used as follows, relative measurement of $\text{Ct}=\text{Ct}$ of the desired gene without the presence of intervention-Ct of the

Table 1. The oligonucleotides sequence of primers used in real time PCR reaction in this study

Gene	Primer sequence (5' to 3')	Product size (bp)	Reference	Annealing temperature
<i>rpoB-F</i>	5'-GAG TCT AAT GGC GGT GGT TC-3'	110	(11)	60
<i>rpoB-R</i>	5'-ATT GCT TCA TCT GCT GGT TG-3'			56
<i>AdeB-F</i>	5'-AAC GGA CGA CCA TCT TTG AGT ATT-3'	84	(11)	62
<i>AdeB-R</i>	5'-CAG TTG TTC CAT TTC ACG CAT T-3'			58

Effect of Thioridazine on *adeB* Gene

Table 2. The MIC ($\mu\text{g/ml}$) of ciprofloxacin, berberine, thioridazine on clinical and standard strain of *A. baumannii* isolates

Strains	Ciprofloxacin	Berberine	Thioridazine	Ciprofloxacin + thioridazine	Thioridazine + berberine	Ciprofloxacin + berberine	Thioridazine + berberine + ciprofloxacin
Standard strain PTCC177	64	>256	128	128	128	128	128
Clinical strain 1	>256	>256	64	128	128	256	128
Clinical strain 2	128	>256	64	128	128	256	128
Clinical strain 3	>256	>256	64	128	256	256	128
Clinical strain 4	128	>256	64	128	256	256	128
Clinical strain 5	>256	>256	64	128	256	256	128

desired gene in the presence of the intervention and a fold change of expression = $2^{-\Delta\text{Ct}(\text{Treated} - \text{Untreated})}$. A reference gene (*rpoB* gene) as a housekeeping gene was used to normalize the results.

Data analysis

SPSS software v21 (IBM, USA) was used for statistical analysis. The results were analyzed by t-test, one-way analysis of variance, and multiple comparisons. A p-value of ≤ 0.05 was considered statistically significant.

Results

The MIC of thioridazine was $64 \mu\text{g/ml}$ and ciprofloxacin in combination with thioridazine was $128 \mu\text{g/ml}$. The MIC of ciprofloxacin, berberine, thioridazine, ciprofloxacin+berberine, ciprofloxacin+thioridazine, and ciprofloxacin+thioridazine+berberine on MDR *A. baumannii* isolates is shown in table 2. The Minimum Bactericide Concentrations (MBCs) of ciprofloxacin, thioridazine, berberine, and ciprofloxacin+berberine on clinical isolates of *A. baumannii* were 256, 128, 256, and $256 \mu\text{g/ml}$, respectively.

According to multiple comparison tests, there were significant differences in the expression of *adeB* gene among different treatments of *A. baumannii*. In different treatments, the expression of *adeB* gene decreased in the presence of thioridazine alone or in combination with ciprofloxacin and berberine. The expression of *adeB* gene in thioridazine group in comparison with the ciprofloxacin, ciprofloxacin+ berberine, and ciprofloxacin+berberine+thioridazine decreased significantly. The results of changing the expression of *adeB* efflux pump gene by different treatments in *A. baumannii* strains compared to the control group are shown in figure 1. In clinical isolates and standard strains, after treatment with thioridazine ($p < 0.000$), ciprofloxacin+thioridazine ($p < 0.031$), berberine+thioridazine ($p < 0.000$), a significant decrease in efflux pump gene expression was observed.

Discussion

In the present study, it was shown that thioridazine alone and in combination with ciprofloxacin and berberine can cause decreased *adeB* efflux pump gene expression in *A. baumannii* strains. In a study conduct-

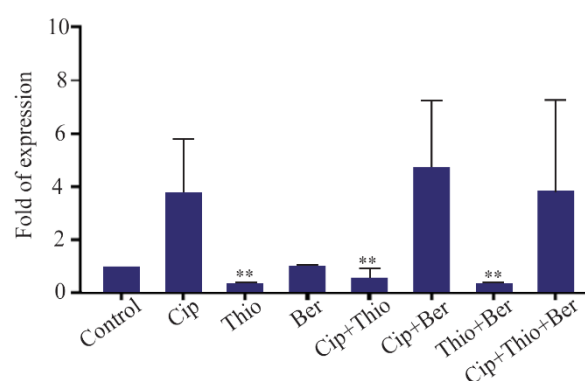


Figure 1. The *adeB* gene expression of *A. baumannii* in different treatment groups; treatment with thioridazine, ciprofloxacin+ thioridazine, and thioridazine+berberine significantly decreased *adeB* gene expression in clinical and standard strains of *A. baumannii* (Cip: Ciprofloxacin, Ber: Berberine, Thio: Thioridazine).

ed by Abdi *et al* using quantitative real time PCR method, they concluded that peptide nucleic acids (PNAs) reduced the expression level of *adeB* gene in *A. baumannii* strain. This may be due to inhibition of translation and transcription of the target gene ¹². In a study by Ruzin *et al* using RT-PCR method, it was shown that there was a significant relationship between the expression of the *AdeABC* efflux pump gene and the MIC of ethidium bromide ¹³. In our study, thioridazine in combination with ciprofloxacin and thioridazine in combination with berberine decreased the expression of the *adeB* efflux pump gene. In a study by Jorgensen *et al*, the effect of thioridazine T5 derivative on methicillin-resistant *Staphylococcus aureus* was examined and they concluded that MIC of T5 ($1-2 \mu\text{g/ml}$) inhibited bacterial growth. However, in our study, MIC of thioridazine at a concentration of $64 \mu\text{g/ml}$ inhibited the growth of MDR *A. baumannii* isolates. This may be due to the use of different thioridazine derivatives or due to the impermeable outer membrane in gram-negative bacteria ¹⁴. In our study, thioridazine in combination with ciprofloxacin reduced the MIC. The reason is that thioridazine can reduce ciprofloxacin sensitivity by blocking efflux pumps. Thioridazine may impair its function by binding to the transport structure of the efflux pump ¹⁵. In the present study, the effect of

berberine alone and in combination with the ciprofloxacin and thioridazine was investigated. According to a study conducted by Mahmoudi *et al*, it was found that berberine reduced the MIC of imipenem and ciprofloxacin by an average of 0 to 2 fold and 0 to 1 fold, respectively in *A. baumannii* isolates⁶. In our study, berberine in combination with ciprofloxacin reduced the MIC of ciprofloxacin between zero and 2 fold. Aghayan *et al* reported that berberine and palmatine reduced the MIC of ciprofloxacin in *Pseudomonas aeruginosa*, but according to PCR results, no significant correlation was observed between the gene patterns of the efflux pump, *MexAB-OprM*, and berberine and palmatine¹⁶. According to Jian J *et al* studies, gram-positive bacteria were more sensitive to berberine than gram-negative bacteria. In our study, berberine alone had no antibacterial effect on *A. baumannii*, which may be due to an impermeable outer membrane in gram-negative bacteria⁵. However, in our study, berberine alone had no inhibitory effect on *A. baumannii*, which may be due to the use of bacterial strains from different sources or due to the use of different berberine derivatives, which may affect the MIC¹⁷. Thioridazine alone and in combination with berberine and ciprofloxacin can reduce resistance in MDR strains of *A. baumannii* by reducing the expression of *adeB* efflux pump gene.

Conclusion

Due to the inhibitory effects of thioridazine on bacterial isolates and *adeB* efflux pump gene, this compound can be used as a potential antimicrobial agent against MDR *A. baumannii*.

Acknowledgement

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Ethics Approval

The study was conducted in accordance with the Declaration of Helsinki. This study has been approved by Research Ethics Committee of Yasuj University of Medical Sciences (IR.YUMS.REC.1399.084).

Conflict of Interest

The authors declare that they have no competing interests.

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