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ORIGINAL ARTICLE

Novel Antimicrobial Target in *Acinetobacter Baumannii*

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SUMMARY

Background: **Resistance to multiple drugs is one of the biggest challenges in managing infectious diseases. *Acinetobacter baumannii* is considered a nosocomial infection. According to the multiple roles of the toxin-antitoxin system, this system can be considered an antimicrobial target in the presence of bacteria. With the impact on bacterial toxin, it can be used as a new antibacterial target. The purpose of this study was to determine the *mazEF* genes as a potent antimicrobial target in *A. baumannii* clinical isolates.**
Methods: **The functionality of mazEF genes was evaluated by qPCR in fifteen *A. baumannii* clinical isolates. Then, the *mazE* locus was targeted by peptide nucleic acid (PNA).**
Results: **The results showed a significant difference in the mean number of copies of *mazF* gene in normal and stress conditions. Also, we found that at a concentration of 15 µM of PNA the bacteria were killed and confirmed by culture on LB agar.**
Conclusions: **This research is the first step in introducing *mazEF* TA loci as a sensitive target in *A. baumannii*. However, more studies are needed to test the effectiveness *in vivo*. In addition, the occurrence and potential for activation of the TA system, *mazEF* in other pathogenic bacteria should be further investigated.**
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KEY WORDS

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INTRODUCTION

The use of antibiotics to treat infections in humans and animals over the past decades has led to the development of antibiotic-resistant microbes. Antibiotic resistance is caused by mutations to target sites or the acquisition of resistance genes from other pathogens. Today, resistance to multiple drugs is one of the biggest challenges in managing infectious diseases. Although studies on new antibiotics are accelerating, identifying new drug targets in microbes is also important for controlling infections. Antibiotic resistance is currently a global problem [1]. *Acinetobacter baumannii* is a gram-negative bacterium known as an opportunistic pathogen in immunocompromised individuals. It is also considered a nosocomial infection. Imipenem and Meropenem are traditionally used to treat infections caused by *A. bau-*

mannii, but unfortunately resistance to these antibiotics is increasing in *A. baumannii* [2].
Today, the toxin-antitoxin system (TA system*)* has two components: a persistent and stable component of toxin and an unstable component of antitoxin. It is involved with deadly toxin action. With regard to physiology, antitoxin binds to toxin and neutralizes it, thus it prevents against bacterial death [3,4].
In order to evaluate a suitable antimicrobial target, this target must be present in pathogenic and antibiotic-resistant bacteria. Also, the study by Ghafourian et al. in Malaysia [5] showed the prevalence of the mazEF is high in *A. baumannii* isolates and all were functional. In the current study, using gene antisense therapy of *mazEF* TA loci as a suitable target was investigated. This study used peptide nucleic acid (PNA) as a specific response to the target was considered.
Currently there is no useful information regarding the potential of TA systems as antimicrobial targets in the *A. baumannii*. According to the multiple roles of the toxin-antitoxin system, this system can be considered as an antimicrobial target in the presence of bacteria. With the impact on bacterial toxin, it can be used as a new antibacterial target. The purpose of this study was to investigate the *mazEF* genes as a potent antimicrobial target in *A. baumannii* clinical isolates.

MATERIALS AND METHODS

Bacterial isolates

Fifteen clinical isolates of *A. baumannii* that were multidrug resistant were selected. The *mazEF* TA loci were evaluated by PCR.

Determine whether TA system is active

In order to determine if the TA system is active under normal conditions and under stress with antibiotics, RNA was extracted from all bacterial strains and converted to cDNA and evaluated by qPCR.
RT-qPCR was used to evaluate the expression of the MazE and MazF TA system genes. For this purpose, bacterial RNA was extracted under normal and stress conditions. According to the instructions, RNA was extracted and converted to cDNA using RevertAidcDNA Synthesis Kit. Finally, RT-qPCR was performed to show the functionality.

Antisense PNA therapy

Initially, a primer-specific sequence for the *mazE* gene via http://pnabio.com/support/PNA_Tool.htm was designed. The designed PNA sequence was then synthesized by Panagene Korea in the following order:
mazE-PNAatgattctcaac-o-KFFKFFKFFK
Initially, the bacterium was inoculated into LB broth medium up to an OD600 = 1 - 2. Then, 100 µL of overnight bacterial culture was added to a Falcon flask containing 5 µL LB broth and brought to an OD600 = 0.5. It was then placed in a shaker incubator for 4 hours at 37°C. Finally, a concentration of half McFarland was prepared from the bacteria. PNA was prepared at different concentrations of five, ten, and fifteen micromolar. RT-qPCR was performed to check the expression level of *mazE* gene after adding PNA to the culture. This method demonstrated the ability of PNA to control *mazE* expression in *A. baumannii*. The results were then analyzed. Note: Only one isolate was treated with PNA.

RESULTS

In order to investigate the mazEF TA system, it needs to be active under normal and stress conditions with imipenem under sub-MIC. RNA was extracted and converted to cDNA and evaluated by qPCR. The expression level of each *mazE* and *mazF* gene was examined in terms of stress compared to control cells and the results were compared with normal conditions. The results obtained under normal conditions showed approximately the same amount of toxin and antitoxin. Analysis of qPCR results showed that the mean number of copies of mazE gene in normal and stress states were 3,728 and 3,338 copies, respectively. Analysis of qPCR results showed that the mean number of copies of *mazF* gene in normal and stress states were 3,803 and 4,652 copies, respectively (Figure 1).
The results of qPCR were analyzed by SPSS software. The results were analyzed using paired samples *t*-test, and the results show that there was no significant difference in the mean number of *mazE* copy number in normal and stress condition ($p = 0.612$). However, there was a significant difference in the mean number of copies of *mazF* genes in normal and stress conditions ($p = 0.045$).
PNA results showed that mazE-PNA could affect and inhibit *A. baumannii* at different concentrations of 5, 10, and 15 µM. The results showed that at a concentration of 15 µM the bacterium was killed and confirmed by culture on LB agar (Figure 2).

DISCUSSION

Bacterial infections are currently one of the most important public health issues, including the appearance of emerging bacterial diseases, water and foodborne infections, nosocomial infections, and antibiotic resistance. According to the World Health Organization, infectious diseases account for 18% of deaths per year worldwide [6]. In 2014, the World Health Organization cited drug resistance to antibiotics as a "major global threat." The organization reported an increase in drug resistance in all parts of the world by examining statistics from 114 countries. On April 30, 2014, the United Nations released a report announcing that the world had entered a "post-antibiotic" era; Periods in which simple infections that had been treatable for many years were fatal [6]. The toxin-antitoxin system is a collection of two or

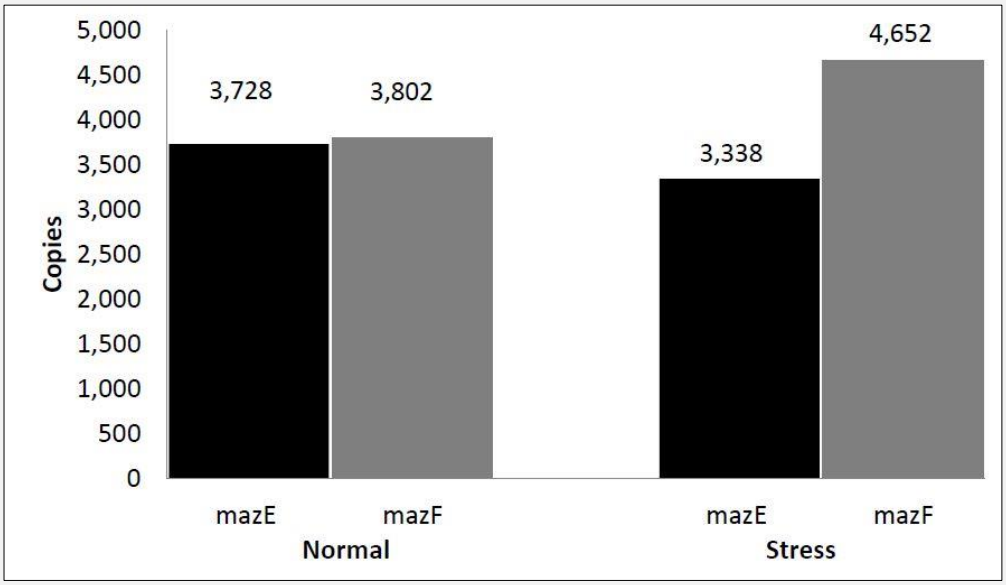

Figure 1. Mean number of *mazE* and *mazF* gene copies in normal and stress conditions. The mazF expression was meaningful in stress condition.

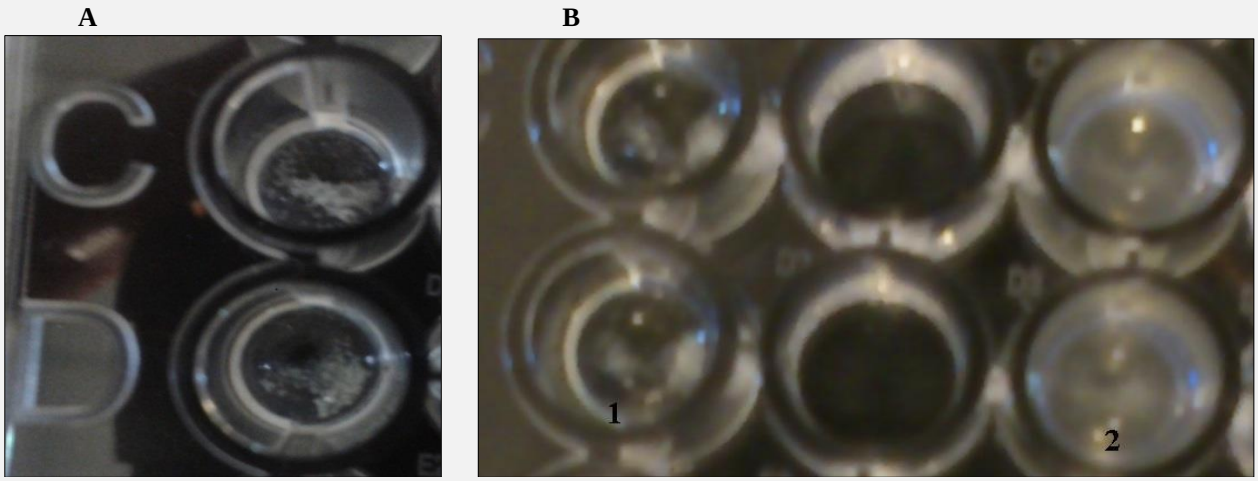

Figure 2. A) Treated *A. baumannii* containing *mazEF* with anti*mazE*-PNA after 24 hours. The white precipitate are the dead cells. The toxin caused death due to inhibition of antitoxin by PNA. The results were confirmed by sub-culture; B) lane 1: *A. baumannii* containing *mazEF* plasmid treated with PNA after 24 hours. The dead cells are the precipitate; lane 2: positive control after 24 hours of normal bacterial cell growth.

more closely related genes that together encode the "poison" and "antidote" proteins. A detailed study of the mechanisms of action of this system over the past two decades has led to some interesting conclusions about the importance of this system for bacterial physiology. TA systems contain a toxin and an antitoxin protein.

When the toxin is expressed alone, it kills the bacteria quickly [5]. In normal bacterial physiology, the antitoxin binds to the toxin and neutralizes it, thus preventing bacterial suicide. Protein toxin is always stable, while antitoxin is unstable. The unstable nature of the antitoxin makes it very sensitive to proteolytic degradation. When destructive antitoxin is inactivated, the toxin produced by the bacterium causes the death of the bacterium through a mechanism called programmed cell death [7]. Therefore, this system has recently received special attention, especially as a target for antimicrobial agents.
Considering that the expression of *mazEF* genes was examined in normal and stress states, the results show that in stress, the amount of toxin increases and other factors in the cell are also affected. The results of our study are in line with Ghafourian et al. [5] and Wang et al. [8].
One of the new ways to kill pathogenic bacteria is to turn off essential genes using antisense therapy mechanisms. It can therefore specifically inhibit the target gene. Therefore, in this study, PNA was used as one of the antisense therapy methods to turn off the target gene. The salient properties of PNA, such as its stability against proteases and nucleases, as well as its stability at high salt and temperature concentrations, make it unique compared to other analogues.

CONCLUSION

In this study, the importance of the *mazEF* toxin and antitoxin system was demonstrated as a potent new target in clinical isolates of *A. baumannii*. Inactivation of mazE antitoxin can be used as a new antimicrobial strategy. This research is the first step in introducing mazEFTA loci as a sensitive target. However, more studies are needed to test the effectiveness *in vivo*. In addition to the occurrence and potential for activation of the TA system, mazEF in other pathogenic bacteria should be further investigated.

Ethical Approval:
Ethical Committee members in Ilam University of Medical Sciences approved this study with ID NO. 949001/2.

Declaration of Interest:
The authors declare that they have no conflicts of interest.

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