Effect of mazEF, higBA and relBE toxin-antitoxin systems on antibiotic resistance in Pseudomonas aeruginosa and Staphylococcus isolates

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Introduction

Bacterial infections are increasingly prevalent due to rapid changes in the patient population and increased number of chronic diseases and immunosuppressed patients. The increase in antibiotic resistance complicates the treatment of Pseudomonas aeruginosa (P. aeruginosa), coagulase-negative Staphylococcus (CNS) and especially S. aureus infections1. S. aureus is a pathogen that can cause invasive infections such as endocarditis, osteomyelitis and sepsis as well as skin and soft tissue infections that can be colonized in humans and animals2. Although the pathogenicity of CNS is lower than that of S. aureus, it has been observed to be more frequently isolated in invasive infections in recent years. In the United States, 80,461 invasive methicillin-resistant S. aureus (MRSA) infections were reported in 2011, of which 11,285 resulted in death. In the same study, it was found that approximately 51,000 hospital-acquired P. aeruginosa infections occurred, of which 13% were due to multi drug resistance (MDR) P. aeruginosa and 400 resulted in death. Along with a reduction in the number of antibiotics that can be used in the treatment of infections, infections that cannot be controlled indicate a universal danger1.

A toxin-antitoxin (TA) system is a set of two or more closely linked genes that are encoded as a poison and a corresponding antidote on a protein. In typical bacterial physiology, an antitoxin binds to a toxin and neutralizes it, which prevents the bacterium from killing itself. TA systems can be used to develop new antibiotics3,4.

Background

A toxin-antitoxin (TA) system is a set of two or more closely linked genes that are encoded as a poison and a corresponding antidote on a protein. In typical bacterial physiology, an antitoxin binds to a toxin and neutralizes it, which prevents the bacterium from killing itself. When the antitoxin is degraded or not functional, the toxin kills the bacterium; this is known as a programmed cell death4.

TA systems are genes encoded on chromosomes and plasmids5 that can be found in both Gram-negative and Gram-positive bacteria. Studies over the past 30 years have revealed detailed information about the functions and movement mechanisms of TA systems as well as various interesting results regarding the importance of such systems for bacterial physiology7,8.

Generally, toxin molecules act as negative regulators for cell life, whereas antitoxin molecules act as positive regulators. The interaction between toxin and antitoxin gene expression levels in stressful conditions is vital for the life of the bacteria. Therefore, studies are being conducted on the possibility that TA systems can be used to develop new antibiotics9,10.

Bacteria often have more than one TA system in their genome11. The presence and type of TA systems and whether effects on antibiotic resistance ...

Methods

This study included 92 P. aeruginosa and 148 S. aureus isolates. RelBE, higBA genes were investigated in P. aeruginosa by multiplex polymerase chain reaction (PCR). The mazEF gene and the all TA genes expression were detected by real time PCR.

Results

RelBE and higBA genes were detected in 100% of P. aeruginosa. It was found that the level of relBE TA gene expression is increased in isolates sensitive to aztreonam compared to resistant isolates (p<0.05). The mazEF gene was detected in 89.1% of Staphylococcus isolates. In terms of MazEF gene expression level there was no significant difference between methicillin-sensitive Staphylococcus aureus (MSSA) and methicillin-resistant S. aureus (MRSA) isolates (p>0.05) whereas there was a significant difference between MSSA and coagulase-negative Staphylococcus (CNS) isolates, MRSA and CNS isolates (p<0.05). The levels of mazEF gene expression were found to be higher in isolates sensitive to gentamicin, ciprofloxacin, levofloxacin, clindamycin, phosphomycine, nitrofurantoin, fusidic acid, cefoxitin compared to resistant isolates (p<0.05).

Conclusion

Studies on the prevalence and functionality of TA systems emphasize that it may be possible to have new sensitive regions in bacteria by activating TA systems. The results of this study lead to the idea that resistance to antibiotics can be reduced by increasing TA gene expression levels. But there is need for further studies to support and develop this issue.

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they are encoded on a plasmid or on a chromosome varies between bacteria. To our knowledge, there is no study showing the existence of TA system in methicillin-sensitive Staphylococcus aureus (MSSA) and CNS isolates. The aim of this study was to determine whether *Pseudomonas aeruginosa* isolates from clinical specimens have relBE and higBA TA systems and whether *Staphylococcus* isolates have mazEF TA systems, and to investigate whether there is a relationship between the expression levels of TA genes and resistance to antibiotics.

**Methods**

**Ethical Information**

This study was approved by the Scientific and the Gaziomans University Clinical Research Ethics Committee (Tokat, Turkey). (16.KAEK-061/03.03.2016).

**Bacterial strains and antimicrobial susceptibility testing**

This study included 92 *P. aeruginosa* isolates from various specimens sent to the Microbiology Laboratory at Recep Tayip Erdogan University Training and Research Hospital between December 2013 and March 2015 and a total of 146 *Staphylococcus* isolates (58 MRSA, 49 MSSA and 41 CNS), isolated from various clinical samples sent to the Microbiology Laboratory at Gaziomans University between January and August 2016 as well as to the Microbiology Laboratory at Amasya Training and Research Hospital between January 2015 and August 2016. For the identification and antimicrobial susceptibility, tests were performed in accordance with the CLSI recommendations using the Vitek-2 (BioMérieux) or the BD Phoenix automated microbiology system (Becton Dickinson Diagnostic Systems, Sparks, Md.). The susceptibility of *P. aeruginosa* isolates to meropenem, imipenem, cefoperazone-sulbactam, ceftazidime, piperacillin-tazobactam, ciprofloxacin, gentamicin, tobramycin, amikacin, colistin, minocycline, trimethoprim-sulfamethoxazole, aztreonam, and teicoplanin were investigated.

**Detection of TA gene expression in *P. aeruginosa* and *Staphylococcus* isolates**

**Toxin-antitoxin systems on antibiotic resistance**

To determine whether there is a relationship between the expression levels of TA genes and resistance to antibiotics, the mazEF and relBE genes were prepared by adding 12.5 μL of reverse transcriptase (RT), to a reaction mix and 2 μL of 5 μg/mL ethidium bromide and were subsequently visualized under UV light. The primers used in multiplex PCR and real-time PCR are shown in Table 1. The gel images of relBE and higBA TA genes are given in Figure 1.

**Preparation of cDNA from total RNA in *P. aeruginosa* and *Staphylococcus* isolates**

The cDNA was prepared by adding 10 μL of water, 8 μL of reaction mix and 2 μL of reverse transcriptase (RT), to a final volume of 20 μL. The cDNA was prepared for a total of 40 minutes with the amplification steps of 5 minutes at 22°C, 30 minutes at 45°C, 5 minutes at 85°C. The identity of the cell number of the resulting cDNAs was confirmed by measuring with NanoDrop spectrophotometer. The activity of the gene region was proven by the detection of the cDNA using SYBR green dye.

**Detection of TA gene expression in *P. aeruginosa* and *Staphylococcus* isolates by real-time PCR**

The mazEF and relBE genes were prepared by adding 12.5 μL of Super SYBR Mix and for the relBE and higBA genes, 0.5 μL (++) and (−) of primer and for the mazEF gene, 0.25 μL (++) and (−) of primer, 6.5 μL of water, and 3 μL of cDNA, to obtain a total of 20 μL mix. The amplification program included 3 minutes of denaturation at 95°C and 45 cycles of 15 seconds denaturation at 95°C for the RelBE primer binding at 52°C for 45 seconds, and for the MazEF primer binding at 54°C for 50 seconds and then elongation at 72°C for 30 seconds, followed by a final elongation step by increasing from 60°C to 90°C.

**Table 1: Primers used for detection of TA genes and analysis of real time PCR in *P. aeruginosa* and *Staphylococcus* clinical isolates.**

| Primer name | Primer sequence (5'-3') | Expected fragment (bp) | Application Reference |
|-------------|------------------------|------------------------|-----------------------|
| mazEF F     | ATACGATCGAGGCTGCTAGT   | 408                   |                      |
| mazEF R     | AGGGAGATTTCCAAATTGCTGA| 408                   |                      |
| relBE F     | CAGGGGTATTTCCAGCTCTC  | 505                   |                      |
| relBE R     | ATGACGGCTAGTCTGCTTC   | 505                   |                      |
| higBA F     | CTCATGTCGACTGTCGTC    | 469                   |                      |
| higBA R     | GATGGCTGGGATGAGTTCTG  | 469                   |                      |

**Table 2: Distribution of clinical specimens of *P. aeruginosa* and *Staphylococcus* isolates.**

| Microorganism | *P. aeruginosa* | *Staphylococcus* isolates |
|---------------|------------------|-------------------------|
| Sample type   | n / %            | n / %                   |
| Endotracheal  | 34 / 37          | 4 / 8.3                 |
| Bacteriuria   | 23 / 25          | 87 / 58.8               |
| Wound         | 10 / 10.8        | 24 / 16.2               |
| Tissue        | 9 / 9.7          |                         |
| Joint fluid   | 5 / 5.4          |                         |
| Conunctiva    | 4 / 4.3          | 1 / 0.7                 |
| Cerebrospinal fluid | 3 / 3.3 | 6 / 4                  |
| Spumut        | 15 / 10.1        |                         |

**Figure 1: Multiplex PCR images of *P. aeruginosa* relBE and higBA TA genes.**

**Figure 2: Genomic DNA isolation of *P. aeruginosa* by multiplex PCR.**

The prepared 30± 5 ng/ µL bacterial suspension was centrifuged at 12,500 g for 5 minutes. Then, 200 μL of lysozyme was added onto the pellet and incubated at 37°C for 30 minutes. The activity of the gene region was proven by the detection of the cDNA using SYBR green dye.
### Table 4: Antibiotic resistance status and mazEF CT values of Staphylococcus isolates

| Factors          | mazEF CT Values | p-value
|------------------|-----------------|---------|
|                  | Median[Q1,Q3]   |         |
| Group            | MSSA            | MRSA    | CNs    |
|                  | 23.35±6.42      | 21.37±8.05 | 32.04±6.63 |
| Penicillin       | Sensitive 60%   | Resistant 22.81±6.88 | <0.001 |
| Gentamicin       | Sensitive 96%   | Resistant 27.19±7.68 |         |
| Ciprofloxacin    | Sensitive 88%   | Resistant 23.3±5.42 |         |
| Levofloxacin     | Sensitive 101%  | Resistant 27.17±6.81 |         |
| Erythromycin     | Sensitive 91%   | Resistant 22.81±6.88 |         |
| Clindamycin      | Sensitive 91%   | Resistant 23.75±6.69 |         |
| Linezolid        | Sensitive 102%  | Resistant 23.82±6.35 |         |
| Daptomycin       | Sensitive 132%  | Resistant 27.72±6.92 |         |
| Teicoplanin      | Sensitive 132%  | Resistant 27.72±6.92 |         |
| Vancomycin       | Sensitive 132%  | Resistant 27.72±6.92 |         |
| Tetracycline     | Sensitive 87%   | Resistant 24.83±6.72 |         |
| Fusidic acid     | Sensitive 97%   | Resistant 24.94±6.48 |         |
| Trimethoprim     | Sensitive 110%  | Resistant 24.86±6.86 |         |
| Sulfamethoxazole | Sensitive 48%   | Resistant 24.15±7.15 |         |
| Cefotaxime       | Sensitive 85%   | Resistant 25.56±7.51 |         |
| Inducible        | Sensitive 87%   | Resistant 25.96±7.15 | <0.002* |
| clindamycin      | Sensitive 85%   | Resistant 35.96±8.36 |         |

**Discussion**

Bacteria often have more than one TA system in their genome. The presence and type of TA systems and whether they are encoded on a plasmid or on a chromosome varies between bacteria. TA systems are thought to be conserved on chromosomes in the *P. aeruginosa* genome. Studies have also revealed the existence of new TA systems. The genes of seven known TA gene families for *S. aureus* have been identified, with the majority of the investigations involving the mazEF TA gene. The mazEF, an operon that is called a "plasmid addiction system" and which stabilizes plasmids, was first identified on the chromosome of *E. coli* in 1993. MazEF is the most studied TA system in *E. coli* and has been reported to be an irreversible mediator in cell death in stress conditions as well as a modulator in the translation process.

RelBE is also one of the most studied TA systems in *E. coli*. It modulates the response induced in the case of amino acid starvation, which leads to the inhibition of translation and consequently to bacteriocin. In the literature, *E. coli* appear to be the most frequently investigated bacteria for TA systems. MazEF has been first described on *Paenibacillus* and it has been shown that TA systems can be encoded on the chromosome of *P. aeruginosa* and *S. aureus* isolated from clinical samples collected from three centers and that 50% of the 78 MRSA isolates had higBA and relBE whereas 30% had parDE and all 42 *P. aeruginosa* isolates had relBE and higBA TA genes. The authors of the study confirmed the PCR products by DNA sequencing, without the result that 100% of the PCR products were compatible with the DNA sequencing. In addition, they also emphasized that these genes are transcribed and that the activation of toxin genes would be an effective antibacterial strategy. The presence of MazEF genes in bacterial DNA was first identified on the chromosome of *P. aeruginosa* and *S. aureus* isolated from clinical specimens were found to have relBE and parDE TA genes at a rate of 100%, 100% and 50%, respectively in *P. aeruginosa*. Another study conducted in Iran by Hemati et al. showed that *P. aeruginosa* isolates had relBE TA genes at a rate of 85.7% and that the isolates were resistant to gentamicin (65%), meropenem (60%), piperacillin (59.28%), fusidic acid and amikacin (52.14%). The authors found a correlation between mazEF gene resistance and the type of resistance to gentamicin, meropenem, piperacillin and amikacin.

In this study, higBA and relBE genes were found in all (100%) of *P. aeruginosa* isolates, while the mazEF gene was found in 89.1% of the *Staphylococcus* isolates. The results of the study are consistent with the literature and show that TA genes are present at high rates in *P. aeruginosa* and *Staphylococcus* isolates, the levels of mazEF gene expression were found to be higher in isolates sensitive to aztreonam compared to resistant isolates (p<0.05). In *P. aeruginosa* isolates, in contrast to the study by Hemati et al. the relBE TA gene expression was found to be increased in isolates sensitive to aztreonam compared to resistant isolates (p<0.05). It has been shown that *P. aeruginosa* isolates, in contrast to the study by Hemati et al., the relBE TA gene expression was found to be increased in isolates sensitive to aztreonam compared to resistant isolates (p<0.05). It has been shown that *P. aeruginosa* isolates, in contrast to the study by Hemati et al., the relBE TA gene expression was found to be increased in isolates sensitive to aztreonam compared to resistant isolates (p<0.05).

**Conclusion**

Studies on the prevalence and functionality of TA systems emphasize that it may be possible to have new resistant bacteria by activating TA systems. The results of this study lead to the idea that resistance to antibiotics can be reduced by increasing TA gene expression levels. But there is need for further studies to support and develop this issue.

**Acknowledgements**

This work was supported by Gaziogranpa University Research Grants (GAP-2015/25).

**Conflict of interests**

All authors declare that they have no competing interests related to this reference.
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