ORIGINAL RESEARCH

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

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Abstract

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. We aimed to determine whether *P.aeruginosa* and *Staphylococcus* isolates have TA genes and to investigate whether there is a relationship between the expression levels of TA genes and resistance to antibiotics.

Methods

This study included 92 *P. aeruginosa* and 148 *Staphylococcus* 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.

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* infections[1].

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 animals[2]. 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 danger[3].

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 death[4].

TA systems are genes encoded on chromosomes and plasmids[5] that can be found in both Gram-negative and Gram-positive bacteria[6]. 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 physiology[7,8].

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 antibiotics[9-11].

Bacteria often have more than one TA system in their genome[12]. The presence and type of TA systems and whether

they are encoded on a plasmid or on a chromosome varies between bacteria[9].

To our knowledge, there is no study showing the existence of TA systems in methicillin-sensitive *Staphylococcus aureus* (MSSA) and CNS isolates. The aim of this study was to determine whether *P.aeruginosa* isolates isolated 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 Ethical Committee of the Gaziosmanpasa University Clinical Research Ethics Committee (Tokat, Turkey), (16-KAEK-061/03.03.2016).

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

Primer name	5'-3' Sequence	Expected Fragment, bp	Application Reference
mazEF			
	F-ATCATCGGATAAGTACGTCAGTTT	408	18
	R-AGAAGGATATTCACAAATGCTGA		
relBE			
	F-CAGGGGGTAATTTCGACTCTG	505	18
	R- ATGAGCACCGTAGTCTCGTTC		
higBA			
	F-CTCATGTTCGATCTGCTTGC	469	18

Bacterial strains and antimicrobial susceptibility testing

This study included 92 *P. aeruginosa* isolates from various specimens sent to the Microbiology Laboratory at Recep Tayyip Erdogan University Training and Research Hospital between December 2013 and March 2015 and a total of 148 *Staphylococcus* isolates (58 MRSA, 49 MSSA and 41 CNS), isolated from various clinical samples sent to the Microbiology Laboratory at Gaziosmanpasa 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.)13. The susceptibility of P. aeruginosa isolates to meropenem, imipenem, cefoperazone-sulbactam, ceftazidime, piperacillin-tazobactam, ciprofloxacin, cefepime, aztreonam and the susceptibility of *Staphylococcal* isolates to penicillin, cefoxitin, gentamicin, erythromycin, clindamycin, linezolid, daptomycin, teicoplanin, vancomycin, ciprofloxacin, levofloxacin, tetracycline, fusidic acid, and trimethoprim-sulfamethoxazole were investigated. Isolates with moderate sensitivity were considered resistant. *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 were used as quality control strains. Stock cultures were stored at -80ºC in Luria Broth (LB)14 or bead stock medium.

Genomic isolation of relBE and higBA genes from P. aeruginosa isolates by multiplex PCR

RelBE, higBA genes were investigated in *P.aeruginosa* strains by multiplex polymerase chain reaction (PCR). A total of 1.5 ml (30± 5 ng/ µL) of bacterial cultures prepared for mold DNA isolation was placed into a 1.5 mL microcentrifuge tube and precipitated for 5 minutes at 13.000 xg. The upper part of the centrifuged liquid was discarded and washed with sterile distilled water. PCRs were performed on a final volume of 50 µL and included 5 µL of genomic DNA, 20 pM of each primer, 10 µL reaction buffer (Promega), 3 µL 25 mM MgCl2, 200 µM of each dNTP and 1.5 U of Taq Polymerase (Promega, USA). PCR amplification conditions were as follows: initial denaturation at 94°C for 3 minutes followed by 30 cycles of 25 seconds at 94°C, 40 seconds at 52°C and 50 seconds at 72 °C with a final extension of 5 minutes at 72°C. All PCR results were analyzed on 1% agarose containing 0.5 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).

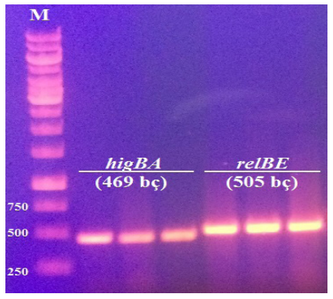

Figure 1: Multiplex PCR images of *P. aeruginosa* relBE and higBa TA genes

Genomic DNA isolation of mazEF gene from Staphylococcus isolates by real-time PCR

The prepared 30± 5 ng/ µL bacterial suspension was centrifuged at 12.500 xg for 5 minutes. Then, 200 µL of lysozyme was added onto the pellet and incubated at 37ºC for 30 minutes. For the degradation of RNA, 4 µL RNase A (50mg/ml) was added to the sample and was vortexed for 10 minutes at room temperature. Then 40 µL proteinase K was added and the DNA isolation was completed according to the supplier's recommendation (Anatolia Geneworks Turkey).

Total RNA isolation from P. aeruginosa and Staphylococcus isolates

The prepared 30± 5 ng/ µL bacterial suspension was centrifuged at 12.500 xg for 5 minutes. Then 200 µL of RB buffer (RB buffer, β-Mercaptoethanol) was added to the pellet and RNA isolation was performed according to the supplier's recommendation. In addition, 20 µL of 10X Reaction mix, 7.5 µL of DNase I and 172.5 µL of water were added for degradation of the genomic DNA during the protocol and pure RNA was obtained.

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

Microorganism	*P. aeruginosa*	*Staphylococcus* isolates
Sample type	**n / %**	**n / %**
Endotracheal aspirate	34 / 37	4 / 8.3
Blood	23 / 25	87 / 58.8
Wound	10 / 10.8	24 / 16.2
Bronchoalveolar lavage	9 / 9.7	-
Pleural effusion	5 / 5.4	-
Catheter	4 / 4.3	1 / 0.7
Urine	3 / 3.3	6 / 4
Abscess	3 / 3,3	3 / 2
Tissue	1 / 1.1	-
Joint fluid	-	1 / 0.7
Conjunctiva	-	1 / 0.7
Cerebrospinal fluid	-	6 / 4
Sputum	-	15 / 10.1

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 42°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 56°C for 45 seconds, for the HigBA primer binding at 52°C for 45 seconds, and for the MazEF primer binding at 54°C for 30 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 3: Antibiotic resistance rates of P. aeruginosa and Staphylococcus isolates.

P. aeruginosa		Staphylococcus isolates	
Antibiotic	**n / %**	**Antibiotic**	**n / %**
Meropenem	59 / 67	Penicillin	126 / 95.5
Imipenem	52 / 59.8	Sefoksitin	84 / 63.6
Cefoperazone-sulbactam	36 / 42.9	Gentamicin	36 / 27.3
Ceftazidime	20 / 23.8	Erythromycin	101 / 7.6
Piperacillin-tazobactam	19 / 21.8	Clindamycin	41 / 31
Ciprofloxacin	19 / 21.8	Linezolid	0
Cefepime	14 / 15.9	Daptomycin	0
Aztreonam	12 / 13.6	Teicoplanin	0
		Vancomycin	0
		Ciprofloxacin	44/33.3
		Levofloxacin	31/23.5
		Tetracycline	45/34
		Fusidic acid	35/26.5
		Trimethoprim-sulfamethoxazole	22/16.6

Statistical Method

In study, Shapiro-Wilk's test was used to assess the data normality. When the data of the *Staphylococcus* isolates were evaluated, independent samples t test was used to compare the normally distributed independent variables between two groups and Mann-Whitney U test was used to compare the non normally distributed independent variables between two groups. One-way ANOVA test was used in three groups comparisons. For multiple comparisons, the Tukey HSD test was used. The difference between resistance to antibiotics in *P. aeruginosa* isolates and transcription levels of TA genes was investigated with an independent samples t-test. The statistical significance level of p was 0.05. Statistical analysis was performed using commercial software (IBM SPSS Statistics 20, SPSS Inc., an IBM Co., Somers, NY).

Results

P. aeruginosa and isolates were identified in respiratory tract specimens (36 endotracheal aspirates and 9 bronchoalveolar lavages) at a rate of 46.7%, whereas *Staphylococcus* isolates were most frequently identified in blood culture samples at a rate of 58.8%. The sample types in which the isolates were identified are shown in Table 2. In the *P. aeruginosa* isolates, aztreonam was identified as the most sensitive antibiotic type, whereas the highest resistance rates were detected against carbapenems, at a rate of 67% and 59.8% for meropenem and imipenem, respectively. The antibiotics

Table 4: Antibiotic resistance status and mazEF cT values of Staphylococcus isolates

Factors		MazEF Ct Values		p
n		Mean±SD		
Group	MSSA	48	23.3±5.42	<0.001
	MRSA	51	21.37±5.85	
	CNS	33	32.04±4.63	
Penicillin	Sensitive	6	25.9±6.24	0.751*
	Resistant	126	24.68±6.94	
Gentamicin	Sensitive	96	23.98±6.44	0.038
	Resistant	36	26.76±7.7	
Ciprofloxacin	Sensitive	88	23.51±6.14	0.007
	Resistant	44	27.19±7.68	
Levofloxacin	Sensitive	101	23.82±6.35	0.015
	Resistant	31	27.71±7.81	
Erythromycin	Sensitive	31	22.81±6.88	0.074
	Resistant	101	25.33±6.82	
Clindamycin	Sensitive	91	23.75±6.69	0.013
	Resistant	41	26.93±6.9	
Linezolid	Sensitive	132	24.67±6.87	-
	Resistant	0		
Daptomycin	Sensitive	132	24.74±6.89	-
	Resistant	0	-	
Teicoplanin	Sensitive	132	24.73±6.92	-
	Resistant	0		
Vancomycin	Sensitive	132	24.73±6.92	-
	Resistant	0		
Tetracycline	Sensitive	87	24.63±7.12	0.807
	Resistant	45	24.94±6.48	
Fusidic acid	Sensitive	97	23.81±6.42	0.010
	Resistant	35	27.3±7.56	
Trimethoprim-sulfamethoxazole	Sensitive	110	24.86±6.86	0.662
	Resistant	22	24.15±7.15	
Cefoxitin	Sensitive	48	23.3±5.42	0.048
	Resistant	84	25.56±7.51	
Inducible clindamycin-resistance	Sensitive	87	25.57±6.15	0.002*
	Resistant	6	35.96±6.36	

MSSA: Methicillin-sensitive *Staphylococcus aureus*; **MRSA:** Methicillin-resistant *S. Aureus*; **CNS:** Coagulase negative staphylococcus,

most resistant against *Staphylococcus* isolates were penicillin (95.5%) and erythromycin (76.5%). Resistance rates against antibiotics are shown in Table 3. HigBA and relBE genes were detected in 100% of the isolates (n=92). For each screened gene, the sample was displayed by running on the agarose gel (Figure 1). The mazEF gene was detected in 132 (89.1%) of 148 *Staphylococcus* isolates. The resistance rates of isolates to antibiotics and the average cycle threshold (Ct) values for the mazEF gene are shown in Table 4. The mean Ct values were 23.9 ± 8.8 for the relBE gene and 18.24 ± 5.8 for the higBA gene. The RelBE TA gene expression level was found to be increased in isolates sensitive to aztreonam compared to resistant isolates ($p<0.05$). The distribution of MazEF values by groups is shown in Figure 2. In terms of MazEF gene expression levels, there was no significant difference between MSSA and MRSA isolates ($p=0.181$), whereas there was significant difference between the MSSA and CNS isolates ($p<0.001$) and 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, nitrofurantoin, fusidic acid and cefoxitin compared to resistant isolates ($p<0.05$). The level of gene expression was found to be higher in isolates without inducible clindamycin-resistance compared to thoswith inducible clindamycin resistance ($p<0.05$).

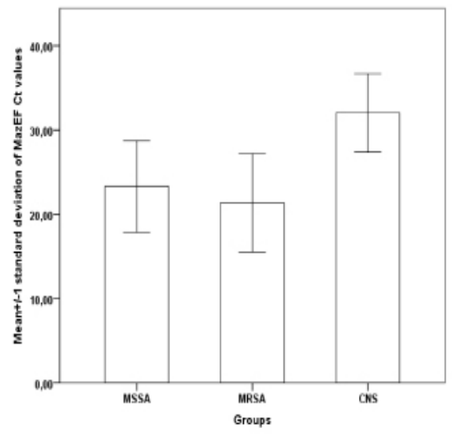

Figure 2: The distribution of MazEF values by groups in Staphylococcus isolates

Discussion

Bacteria often have more than one TA system in their genome[12]. The presence and type of TA systems and whether they are encoded on a plasmid or on a chromosome varies between bacteria[4]. TA systems are thought to be encoded on chromosomes in the *P. aeruginosa*[15,16]. Based on bioinformatic data, four TA systems were identified on P. aeruginosa, including PAQ1, relBE, higBA, and parDE[17,18]. However, a study by Gang Li et al. in 2016 identified a new TA gene named hibAB in the P. aeruginosa[19]. The 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[17,18]. 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[20] as well as a modulator in the translation process[21,22].

RelBE is also one of the most studied TA systems in *E. coli*[20]. It modulates the response induced in the case of amino acid starvation[22], which leads to the inhibition of translation and consequently to bacteriosis[21]. In the literature, *E. coli* appears to be the most frequently investigated bacterium for TA systems. HigBA was first described on *Proteus vulgaris*[23]. In a study conducted in the United States, it was reported that TA genes were encoded on the chromosome in *P. aeruginosa* and *S. aureus* isolated from clinical samples collected from three centers and that 100% 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 authers of the study confirmed the PCR products by DNA sequencing, with the result that 97.8% to 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[15]. In a study conducted in 2016, 174 *P. aeruginosa* isolates isolated from clinical specimens were found to have relBE, higBA and parDE TA genes at a rate of 100%, 100% and 30%, respectively in Iran[16]. Another study conducted in Iran by Hemati et al. showed that *P. aeruginosa* isolates had mazEF TA genes at a rate of 85.7% and that the isolates were resistant to gentamicin (65%), meropenem (60%), piperacillin (59.28%), and amikacin (52.14%). The authors found a correlation between mazEF genes and resistance to gentamicin, meropenem, piperacillin and amikacin[24]. 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. In Staphylococcus isolates, the levels of mazEF gene expression were found to be higher in isolates sensitive to gentamicin, ciprofloxacin, levofloxacin, clindamycin, phosphomycin, nitrofurantoin, fusidic acid and cefoxitin compared to resistant isolates ($p<0.05$). In *P. aeruginosa* isolates, in contrast to the study by Hemati et al.[24], the relBE TA gene expression level was found to be increased in isolates sensitive to aztreonam compared to resistant isolates ($p<0.05$). For *Staphylococcus* isolates, the existence of TA systems has been proven only in MRSA isolates, while these systems remained unexplored in CNS and MSSA isolates. In this study, we evaluated the presence of TA systems in MSSA and CNS and observed the transcription of the mazEF TA gene in MRSA and MSSA isolates is higher than in CNS ($p<0.05$). This suggests that the mazEF TA gene may be related to virulence in *S. aureus*. To our knowledge, this is the first study investigating TA genes in the CNS. The determination of the prevalence of TA genes in strains with less virulence will also contribute to the studies investigating the possibility that TA systems are antibacterial targets. Bukowski et al.[25] suggests that TA systems induce bacteriosis, which in the long run result in the death of bacterial cells. The results of Bukowski et al.[25] and Hemati et al.[24] also point out that drugs that can activate silent toxins in TA systems can be used as antibiotics. The present study is consistent with the results of Bukowski et al.[25] and Hemati et al.[24] in terms of the finding that isolates with higher mazEF gene expression levels were more sensitive to gentamicin, ciprofloxacin, levofloxacin, clindamycin, phosphomycin, nitrofurantoin, fusidic acid and cefoxitin. In the present study, the toxin and antitoxin genes were evaluated together because they were located on the same operon. Bacteria were not exposed to any stress factors, such as heat, amino acid starvation, or antibiotic exposure. We investigated only the presence of these TA systems and the association between their presence and antibiotic resistance. Only the statistical determination of these data constitutes a limitation of this study. The studies on the prevalence and functionality of TA systems emphasizes that it may be possible to have new sensitive regions in bacteria by activating TA systems (by degrading antitoxins or increasing toxin expression)[6,18]. As a matter of fact the results of this study lead to the idea that resistance to antibiotics can be reduced by increasing TA gene expression levels. The most important step at this stage is to investigate the presence and functionality of TA genes in microorganisms that cause infections. Therefore, there is require to investigate the presence and frequency of TA genes in bacteria to support and develop this strategy and to determine which strategies are suitable for this target. Our study is the first study in Turkey investigating TA systems in *P. aeruginosa* and *Staphylococcus* isolates isolated from clinical specimens.

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.

Acknowledgements

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Conflict of interests

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

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