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Evaluation of toxin-antitoxin genes, antibiotic resistance, and virulence genes in *Pseudomonas aeruginosa* isolates

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SUMMARY

OBJECTIVE: Toxin-antitoxin genes *RelBE* and *HigBA* are known to be involved in the formation of biofilm, which is an important virulence factor for *Pseudomonas aeruginosa*. The purpose of this study was to determine the presence of toxin-antitoxin genes and *exoenzyme S* and *exotoxin A* virulence genes in *P. aeruginosa* isolates and whether there is a relationship between toxin-antitoxin genes and virulence genes as well as antibiotic resistance.

METHODS: Identification of the isolates and antibiotic susceptibilities was determined by a VITEK 2 (bioMérieux, France) automated system. The presence of toxin-antitoxin genes, virulence genes, and transcription levels were detected by real-time polymerase chain reaction.

RESULTS: *RelBE* and *HigBA* genes were detected in 94.3% (82/87) of *P. aeruginosa* isolates, and *exoenzyme S* and *exotoxin A* genes were detected in all of the isolates (n=87). All of the isolates that harbor the toxin-antitoxin and virulence genes were transcribed. There was a significant increase in the *RelBE* gene transcription level in imipenem- and meropenem-sensitive isolates and in the *HigBA* gene transcription level in amikacin-sensitive isolates ($p<0.05$). There was a significant correlation between *RelBE* and *exoenzyme S* ($p=0.001$).

CONCLUSION: The findings suggest that antibiotic resistance may be linked to toxin-antitoxin genes. Furthermore, the relationship between *RelBE* and *exoenzyme S* indicates that toxin-antitoxin genes in *P. aeruginosa* isolates are not only related to antibiotic resistance but also play an influential role in bacterial virulence. Larger collections of comprehensive studies on this subject are required. These studies should contribute significantly to the solution of the antibiotic resistance problem.

KEYWORDS: *Pseudomonas aeruginosa.* Toxin-antitoxin systems. Virulence. Anti-bacterial Agents.

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the major pathogens causing hospital-acquired infections, particularly affecting patients with immunocompromised or prolonged stay in the intensive care unit[1]. As a pathogen, *P. aeruginosa* is of growing clinical significance as a result of its inherent resistance to multiple antimicrobials and its ability to develop high-level multidrug resistance (MDR) due to the presence of a lot of virulence factors expressed in its genome[2].

These virulence factors allow *P. aeruginosa* to easily reproduce and live in both the host cell and the environment. Virulence factors can cause a number of harmful effects, including damage to tissues, the spread of infection to blood and tissue, the escape of bacteria from the host cell defense, and disease progression. In addition, they can induce antibiotic resistance in *P. aeruginosa*, making treatment difficult[3].

In recent years, studies on toxin-antitoxin (TA) genes have shown that they are associated with virulence regulation, biofilm formation, plasmid maintenance, and antibiotic resistance[4-6]. TA genes are small operons composed of both a growth-inhibitory toxin and an antitoxin that regulates toxin activity by direct inhibition. This antitoxin also plays a role in cell physiology by acting as a regulator of transcription[7-9]. Previous studies have shown that TA genes play several important physiological roles and, therefore, may be able to treat infections caused by MDR bacteria[6].

It has been indicated that TA genes are involved in the formation of biofilm, which is an important virulence factor for *P. aeruginosa*[10-12]. The purpose of this study was to determine the presence of *RelBE* and *HigBA* TA genes and *exoenzyme S* (*ExoS*) and *exotoxin A* (*ToxA*) virulence genes in *P. aeruginosa* isolates. In addition, we aimed to investigate

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whether there is a relationship between TA genes and virulence genes as well as whether TA genes are associated with antibiotic resistance.

METHODS

Bacterial isolates and antimicrobial susceptibility testing

This study included 87 *P. aeruginosa* isolates from various samples sent to the Microbiology Laboratory at Tokat Gaziosmanpasa University Training and Research Hospital between January 2016 and March 2017. Identification and antibiotic resistance profile of *P. aeruginosa* isolates were determined by a Vitek 2 (bioMérieux, France) automated system according to Clinical and Laboratory Standards Institute criteria[13]. The total number of susceptible and resistant antibiotics is not equal to all antibiotics because not all antibiotics have been studied in every isolate. Only one isolate was obtained from each patient. *P. aeruginosa* ATCC 27853 isolates were used as quality controls.

Genomic DNA isolation of *RelBE*, *HigBA*, *ExoS*, and *ToxA* genes in *Pseudomonas aeruginosa* isolates by real-time polymerase chain reaction

A volume of 10^5 McFarland bacterial suspension (1.5 µL) was centrifuged at 12,500 × *g* for 5 min. Then, 200 µL of lysozyme was added to the pellet and incubated at 37°C for 30 min. To degrade the RNA, 4 µL of RNase A (50 mg/mL) was added to the sample, which was vortexed for 10 min at room temperature. In addition, 40 µL of proteinase K was added, and the DNA isolation was completed according to the manufacturer's recommendations using a Magnesia 16 isolation device (Anatolia Geneworks, Turkey).

Total RNA isolation from *Pseudomonas aeruginosa* isolates

The prepared bacterial suspension (1.5 µL) was centrifuged at 12,500 × *g* for 5 min and 200 µL of RB buffer prepared with mercaptoethanol was added to the pellet, and RNA isolation was performed by using the Magnesia 16 Cultured Cell and Tissue Total RNA Extraction Kit. To degrade the genomic DNA, 20 µL of 10´ reaction mix, 7.5 µL of DNase I, and 172.5 µL of water were added to each sample, and pure RNA was obtained. RNA isolation was performed using a Magnesia 16 isolation device (Anatolia Geneworks, Turkey).

Preparation of cDNA from total RNA in *Pseudomonas aeruginosa* isolates

The cDNA mixture was prepared by adding 10 µL of water, 8 µL of the reaction mix, and 2 µL of reverse transcriptase (RT) to the final volume of 20 µL. The cDNA was prepared using a Montania 4896 real-time PCR device (Anatolia Geneworks, Turkey) for a total of 40 min as follows: 5 min at 22°C, 30 min at 42°C, and 5 min at 85°C. The activity of the gene region was proven by the detection of the cDNA using SYBR green dye.

Detection of *RelBE*, *HigBA*, *ExoS*, and *ToxA* genes expression in *Pseudomonas aeruginosa* by real-time polymerase chain reaction

All of the genes were prepared by mixing 12.5 µL of Super SYBR Mix, 0.5 µL of forward and reverse primers, 6.5 µL of water, and 3 µL of cDNA for a total volume of 20 µL. The gene expression levels were detected by a Montana 4896 real-time PCR device (Anatolia Geneworks, Turkey).

The amplification programs for *RelBE*, *HigBA*, *ExoS*, and *ToxA* were as follows: 3 min of denaturation at 95°C and 45 cycles of 15-s denaturation at 95°C; for the *RelBE* primer, binding at 56°C for 45 s; elongation at 72°C for 30 s, followed by a final elongation step in which the temperature was increased from 60 to 90°C; for the *HigBA*, *ExoS*, and *ToxA* primers, binding at 52°C for 45 s; elongation at 72°C for 30 s, followed by a final elongation step in which the temperature was increased from 60 to 90°C. The primers were used in the PCR step according to the previous study[12,14].

Statistical analysis

Statistical analysis was performed by using commercial software IBM SPSS Statistics version 20 (SPSS Inc., an IBM Corp., Somers, NY, USA). The differences between antibiotic resistance in the *P. aeruginosa* isolates and the transcription levels of the TA and virulence genes were investigated with independent samples t-test and Mann-Whitney U test. The relationship between *RelBE* and *HigBA* genes and *ExoS* and *ToxA* genes was investigated with the Pearson's correlation test. The values of $p \leq 0.05$ were considered significant.

Ethics

This study was approved by the Ethics Committee of Tokat Gaziosmanpasa University (number 17/KAEK/022).

RESULTS

The isolates were obtained from respiratory samples (40.3%, n=35), wound samples (26.4%, n=23), urine (20.7%, n=18),

blood (11.5%, n=10), and sterile body fluid samples (1.1%, n=1). The *P. aeruginosa* isolates had the highest rates of antibiotic resistance to aztreonam 64.4% (47/73), piperacillin-tazobactam 64% (55/86), imipenem 42.7% (35/82), and meropenem 36.8% (32/87). While the *RelBE* and *HigBA* genes were detected in 94.3% (82/87) of the *P. aeruginosa* isolates (n=87), the *ExoS* and *ToxA* genes were detected in all of the isolates (n=87). It is shown that TA genes (82/82) and virulence genes (87/87) are involved.

There was a significant increase in the *RelBE* gene transcription level in imipenem- and meropenem-sensitive isolates ($p<0.05$). There were no correlations between *RelBE* and *HigBA* gene transcription levels with any of the other antibiotics ($p>0.05$). Antibiotic susceptibility rates of isolates and the relationship between antibiotic susceptibilities and transcription levels of *RelBE* and *HigBA* TA genes are shown in Table 1. There was a significant correlation between *RelBE* and *ExoS* (p=0.001); none of the other correlations were significant.

DISCUSSION

The MDR *P. aeruginosa* caused 32,600 estimated infections among hospitalized patients and 2,700 estimated deaths in the United States. Some types of MDR *P. aeruginosa* are resistant to nearly all antibiotics, including carbapenems, which means that several classes of antibiotics including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems may not cure these infections[1]. In recent years, the increase in carbapenem-resistant frequency among *P. aeruginosa* is becoming a major challenge. The reported rates of carbapenem resistance seem to be considerably different (12–67%) in various regions[15-17]. In this study, it was observed that the carbapenem's resistance rates were consistent with the previous studies. It is seen that carbapenems still maintain their importance among the antibiotics used in the treatment of *P. aeruginosa* infections.

Pseudomonas aeruginosa have virulence factors including biofilm, *ToxA*, *ExoS*, pigments, mucoid exopolysaccharide, lipopolysaccharide, protease, leucocidin, and hemolysins. *ToxA* is secreted outside the cell and causes cell death and cell damage as well as suppression of host response by inhibiting protein synthesis[18]. Nikbin et al. indicated *ExoS* and *ToxA* genes existed in wound samples at rates of 62 and 90% and in respiratory system at rates of 47.4 and 46.6%, respectively. They indicated that the prevalence of *Tox*A gene was significantly higher in the pulmonary tract and burn isolates. In addition, the difference between *ExoS* prevalence in isolates from the pulmonary tract and burn isolates was statistically significant[19].

Table 1. Antibiotic susceptibility rates of isolates and the relationship between antibiotic susceptibilities and expression levels of *RelBE* and *HigBA* Toxin-antitoxin genes.

Antibiotic		n	%	*RelBE* gene p	*HigBA* gene p
Piperacillin-tazobactam	Sensitive	31	36	0.436[a]	0.723[a]
Seftazidime	Sensitive	62	71.3	0.154[a]	0.636[a]
Sefepime	Sensitive	55	66.3	0.331[a]	0.431[a]
Aztreonam	Sensitive	26	35.6	0.449[a]	0.664[a]
Imipenem	Sensitive	47	57.3	**0.002**[a]	0.365[a]
Meropenem	Sensitive	55	63.2	**0.043**[a]	0.367[a]
Amikacin	Sensitive	80	92.0	0.627[a]	0.050[a]
Gentamycin	Sensitive	75	86.2	0.756[a]	0.181[a]
Netilmicin	Sensitive	64	87.7	0.848[a]	0.228[a]
Tobramycin	Sensitive	67	93.1	0.406[b]	0.086[b]
Siprofloxacin	Sensitive	67	77.9	0.251[a]	0.144[a]
Levofloxacin	Sensitive	52	72.2	0.236[a]	0.323[a]
Colistine	Sensitive	82	100	-	-

TA: toxin-antitoxin. The total number of susceptible and resistant antibiotics is not equal to all antibiotics because not all antibiotics have been studied in every isolate. Differences in antibiotic resistance in *P. aeruginosa* isolates and transcription levels of TA genes were investigated with the independent samples t-test and Mann-Whitney U test. The relationship between *RelBE* and *HigBA* genes and *ExoS* and *ToxA* genes was investigated with a Pearson's correlation test. [a]Independent samples t-test. [b]Mann-Whitney U-test. Bold indicates statistically significant p-value.

Faraji et al. in 2016 reported that *ExoS* and *ToxA* genes were detected in cystic fibrosis isolates at rates of 70.8 and 63.1%, respectively[20]. Wolska et al. indicated that the *ExoS* gene was present in 78.5% of 62 *P. aeruginosa* isolates, while the *ToxA* gene was found in 88.7%[14]. In this study, all the *P. aeruginosa* isolates had *ExoS* and *ToxA* genes. Therefore, the statistical distribution of virulence genes according to the samples has not been studied. However, most isolates were isolated from respiratory tract samples. The results of this study determined that *ExoS* and *ToxA* virulence factors are found at high rates in *P. aeruginosa* isolates, which is consistent with previous studies.

Hemati et al. observed biofilm formation in 87.5% of 140 *P. aeruginosa* isolates; furthermore, the TA genes *MazEF*, *RelBE*, *HigBA*, *CcdAB*, and *MqsR* were found at rates of 85.71, 100, 1.42, 100, and 57.14%, respectively. In addition, they reported a relationship between biofilm formation and TA gene expression[9]. In 2016, Wood et al. detected the *HigBA* gene in *P. aeruginosa* PA14 isolate and investigated the biofilm formation by using crystal violet, pyocyanin production by using acetic acid, and dichloromethane and pyoverdine production by using chrome azurol S agar plate method. They indicated that the *HigBA* TA gene is effective not only on biofilm formation but also on pyoverdine production[10].

Previous studies have determined that *P. aeruginosa* isolates have *RelBE* and *HigBA* TA genes[9,13,21,22]. Guo et al. demonstrated the antitoxin *HigA* regulates virulence in *P. Aeruginosa* by binding especially to the promoter region of the *MvfR* gene that regulates pyocyanin synthesis[5]. Song et al. indicated that *HigA*-mediated transcriptional inhibition on stress stimulation could affect virulence genes and also take attention to the potential of the *HigBA* TA system as an antibacterial treatment target[6]. In 2022, Zadeh et al. determined that ciprofloxacin and colistin may induce persister cell formation by enhancing the expression of type II TA systems during stationary and exponential phases[23]. Also, it was shown that there was a strong correlation between the *mazEF* TA gene and resistance against gentamicin, meropenem, and amikacin[9].

Even though TA genes and virulence genes were considered to be associated with antibiotic resistance, we observed the level of *RelBE* gene expression was higher in imipenem- and meropenem-sensitive isolates, and the level of *HigBA* gene expression was higher in amikacin-susceptible isolates. In our previous study, we investigated the relationship between toxin genes and antibiotic resistance in a different bacterial collection consisting of 92 *P. aeruginosa* and 148 staphylococci isolates. It was found that in *P. aeruginosa*, the level of *RelBE* TA gene expression is increased in isolates sensitive to aztreonam compared to those resistant to aztreonam. Also, in staphylococci, the levels of mazEF gene expression were found to be higher in isolates sensitive to gentamicin, ciprofloxacin, levofloxacin, clindamycin, phosphomycine, nitrofurantoin, fusidic acid, and cefoxitin compared to those resistant to the above antibiotics[21]. In the present study, we observed that toxin genes were associated with antibiotic resistance, and *RelBE* TA gene expression was associated with the exoS virulence gene in *P. aeruginosa* isolates.

CONCLUSION

The fact that TA genes are expressed more in strains sensitive to carbapenems should draw attention to these strains, which may cause serious infections that are difficult to treat in the future. The relationship between *RelBE* and *ExoS* indicates that TA genes in *P. aeruginosa* isolates are not only related to antibiotic resistance but also play important roles in bacterial pathogenesis and virulence. Further studies including larger numbers of genes are necessary to illustrate the role of TA genes in the pathogenesis of *P. aeruginosa* and to elucidate their connection with antibiotic resistance. These studies should make a significant contribution to the solution of the antibiotic resistance problem.

ETHICAL APPROVAL

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AUTHORS' CONTRIBUTIONS

USSC: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **YD:** Data curation, Methodology, and Writing – review & editing.

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