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Original scientific article

TYPE II TOXIN- ANTITOXIN SYSTEMS IN CLINICAL ISOLATES OF ANTIBIOTIC RESISTANT *Acinetobacter baumannii*

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The over use of antibiotics to treat infections in humans and animals made a phenomenon of the antibiotic-resistant bacteria. While studies focused to find on new antibiotics but, identification of novel antibacterial targets in bacteria is very important. By Toxin antitoxin systems this hypothesis could be done, whereas by the activation of a toxin or inactivation of an antitoxin, the raised toxin kills the bacterium. These systems are attractive target for antimicrobial therapy. However, the most important step for potency of TA system, as an antibacterial target, is to identify a TA system that is prevalent in all resistant clinical isolates. So, the prevalence of different TA systems among clinical isolates of *Acinetobacter baumannii* in Emam khomeini hospital, Ilam, Iran was evaluated to determine which TA system is prevalent in all antibiotic resistant *A. baumannii*. So, one hundred *A. baumannii* clinical isolates were identified during one-year period in Emam khomeini hospital, Ilam, Iran. *A. baumannii* clinical isolates were isolated from hospitalized patients in ICU and burn patients. All isolates were resistant to at least one antibiotic. Then, the isolates were subjected to evaluation to find *mazEF* and *higBA* TA genes by PCR. The results showed the frequency of *mazEF* and *highBA* TA genes in all isolates was 72% and 39%, respectively. *mazEF* or *higBA* TA systems are not presented in all isolates. So, the potency of these two TA systems are in challenged.

Also, all isolates were not positive for one TA gene. So, more research in different geographical area should be done with functionality of TA genes.

Key words: Acinetobacter baumannii, antimicrobial resistance, toxin antitoxin system

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INTRODUCTION

Acinetobacter baumannii is a gram-negative bacterium known as an opportunistic pathogen in immunocompromised individuals. It is also considered as a nosocomial infection. Imipenem and Meropenem are traditionally used to treat infections caused by *A. baumannii*, but unfortunately resistance to these antibiotics is increasing (HOWARD *et al.*, 2012). Resistance to antibiotics develops through mutations of target sites or the acquisition of antibiotic resistant genes from other pathogens. To date, multiple drug resistance is very challenging and make the antimicrobial therapy failure. While studies focused to find on new antibiotics but, identification of novel antibacterial targets in bacteria is very important. The dissemination of antibiotic-resistance genes among nosocomial pathogens has led to many cases of antimicrobial therapy failure. The identification of the novel antimicrobial targets in bacteria is an important step in the effort to develop new drugs (DREWS, 1996). Toxin antitoxin (TA) system could be a potent target for antibiotic therapy. Generally, by inactivation of antitoxin, it is an marvelous strategy for antimicrobial therapy (DENAP and HERGENROTHER, 2005; ENGELBERG-KULKA *et al*., 2005). AMITA *et al*. (2004) showed by activation of a toxin 95% were killed, because of the raised toxin could not be neutralized by antitoxin(AMITA *et al.*, 2004). Hence, artificial disruption of antitoxin can lead to bacterial cell killing. But it should be noted be for inactivation a toxin, because of presence many TA genes in bacteria, firstly, the prevalence of different TA genes should be evaluated to find the reliable frequent TA gene. So, the current study aimed to evaluate the prevalence of *mazEF* and *higBA* TA loci in all *A. baumannii* clinical isolates, Ilam, Iran.

METHODS

Bacterial isolates

One hundred *A. baumannii* clinical isolates were identified during one-year period in Emam khomeini hospital, Ilam, Iran. *A. baumannii* clinical isolates were isolated from hospitalized patients in ICU and burn patients. After transferring all samples to the Clinical Microbiology research center, Ilam University of Medical Sciences, Ilam, Iran, *A. baumannii* isolates were recognized based on conventional biochemical and microbiological tests including Gram staining, oxidase and catalase test, motility, oxidation of glucose, hydrolysis of esculin, decarboxylation of lysine, hydrolysis of arginine, reduction of nitrate, citrate utilization, oxidative/fermentative glucose (O/F) test, and growth ability at 44°C. Negative result for oxidase test, no motility, non-fermentation, and growth in temperature of 42–44°C will be considered as the elementary criteria for *A. baumannii* recognition.

All isolates were evaluated against gentamicin (10μg), amikacin (30 μg), kanamycin (30 μg), tobramycin (10 μg), tetracycline (30 μg), minocycline (30 μg), Imipenem (10 μg), Ciprofloxacin (5 μg), Ticarcillin (75μg), Ceftazidime (30 μg), Clindamycin (2 μg) and doxycycline (30 μg) (Padtan Teb Co, Iran).

DNA extraction of A. baumannii

A. baumannii were cultured into LB broth at 35°C overnight, and then DNA was extracted using the DNA extraction KIT (Gene ALL, South Korea) and evaluated method described by Sambrook (SAMBROOK, 2001). The DNA was extracted and stored at -70ºC.

Evaluation of toxin-antitoxin systems

All clinical isolates of *A. baumannii* were subjected to Polymerase Chain Reaction (PCR) by specific primers of *mazEF* and *higBA* TA genes listed in table 1. PCR amplification was carried out in a DNA thermo cycler (Bio-Rad) using the amplification parameters with initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 58°C for 10 seconds, and extension at 72°C for 1 minutes, with a final extension at 72°C for 5 minutes. PCR amplified products were analyzed by 1% agarose gel electrophoresis.

Table 1. The primers for identification of mazEF and higBA TA genes.

Primer name	Sequence 5'-3'	Accession No.	Product size (bp)
mazE	F:GTCCGCCTACCATCCATTTT R: TTCCTTACCTACAGCATGTCCA	UFIO01000063	192bp
mazF	F: TTGCTGTCCAATGACAACGC R: GCTTGTCCCTTTTTCGTTGCT	VHFV01000164	138bp
higA	F: TGGCACTACGTTGCACCATT R: GCTTGTCCCTTTTTCGTTGCT	CP042210	202bp
higB	F: CACAGGCTGAAACGGCATTAG R: GGAGCAACCAAATGAGCAGT	WBIX01000026	250bp

Statistical analysis

The relationship between isolates and TA genes evaluated by SPSS and chi-square program.

RESULTS

In this study, which is a cross-sectional study, 100 isolates of *A. baumannii* were collected from patients admitted to ICU and burn wards in Emam Khomeini hospital, Ilam, Iran during one-year period.

Antibiotic susceptibility assay by disk diffusion method was performed on all isolates. the results are presented in Table 1. The highest antibiotic resistance was observed to gentamycin (70%) and the lowest resistance identified to minocycline (28%) (Table2).

A. baumannii were evaluated using specific primers to identify *mazE*, *mazF*, *higA* and *higB* TA genes. The results of this study showed that the overall frequency of *mazEF* and *higBA* TA genes in all isolates was 72% and 39%, respectively (figure 1-4), and there was no significant difference in the presence of these genes in the ICU and burn wards. Among them 12% possessed both TA systems.

Out of 100 clinical isolates of *A. baumannii* collected in this study, 36 isolates (36%) were from wounds infection and 64 isolates (64%) were from patients admitted to the intensive care unit with respiratory infections. The results showed that there was no significant relationship between the presences of TA genes with the origin of the isolates.

Table 2. Antibiotic susceptibility assay of A. baumannii clinical isolates. The highest resistance observed for gentamycin

Antibiotic	*susceptible*		*Intermediate*		*resistant*	
	No	%	No	%	No	%
Tetracycline	31	31	2	2	67	67
Gentamycin	24	24	6	6	70	70
Doxycycline	48	48	3	3	49	49
Minocycline	62	62	0	0	28	28
Tobramycin	44	44	0	0	66	66
Amikacin	37	37	1	1	62	62
Kanamycin	31	31	0	0	69	69
Imipenem	55	55	0	0	45	45
Ceftazidime	53	53	0	0	47	47
Ciprofloxacin	64	64	0	0	36	36
Ticarcillin	39	39	0	0	61	61
Clindamycin	46	46	0	0	54	54

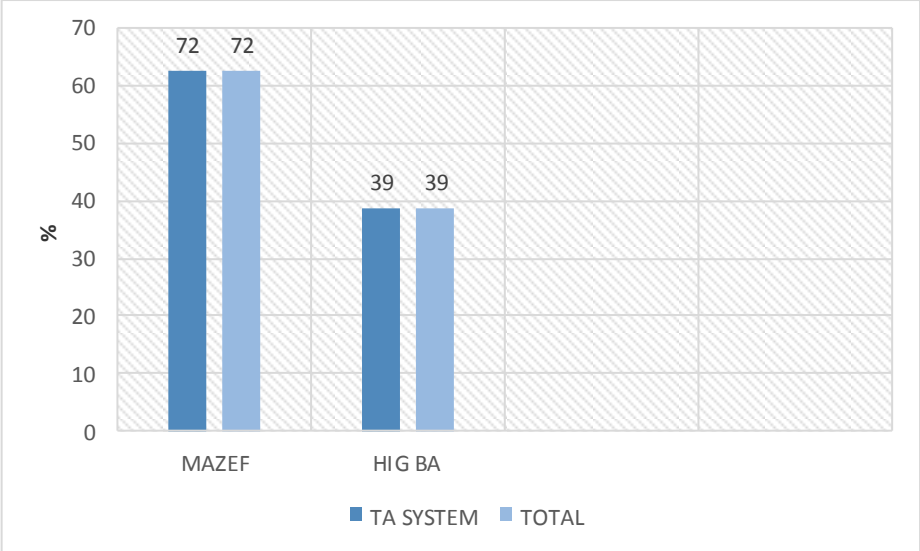

Figure 1. Abundance of toxin and antitoxin genes in A. baumannii clinical isolates

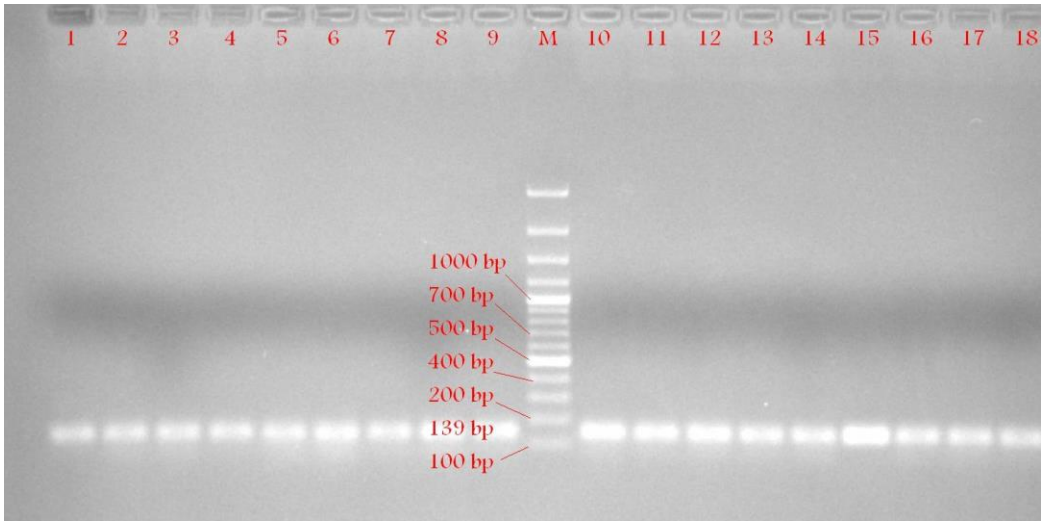

Figure 2. Agarose Gel Electrophoresis (1% agarose, 5-10 V/cm for 40 min) of *mazF*, gene, 139bp. Lane M 100bp DNA Ladder, Lanes 1-18 Represent of Isolates Bands.

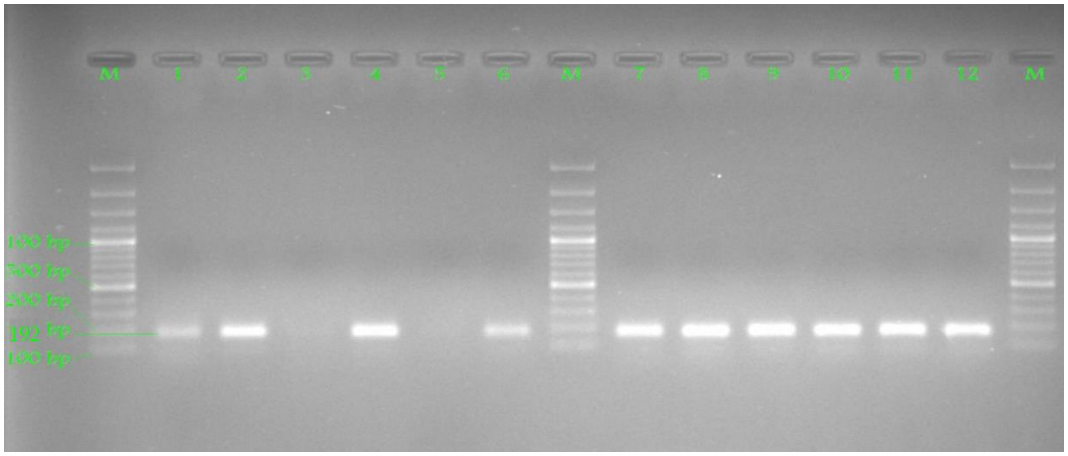

Figure 3. Agarose Gel Electrophoresis (1% agarose, 5-10 V/cm for 40 min) of *mazE*, gene, 192bp. Lane M 100bp DNA Ladder, Lanes 1-6 and 7-12 Represent of Isolates Bands.

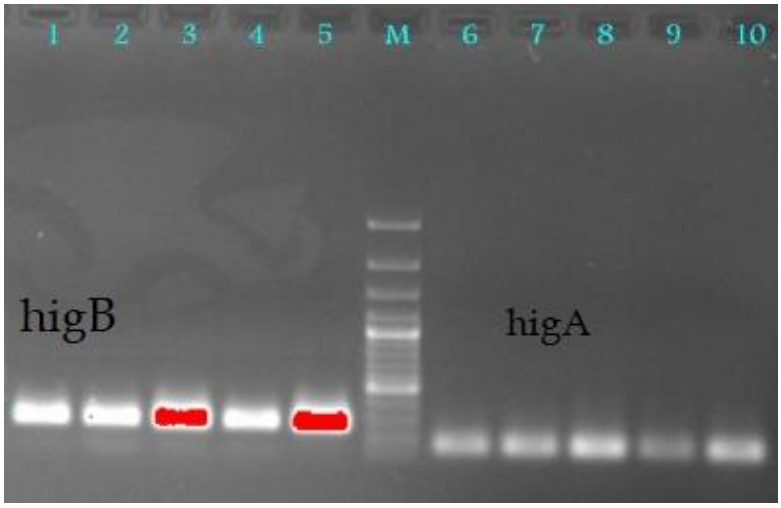

Figure 4. Agarose Gel Electrophoresis (1% agarose, 5-10 V/cm for 40 min) of *higB*, gene, 250bp. Lane M 100bp DNA Ladder, Lanes 1-5 Represent of Isolates Bands of *higB; higA*, gene, 202bp, Lanes 6-10 Represent of Isolates Bands of *higA*.

DISCUSSION

The toxin-antitoxin system is a collection of two or more closely related genes that together encode the "toxin" and "antitoxin". The past research about these systems showed the importance of TA systems for bacterial physiology. When there is only the toxin, possibility in the absence of antitoxin is able to kill the bacteria (GHAFOURIAN *et al.*, 2014). In the normal condition, the antitoxin binds to the toxin and neutralizes it, so the cells will be alive. The toxin is almost stable and the antitoxin is unstable. When the antitoxin is inactivated, the toxin kills the bacterium through a mechanism called programmed cell death (CHERNY *et al.*, 2007). Therefore, by inactivation of antitoxin, it will be possible that the toxin kills the bacterium. So, the focus in the researches could be on antitoxin as a novel antimicrobial strategy. But it should be noted be for inactivation a toxin, because of presence many TA genes in bacteria, firstly, the prevalence of different TA genes should be evaluated to find the reliable frequent TA gene.

The results of this study show that the overall frequency of *mazEF* and *highBA* toxin and antitoxin genes in isolates were 72% and 39%, respectively, and there was no significant difference in the presence of these genes in clinical isolates. In a study by GHAFOURIAN *et al.* (2014), the prevalence of toxin and antitoxin systems in clinical isolates of *A. baumannii* showed *mazEF* in 100% of isolates and *higBA* in 4.7% of isolates were observed (GHAFOURIAN *et al.*, 2014).The results of our study in comparison with the study of GHAFOURIAN *et al.* (2014) showed that the prevalence of toxin and antitoxin system is lower in clinical isolates of *A.baumannii* and the difference in the prevalence of this system may due to geographical differences in the study area.

In the studies of GHAFOURIAN *et al.* (2014) the first report of the presence of this system in *A.baumannii* was obtained with the abundance of *mazEF* in Malaysia. *MazEF* was also predominantly reported in *Pseudomonas aeruginosa* strains by Williams (PARK *et al*., 2013). Our findings showed both TA loci were not presented in all antibiotic resistance isolates.

CONCLUSION

Despite the studied TA loci were presented in all antibiotic resistant isolates but it may be silenced or non-functional and more study should be done. So, more research in different geographical area and a bigger sample size should be done with functionality of TA genes.

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TOKSIN TIPA II - ANTITOKSIN SISTEMI U KLINIČKIM IZOLATIMA OTPORNI NA ANTIBIOTIK *Acinetobacter baumannii*

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Izvod

Prekomerna upotreba antibiotika za lečenje infekcija kod ljudi i životinja stvorila je fenomen bakterija otpornih na antibiotike. Dok su se studije fokusirale na pronalaženje novih antibiotika, identifikacija novih antibakterijskih ciljeva u bakterijama je veoma važna. Pomoću sistema protiv toksina ova hipoteza se može postići, dok aktivacijom toksina ili inaktivacijom antitoksina, povišeni toksin ubija bakteriju. Ovi sistemi su atraktivna meta za antimikrobnu terapiju. Međutim, najvažniji korak za potenciju TA sistema, kao antibakterijske mete, jeste da se identifikuje sistem TA koji preovladava u svim rezistentnim kliničkim izolatima. Dakle, procenjena je prevalenca različitih sistema TA među kliničkim izolatima Acinetobacter baumannii u bolnici Emam Homeini, Ilam, Iran da bi se utvrdilo koji TA sistem preovlađuje kod svih *Acinetobacter baumannii* otpornih na antibiotike. Klinički izolati *A. baumannii* izolovani su od hospitalizovanih pacijenata na intenzivnoj nezi i pacijenata sa opekotinama. Svi izolati su bili otporni na najmanje jedan antibiotik. Zatim su izolati podvrgnuti evaluaciji da bi se pronašli geni *mazEF* i *higBA* TA pomoću PCR-a. Rezultati su pokazali da je učestalost gena *mazeEF* i *highBA* TA u svim izolatima bila 72% i 39%, respektivno. *mazEF* ili *higBA* TA sistemi nisu predstavljeni u svim izolatima. Dakle, ispitivanje moći ova dva sistema TA je veliki izazov.

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