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ORIGINAL ARTICLE

Detection of Toxin-Antitoxin System in *Acinetobacter baumannii* Isolated from Patients at Zagazig University Hospitals

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ABSTRACT

Background: *Increased resistance to antibiotics among Acinetobacter baumannii (A. baumannii) isolates is a rising problem, and new alternatives should be provided to overcome this problem. Toxin-Antitoxin system (TA) is considered a promising essential target for antimicrobial drugs.* ***Objective:*** *To detect the prevalence of toxin-antitoxin system in A. baumannii isolated from patients at Zagazig University Hospitals.* ***Methodology:*** *Following isolation, oxidase test and API20NE were used to identify A. baumannii, antibiotic susceptibility testing was performed to all isolates obtained, and amplification and screening of functional mazEF, relBE and higBA toxin-antitoxin genes were done by PCR and RT-PCR, respectively.* ***Results:*** *Out of 252 clinical specimens collected, 27(10.7%) were A. baumannii; 13 (15.3%) isolates were isolated from endotracheal aspirates, 4 (13.3%) from sputum samples, 7 (9.2%) from urine, 2 (4.5%) from pus, and 1 (14.3%) from blood. Most of the isolates were multidrug resistant, and the highest susceptibility was to meropenem (66.7 %) followed by imipenem (63%). Regarding PCR results, 22 isolates (81.5%) had relBE gene, 17 (62.9%)had mazEF gene, and 8(29.6%) had higBA gene. In the RT-PCR results, all genes were functional in all isolates.* ***Conclusion:*** *TA system genes are prevalent among A. baumannii isolates, in particular; relBE and mazEF genes and they are functional.*

INTRODUCTION

The Gram-negative opportunistic coccobacillus recognized as *A.baumannii* is responsible for about 2–10% of all Gram-negative infections in hospitals[1].

Given that *A.baumannii* is an important bacterial pathogen with increasing resistance to antimicrobials, it is considered one of the ESKAPE pathogens named by The Infectious Diseases Society of America (IDSA), which includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A.baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*[2].

For bacteria, toxin-antitoxin (TA) systems are widespread and have many functions such as persistence and genetic stability. They are specific genetic elements carrying two genes; one encoding a toxin and the other encoding its related antitoxin. The toxic protein's action is neutralized by the antitoxin[3].

When the toxin is released by *A.baumannii*, it has the capability of killing the bacteria by inducing bacterial apoptosis; however, the antitoxin is synthesized prior to the toxin synthesis to protect the bacteria by forming a toxin-antitoxin protein complex that is by making the toxin harmless to the bacteria. On one hand, the toxin is a stable protein, while the antitoxin is an unstable molecule making itextremely liable to destruction. If the antitoxin is degraded, the toxin could induce bacterial programmed cell death. For this reason, TA systems acquire their importance as a possible effective target for antimicrobial drugs[4].

Moreover, Soheili and his colleagues[5] supposed that the TA system could be considered as an interesting target for antimicrobial drugs through toxin stimulation or through antitoxin inhibition.

METHODOLOGY

Study design: A cross-sectional study was carried out over one year at the Medical Microbiology and Immunology Department and Zagazig University Hospitals.

Ethical consideration: The institutional review board (IRB), Zagazig Faculty of Medicine approved this work, and consents were taken from all study participants.

Isolation and identification of *A. baumannii*:

Different clinical specimens were collected from patients admitted in different units of Zagazig University Hospitals and were subjected to Gram stain and aerobic culture on MacConkey agar plates (Oxoid, UK) at 37°C for 24 hours. Gram negative, lactose non-fermenting colonies were further identified using oxidase test, triple sugar iron (Oxoid, UK), and API 20 NE (Bio-Merieux, Marcy L Etoile. France). The following discs were used to determine the antibiotic

sensitivity by disc diffusion method according to Clinical Laboratory Standards Institute guidelines[6]: amoxacillin/clavulanic acid 30ug, aztreonam 30ug, amikacin 30ug, ceftriaxone 30ug, ceftazidime 30ug, piperacillin 100ug, cefotaxime 30ug, tetracycline 30ug, sulphamethoxazole-trimethoprim 25ug, ciprofloxacin 5ug, imipenem 10ug, and meropenem 30ug.

Detection of Toxin-antitoxin genes:

PCR:

DNA extraction from isolated colonies was done by the boiling method. PCR amplification for m*azEF, relBE, and higBA* genes was performed in a final volume of "100 µl containing 1–3 µl of purified total DNA, 1X PCR buffer (20 mM TrisHCl/50 mM KCl, pH 8.4), 1.5 mm $MgCl_2$, 0.2mm each deoxynucleoside triphosphate, 0.5M each primer, and 2.5 units of *Taq* polymerase"[7]. PCR was carried out in DNA thermal cycler **(Veriti ® 96 well thermal cycler, Applied Biosystems, Singapore)** and reaction mixtures were subjected to conditions mentioned in table I. PCR amplification products were analyzed by an agarose gel electrophoresis in 1% agarose and stained with ethidium bromide.

RT-PCR:

RNA was extracted from all isolates containing TA genes using TRIzol™ Reagent (Invitrogen, USA), then reverse transcription and PCR were performed in the DNA thermal cycler previously used for PCR using SuperScript One-Step RT-PCR System with a Platinum Taq kit **(Invitrogen, USA)**. RT-PCR was performed in a total reaction volume of 50 µl and reaction mixture were subjected to the conditions mentioned in table II. DNA contamination of RNA extract was excluded by using a control, in which the reverse transcriptase enzyme was not added to the reaction mixtures where no amplification is detected after gel electrophoresis.

Table I: PCR steps of TA genes

Cycling condition	Temperature	Time	Cycles
Initial denaturation	94°C	3 min	1
Denaturation	94°C	1 min	30
Annealing	54°C	1 min	30
Extension	72°C	1 min 30 s	30
Final extension	72°C	10 min	1

Table II: RT-PCR steps of TA genes

Cycling condition	Temperature	Time	Cycles
cDNA synthesis	50°C	30min	1
Predenaturation	94°C	2 min	1
Denaturation	94°C	30 s	35
Annealing	54°C	45 s	35
Extension	72°C	1 min	35
Final extension	72°C	10min	1

Primers used for both PCR and RT-PCR were according to Ghafourian and his colleagues[4]. They were supplied from ThermoFisher Scientific, USA. Their sequences are demonstrated in Table III.

Table III: Primer sequence of TA genes

Gene	Primer sequence
MazEF	**Forward:** ACCTTCGAAGGAACTACGTCAGTAG **Reverse**: ATAGGCGAACATGCAAGAAAAGGCAGC
RelBE	**Forward**: ATGAAGTGAACGGTCAACAATA **Reverse**: ACAGACCTCGGAAAGTGGTCG
HigBA	**Forward**: AGCACATCCGTACGATCTACTGC-3 **Reverse**: TGCACTCCTGCGATGCGGCGAA

Statistical analysis

The data were collected, presented, summarized, tabulated, and analyzed using computerized software. Statistical packages (EPI-info Version 6.04 and SPSS Version 19 inc.Chiago, USA), A P-Value<0.05 was considered to be statistically significant at 95% confidence interval. Chi-square was used to compare proportions.

RESULTS

Out of the 252 clinical specimens collected, 27 (10.7%) *A. baumannii* were isolated. The numbers and percentages of *A. baumannii* isolates from different clinical specimens are presented in table 1; 13 (15.3%) isolates were collected from endotracheal aspirates, 4 (13.3%) from sputum samples, 7 (9.2%) from urine, 2 (4.5%) from pus, and 1 (14.3%) from blood, with no statistically significant difference (P =0.462).

Table 1: Distribution of *A.baumannii* isolates according to type of infection and clinical specimens (No.252).

Type of Infection	Clinical specimens		*A. baumannii isolates*		X^2	P value
	Type	No.	No.	(%)		
Chest infections	Endotracheal aspiration	85	13	15.3	3.6	0.462
	Sputum	30	4	13.3		
Urinary tract infections	Urine	76	7	9.2		
Infected wounds	Swabs from pus	44	2	4.5		
Septicemia	Blood	17	1	14.3		

The antibiogram of isolated *A. baumannii* by disc diffusion method is shown in table 2.All isolates were resistant to amoxicillin/clavulanic acid and tetracycline, while 96.3% of isolates were resistant to sulphamethoxazole-trimethoprim, 96.3% to aztreonam, 92.6% to Cefotaxime, 77.8% to amikacin, and 63% to piperacillin. More than half of the isolates were resistant to ceftriaxone (51.9%), ciprofloxacin (55.6%) and ceftazidime (59.3%). However, the highest susceptibility was recorded for meropenem (66.7%), followed by imipenem (63%).

Table 2: Antibiogram of isolated *A. baumannii* by disc diffusion method

Antibiotic	*A. baumannii*			
	Susceptible		Resistant	
	No.	%	No.	%
Amoxicillin/Clavulanic acid	0	0%	27	100.0
Aztreonam	1	3.7	26	96.3
Amakicin	6	22.2	21	77.8
Ceftriaxone	13	48.1	14	51.9
Ceftazidime	11	40.7	16	59.3
Piperacillin	10	37	17	63
Cefotaxime	2	7.4	25	92.6
Tetracycline	0	0.0	27	100.0
Sulphamethoxazole-Trimethoprim	1	3.7	26	96.3
Ciprofloxacin	12	44.4	15	55.6
Imipenem	17	63	10	37
Meropenem	18	66.7	9	33.3

In the PCR results, 22 isolates (81.5%) had *relBE* gene, 17 (62.9%) had *mazEF* gene, and 8 (29.6%) had *higBA* gene, with a high statistically significant difference ($P < 0.001$) (Table 3).

All the detected genes of TA system were expressed in their corresponding *A. baumannii* isolates when screened by RT-PCR.

Table 3: Frequency of *relBE*, *mazEFand higBA* genes in the obtained *A. baumannii* isolates

Genes / Isolate	*relBE*		*mazEF*		*higBA*		*X2*	*P value*
	No.	*%*	*No.*	*%*	*%*	*%*		
Isolate positive	22	81.5	17	62.9	8	29.6	15.308	<0.001**
Isolate negative	5	18.5	10	37.1	19	70.4		
T0TAL	27	100.0	27	100.0	100.0	100.0		

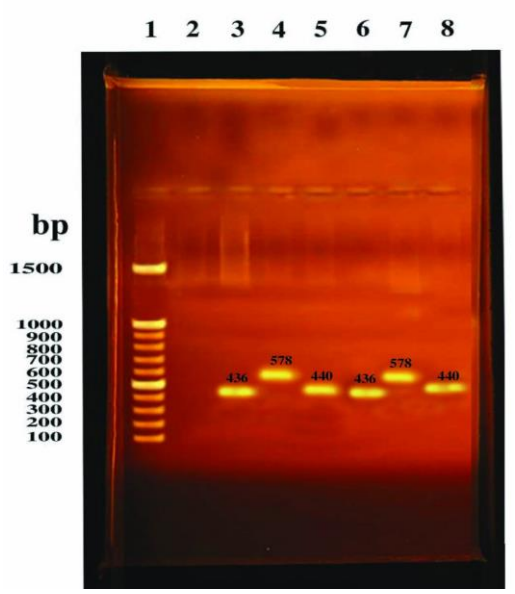

Fig. 1: Results of PCR and RT- PCR for *mazEF, relBE and higBA* genes in the obtained *A. baumannii* isolates: Lane 1: DNA marker (100-1500bp), lane 2: negative control, PCR and RT- PCR result for *mazEF gene* (lane 3,6), *relBE* (lane 4,7) and *higBA* (lane 5,8), respectively.

DISCUSSION

Detection of TA system in isolated bacteria is considered an important approach as TA system might be a promising target for antimicrobial agents especially with the emergence of bacteria with resistance to almost all drugs used.

In our study, out of 252 different clinical specimens, 27 (10.7%) *A. baumannii* were isolated. This was in agreement with Gomaa and his colleagues[8] who isolated (60/631) 9.5% *A. baumannii* from immunecompromised patients. On the other hand**,** Babapour and his colleagues[9] had found that *A. baumannii* were isolated from patients in three hospitals of Tehran with a higher isolation rate (24%). Rani and his colleagues[10] in India reported that 18.7% *A. baumannii* were identified out of 2459 different clinical specimens.Lower prevalence was detected by Nageeb and his colleagues[11] who declared that 2.9% *A. baumannii* were isolated from patient admitted at different ICUs of Suez Canal University Hospital. Castilho and his colleagues[12] in Brazil also, had detected a lower frequency of *A. baumannii* (4.8%). The variable isolation rates recorded in different studies might be due to different sample size, patients' admission in ICU or ordinary hospital units and strict application of infection control measures in some hospitals.

In our study, the highest isolation rate of *A. baumannii* was from chest infection specimens (15.3% of endotracheal aspiration & 13.3% of sputum samples), followed by blood (14.3%), urine (9.2%), and lastly, pus (4.5%). This agrees with Alkasaby and Zaki[13] who reported that *A. baumannii* was isolated mainly from the respiratory tract specimens followed by wound and blood specimens. Amiri and his colleagues[14] had also isolated *A. baumannii* mainly from respiratory tract secretions and blood. However, Al Mobarak and his colleagues[15] found that *A. baumannii* was mainly recovered from wounds followed by respiratory secretions, urine, and blood. Different number of the specimens collected, underlying medical conditions that led to hospitalization and hospital conditions, could explain this variation.

All *A. baumannii* isolates from this study were resistant to amoxicillin/clavulanic acid and tetracycline while 96.3% of isolates were resistant to sulphamethoxazole-trimethoprim, 92.6% to aztreonam, 77.8% to amikacin, and 63% to piperacillin. More than half of the isolates were resistant to ceftriaxone (51.9%), ciprofloxacin (55.6%), and ceftazidime (59.3%). However, a lower level of resistance was detected to imipenem (37%) and meropenem (33.3%).

The widespread resistance of *A. baumannii* to different groups of antimicrobials has been reported previously in different studies. Fazeli and his colleagues[16] had found that all isolates were resistant to cefotaxime and aztreonam. In Saudi Arabia, Al-Mously and Hakawi[17] reported high resistance rates with cefepime (73.8%), piperacillin/tazobactam (72.3%), ciprofloxacin (68%), gentamicin (66%), imipenem (61.3%), and lastly, meropenem (60.7%).In China, Zhao and his colleagues[18] reported high frequency of resistance rates to imipenem, gentamicin, ampicillin/ sulbactam, ceftazidime, and ciprofloxacin. Sarhaddi and his colleagues[19] had also found that all isolates were resistant to ceftazidime, cefotaxime, imipenem and tetracycline. Moreover, 98.1% of isolates were resistant to ciprofloxacin, 96.3% to sulphamethoxazole-trimethoprim, 96.3% to cefepime, 90.7% to amikacin, and 50.0% to gentamicin.

Following PCR amplification of TA system genes, the current study detected that among the examined 27 isolates of *A. baumannii*, 81.5% had *relBE* gene, 62.9% had *mazEF* gene, and 29.6% had *higBA* gene. All detected genes were functional in their corresponding isolates following RT-PCR examination.

As far as we know, only a few studies had been carried out to detect the prevalence and functionality of genes of the TA system in bacterial isolates. Ghafourian and his colleagues[4] had screened for TA loci and showed that all *A. baumannii* isolates were positive for *mazEF*, 88.2% of isolates were positive for *relBE*, and only 4.7% were positive for *higBA*. In addition, in accordance with our study, all genes were functional.

Previous studies were carried out to assess TA system in other bacterial species. In Iran, Savari and his colleagues[20] found both *relBE* and *higBA* genes in all isolates of *P. aueroginosa*. Hemati and his colleagues[21] also found *relBE* gene in all isolates of *P. aueroginosa*. TA system genes were also prevalent in *vancomycin-resistant enterococci*, *mazEF* (100%) and *relBE* (44%), as reported by Moritz and Hergenrother[7]**.** Furthermore, Karimi and his colleagues[22] screened the prevalence of TA system and its association with biofilm formation in *E. coli* and found that TA genes were abundant among isolates, as 80% of isolates had *mazEF* and 85% had *relBE*.

In conclusion, the current study reports the prevalence of functional TA system genes in *A. baumannii*, in particular the *relBE* and *mazEF* genes, which could be promising targets for the development of new classes of antimicrobial drugs affecting this organism.

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Conflict of interest: None

Limitations: None

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