ORIGINAL ARTICLE

Detection of Genes of Efflux Pumps (adeB, adeJ and adeG) in Tigecycline Resistant *Acinetobacter baumannii* Isolated from Benha University Hospital

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ABSTRACT

Background*: Emerging of tigecycline resistant Acinetobacter baumannii (A. baumannii) is a critical global health problem as tigecycline is considered the last-line antibiotic for treatment of carbapenem resistant A. baumannii infections. Overexpression of efflux pumps is a leading mechanism of antibiotic resistance in A. baumannii.* ***Objectives:*** *Detection of the presence of three efflux pump genes; adeB, adeJ and adeG and determination of their expression level in Tigecycline Resistant A. baumannii collected from Benha University Hospital.* ***Methodology:*** *Thirty A. baumannii strains were collected and tested for antibiotic susceptibility. Presence of adeB, adeJ and adeG genes was detected by conventional PCR and their expression levels were assessed by RT-PCR.* ***Results:*** *Tigecycline susceptibility showed 60% (18/30) resistance and 40% (12/30) sensitivity. In tigecycline resistant strains, adeB gene was identified in 13/18 (72.2%) and adeJ gene in 12/18 (66.7%) with statistically significant association (P= 0.023). In tigecycline sensitive isolates adeB and adeJ genes were identified in 7/12 (58.3%) and 2/12 (16.7%) strains respectively. The adeG gene was not identified in any of the isolated strains. Combined adeB and adeJ genes were identified in 50% of tigecycline resistant isolates and only in 8.3% of (tigecycline sensitive) isolates with statistical significance difference (P= 0.018). Comparing adeB and adeJ gene expression revealed that transcription level was increased significantly and associated with tigecycline resistance.* ***Conclusion:*** *This study revealed wide spread of tigecycline resistant A. baumannii strains in Benha University Hospital and elucidated the significant role of (adeB and adeJ) genes in their emergence.*

INTRODUCTION

Acinetobacter baumannii (A. baumannii) is an aerobic, non-motile, non-fermentative, oxidase negative, catalase positive, Gram-negative, coccobacillus[1]. It is a widespread opportunistic pathogen and has been emerged as a clinically relevant pathogen causing a variety of hospital and community-acquired infections[2].

Over the last few decades, *A. baumannii* became important problematic nosocomial pathogens resulting in serious infections with a high mortality rate especially among intensive care units (ICUs) patients due to its tremendous capability to evade the host immunity, resist the cruel environmental conditions and achieve antibiotic resistance[3].

A. baumannii shows mounting antimicrobial resistance; turned from multidrug resistant (MDR) that resists the 3rd generation cephalosporins, aminoglycosides and fluoroquinolone[4] into extensively drug - resistant (XDR) which resists all antibacterial agents except colistin and tigecycline; the two last-resort antibiotics for treatment of extensively drug resistant *A. baumannii* (XDRAB) infections [5].

Increased prescription of the last-resort antibiotics due to growing resistance of *A. baumannii*, limitation of the available antimicrobial options and lack of novel antibiotics led to emergence of pan-drug resistant strains (PDR), against which no antibacterial agents retain activity[6].

Mechanisms of antibiotics resistance expressed by *A. baumannii* are biofilm formation, deactivating enzymes production, poor membrane penetration and permeability defects, alteration of target sites or cellular functions and overexpressing efflux pumps[3].

A. baumannii encodes a collection of efflux pumps responsible for extrusion of antibiotics, dyes, biocides, detergents and antiseptics outside the bacterial cell; of special interest are the resistance-nodulation-division (RND) efflux family members that have been shown to play an important role in *A. baumannii* antimicrobial resistance as they are implicated in efflux of enormous diversity of antibiotics[7].

The structure of RND efflux pumps consists of an outer membrane protein (OMP), an inner membrane

protein (IMP) with a substrate binding site that pumps drugs out utilizing proton motive force and a periplasmic adapter protein (PAP, also known as the membrane fusion protein or MFP) that links the OMP and the IMP[8].

Among Acinetobacter drug efflux pumps is (ade) RND family. Overexpression of three chromosomally encoded members; adeABC, adeIJK and adeFGH have been reported to involve in tigecycline resistance[9].

The objective of the present study was to determine the existence of three Acinetobacter drug efflux (ade) resistance nodulation-cell division (RND) efflux pumps; adeB, adeJ, adeG and their expression level in tigecycline resistant *A. baumannii* collected from different clinical specimens at Benha University Hospital.

METHODOLOGY

The current study was performed at the Medical Microbiology and Immunology Department, Faculty of Medicine, Benha University after approval of the study protocol by the Ethical Committee at Benha Faculty of Medicine, article No. (RC1372022) and a written informed consent was obtained from all study participants or their legal designates.

Study Design and Sampling:

This cross sectional study was carried out on 150 non-duplicate clinical specimens (broncho-alveolar lavage (BAL), sputum, urine, pus and blood) collected following standard collection procedures from patients aged from 20 to 79 years, hospitalized in different Inpatients Departments at Benha University Hospital.

Bacterial Isolation and Identification:

All specimens were inoculated on MacConkey agar media (Oxoid, UK) and incubated for 24 h at 37 °C. Urine samples were inoculated on CLED. Blood samples were incubated in Brain heart infusion (BHI) broth for 7 days at 37 °C, samples showing turbidity were sub-cultured on MacConkey agar. Preliminary identification of Acinetobacter based on colony morphology and Gram-negative staining. On MacConkey agar Acinetobacter strains were identified as pale and rounded colonies[10]. In Gram-stained smears it appeared as tiny, Gram -ve coccobacilli either single or paired[11]. For speciation, the strains underwent hanging drop test, sub-cultivation at 44°C and various biochemical tests including (catalase, oxidase, coagulase, sugar fermentation, indole, methyl red, Voges-Proskauer, citrate, urease, H_2S production, gelatin liquefaction, DNase, bile solubility and esculine hydrolysis). Identification was confirmed using VITEK® 2 Systems cards (BioMerieux, France).

Antimicrobial Susceptibility Test (AST):

Strains confirmed as *A. baumannii* were examined for their antibiotic susceptibility using Ampicillin-Sulbactam (20/10 µg), Ceftazidime (30 µg), levofloxacin (5 µg), Ceftriaxone (30 µg), Trimethoprim/Sulfamethoxazole (1.25/23.75 µg), Amikacin (30 µg), Cefepime (30 µg), Piperacillin/Tazobactam (100/10 µg), Cefotaxime (30 µg), Gentamicin (10 µg), Ciprofloxacine (5 µg), Imipenem (10 µg), Meropenem (10 µg), Tobramycin (10 µg), Tetracycline (30 µg), Colistin (10 µg) and Tigecycline (15 µg) antibiotic discs (Hi-Media, India) by Kirby Bauer's disc diffusion method on Mueller-Hinton (M-H) Agar (Oxoid, UK) as recommended by the clinical and laboratory standards institute (CLSI, 2021)[12] using *A. baumannii* ATCC19606 as a reference strain. Results were recorded and interpreted according to the guidelines of CLSI 2021 except for colistin and tigecycline which are not available in the guidelines for Acinetobacter. The zone sizes were taken as susceptible ≥11 mm and resistant ≤10 mm for colistin[13]. The interpretation for tigecycline was sensitive ≥16 mm, intermediate resistant 13–15 mm and resistant ≤12 mm according to Jones et al.[14].

Multiplex PCR for Detection of adeB, adeJ and adeG Genes:

DNA was extracted by i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Korea). Amplification of adeB, adeJ and adeG genes was carried out with designed specific primers (Table 1) in a reaction mixture of a total volume 50µL composed of 5µL DNA template, 25µL 2×*EasyTaq*® PCR SuperMix (TransGen Biotech, China), 1µL of each primer and 14µL nuclease free water.

The thermal profile was initial denaturation step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing at 47°C for 30s and extension at 72°C for 30s and a final extension step at 72°C for 5 min. Analysis of PCR products were performed on 1% agarose gel stained with ethidium bromide using 100bp DNA ladder (Thermo Scientific, USA) as a molecular weight size standard. AdeB and adeJ genes were visualized at 340 bp and 221 bp respectively while adeG gene couldn't be detected (Figure.1).

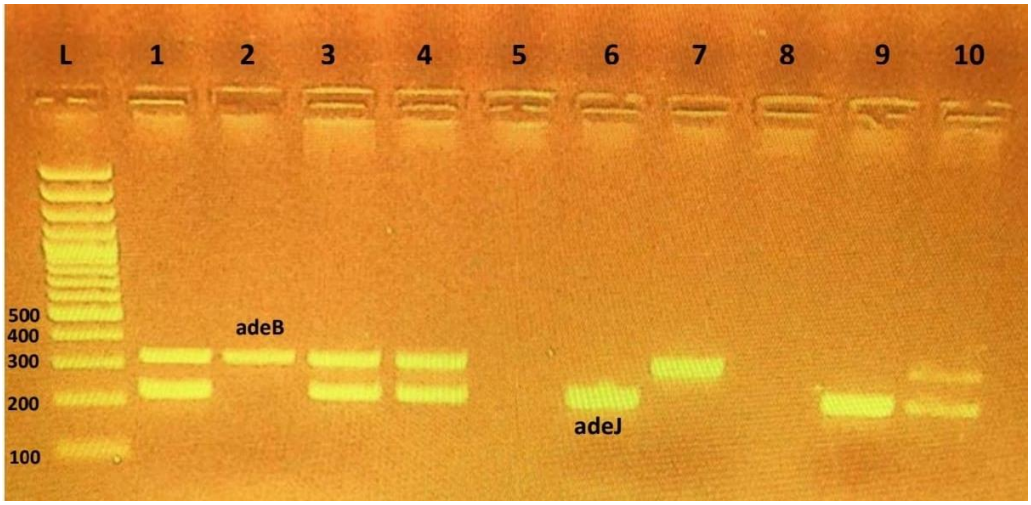


Fig. 1: Ethidium bromide stained 1% agarose gel of the amplified DNA products. AdeB and adeJ genes were detected at 340 bp and 221 bp respectively. Lanes 1, 3, 4 and10 show simultaneous detection of adeB and adeJ genes. Lanes 2 and 7 show adeB gene. Lanes 6 and 9 show adeJ gene. L is a 100 bp DNA ladder.

Quantitative Real-Time PCR (qRT-PCR):

The levels of expression of adeB and adeJ genes were estimated by reverse transcription-PCR (RT-PCR). Total RNA was extracted by EasyRNA™ Bacterial RNA Mini Kit (BioVision, USA) according to manufacturer's instructions. Reverse transcription and cDNA synthesis was carried out by using FastKing RT Kit
with gDNase (TIANGEN Biotech, China) following the manufacturer's protocol. PCR amplifications were performed in a StepOne® Real-Time PCR System (Applied Biosystems, Singapore) using HERA^{PLUS} SYPER® Green qPCR Master Mix (Willowfort, UK) with the 16S rRNA gene was used as a housekeeping gene to normalize the expression levels of target genes. Expression analysis was performed by calculating the relative expression of the mRNA compared with that of *A. baumannii* ATCC 19606. The reaction mixture was 20µL and consists of 5µL cDNA template, 10µL HERA^{PLUS} SYPER® Green qPCR Master Mix, 1µL Forward primer, 1µL reverse primer and 1µL nuclease free water. The used PCR program was 95°C for 2 min, followed by 40 cycles at 95˚C for 10 s, 47°C for 30 s and 72°C for 20 s.

Table 1: **Primers used in the study**

Target genes	Primer sequence (5′–3′)	Product Size (bp)	References
adeB	F: ATTTGGATTGCTGAGCATTC R: GTAAACCTTGCTGACGTACA	340 bp	15
adeJ	F: GGTCATTAATATCTTTGGC R: GGTACGAATACCGCTGTCA	221 bp	16
AdeG	F: TTCATCTAGCCAAGCAGAAG R: GTGTAGTGCCACTGGTTACT	468 bp	17
16S rRNA	F: GACGTACTCGCAGAATAAGC R: TTAGTCTTGCGACCGTACTC	426 bp	18

Statistical Analysis

Data were analyzed using (IBM SPSS) software package version 24 (SPSS Inc., Chicago, Illinois, USA) Qualitative data were described using number and percentage. The Kolmogorov–Smirnov test was used to verify the normality of distribution. Quantitative data were described by using range (minimum and maximum), mean and SD. Mann-Whitney–U test was used to compare between more than two studied groups for abnormally distributed quantitative variables. Chi square test (X^2) was used to compare categorical variables. P value of 0.05 was considered statistically significant.

RESULTS

This study included 150 inpatient; 87 males and 63 females, their ages ranged from 20 to 79 years admitted to various Departments at Benha University Hospital. The collected specimens were bronchoalveolar lavage (BAL) 67 (44.7%), sputum 30 (20.0%), urine 25 (16.7%), pus 18 (12%) and blood 10 (6.6%).

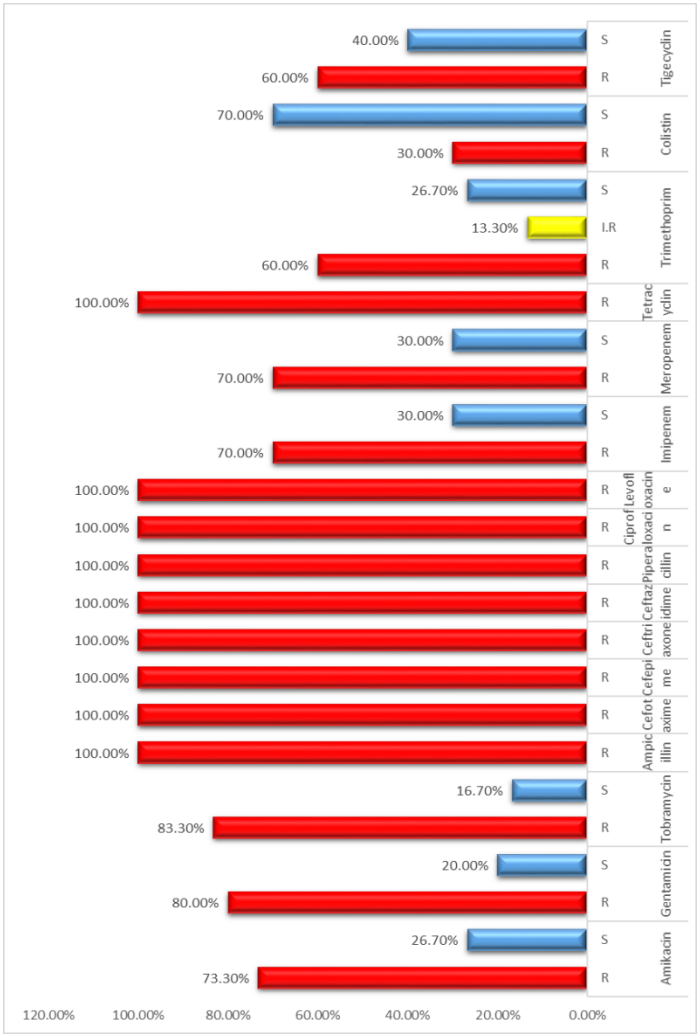
The most commonly isolated organisms were klebsiella 53/150 (35.3%), followed by *Pseudomonas aeruginosa* 32/150 (21.3%), *A. baumannii* 30/150 (20%), Staphylococci 14/150 (9.3%), *E. coli* 10/150 (6.6%) and others 11/150 (7.3%).

A. baumannii was greatly isolated from respiratory specimens; BAL 11/30 (36.7%), sputum 7/30 (23.3%) followed by pus 6/30 (20%), urine 5/30 (16.7%) and blood 1/30 (3.3%) (Table 2).

Table 2: Distribution of *A. baumannii* according to clinical specimens

Specimens	Total specimens		Isolated A. baumannii	
	N=150	%	N=30	%
BAL	67	44.7%	11	36.7%
Sputum	30	20.0%	7	23.3%
Urine	25	16.7%	5	16.7%
Pus	18	12.0%	6	20.0%
Blood	10	6.6%	1	3.3%

Antibiogram of *A. baumannii* isolates displayed that all isolates were MDR (resistant to more than 3 classes of antibiotics) as all were Ampicillin, Cefotaxime, Cefepime, Ceftriaxone, Ceftazidime, Piperacillin, Ciprofloxacin, Levofloxacine and Tetracyclin resistant and exhibited high percentage of resistance to Gentamicin, Amikacin, Tobramycin, Imipenem, Meropenem and Trimethoprim. Eighteen strains out of the 30 isolated strains of *A. baumanii* 18/30 (60%) were tigecycline resistant and 12/30 (40%) were tigecycline sensitive (Figure 2).



S= sensitive (blue) R= resistant (red) IR= intermediate resistant (yellow)

Fig. 2: Antibiogram of isolated *A. baumannii* strains

On investigating factors increasing the isolation of tigecycline resistant *A. baumannii*, statistical significant association occurs between (increasing patients' age, prior antibiotics use, increasing hospital stay length and mechanical ventilation) and isolation of tigecycline resistant strains, while, sex and chronic diseases like DM, hypertension and chronic lung diseases were non - significant risk factors (Table 3).

Table 3: Isolation rate of tigecycline sensitive and tigecycline resistant *A. baumannii* as regards different risk factors

Risk factors		Tigecycline sensitive		Tigecycline resistant		P value
		N=12	%	N=18	%	
Age	**20-40 years**	6	50.0%	0	0.0%	**0.002***
	41-60 years	4	33.3%	7	38.9%	
	> 60 years	2	16.7%	11	61.1%	
Sex	**Male**	8	66.7%	9	50.0%	0.36
	Female	4	33.3%	9	50.0%	
Length of hospital stay	**More than 7**	6	50.0%	16	88.9%	**0.018***
	Less than 7	6	50.0%	2	11.1%	
Prior use of antibiotics**	**Yes**	5	41.7%	14	77.8%	**0.036***
	No	7	58.3%	4	22.2%	
Hypertension	**Hypertensive**	4	33.3%	6	33.3%	1
	Normal	8	66.7%	12	66.7%	
Diabetes mellitus	**Diabetic**	7	58.3%	9	50.0%	0.65
	Non diabetic	5	41.7%	9	50.0%	
Mechanical ventilation	**Yes**	4	33.3%	8	44.4%	**0.033***
	No	8	66.7%	10	55.6%	
Chronic lung diseases	**Absent**	9	75.0%	11	61.1%	0.42
	Present	3	25.0%	7	38.9%	

[#] Chi square test, * significant

**Penicillin, fluoroquinolones, aminoglycosides and carbapenems.

PCR exhibited that adeJ and adeB genes only were detected in *A. baumanii* isolated strains whereas adeG gene was not detected. The adeB gene was found in (13/18) 72.2% of tigecycline resistant strains and (7/12) 58.3% of tigecycline sensitive isolates. AdeJ was shown in (12/18) 66.7% of tigecycline resistant strains with statistically significant association (P= 0.023) and in (2/12) 16.7% of tigecycline sensitive isolates. Combined adeB and adeJ genes were found in 50% of tigecycline resistant isolates and only in (1/12) 8.3% of tigecycline sensitive isolates with statistical significance difference (P= 0.018).

Table 4: Distribution of efflux pump genes adeB, adeJ and adeG among tigecycline resistant and tigecycline sensitive *A. baumannii* isolates

Target gene	PCR result	Tigecycline sensitive		Tigecycline resistant		P value
		N=12	%	N=18	%	
AdeB	**Detected**	7	58.3%	13	72.2%	0.43
	Not detected	5	41.7%	5	27.8%	
AdeJ	**Detected**	2	16.7%	12	66.7%	**0.023***
	Not detected	10	83.3%	6	33.3%	
AdeG	**Detected**	0	0.0%	0	0.0%	-
	Not detected	12	100.0%	18	100.0%	
Combined adeB & adeJ	**No**	11	91.7%	9	50.0%	**0.018***
	Yes	1	8.3%	9	50.0%	

[#] Chi square test, * significant

On comparing adeB and adeJ gene expression, there was a significant difference between tigecycline resistant and tigecycline sensitive isolates (P=0.002, P<0.001 respectively). The transcript level of adeB and adeJ genes increased significantly and was correlated with tigecycline resistance.

Table 5: Comparison between tigecycline sensitive and tigecycline resistant *A. baumannii* isolates regarding gene expression

RT-PCR results of the detected genes		Tigecycline sensitive	Tigecycline resistant	P value
		N=12	N=18	
Ade B	**Mean ±SD**	3.1±1.2	8.5±4.4	**0.002***
	Range	1.462-4.37	3.85-16.35	
Ade J	**Mean ±SD**	1.8±0.9	63.1±16.6	**<0.001***
	Range	1.12-3.13	39.05-87.54	

#Mann-Whitney U-test, * significant

DISCUSSION

Our study was carried out on 150 different specimens, 30 (20%) *A. baumannii* strains were isolated. Lower prevalence rate was detected in Menoufia University Hospitals where 70/614 (11.4%) Acinetobacter were isolated[19]. *A. baumannii* represented 16.1% from the total Gram-negative isolates in Ain Shams University, Cairo[20]. This contrasted with the higher prevalence rate detected by Uwingabiye et al.[21] (24.8%) and Banergee et al.[22] in India (42.9%).

The isolation rates variations among studies could be linked with difference in hospital environment, different number of investigated specimens and change in patients' clinical conditions.

Susceptibility profiles of *A. baumannii* isolates exhibited that all the strains were MDR and showed sufficient susceptibility only to colistin. Seventy percent of the isolates were XDR i.e resistant to carbapenems too. Our results coincide with Nageeb et al.[23], in Ismailia, Egypt who stated that all isolated *A. baumannii* strains were MDR and the same results were obtained by Tolba et al.[20], Cairo, Egypt.

Aladel et al.[19]at Menoufia University Hospitals detected a lower prevalence 61.4% of MDR or XDR *A. baumannii.* Joshi et al.[24] also found that 62% of *A. baumannii* isolated from ICU in Varanasi, India, were Multi and extensively drug resistant.

In the current study tigecycline resistance was evaluated to be 60% (18/30) among *A. baumannii* isolates. Coincides with our results, Khodier et al.[25] in El-Fayoum General Hospital, Egypt, stated that tigecycline resistance was 58.5%. Higher rate was reported by Nazeih et al.[26] in ICU at Zagazig University Hospitals, as tigecycline resistance was 73%. Also Deng et al.[27] in China reported that tigecycline resistance is 86% (64/74).

On the other hand, Rani et al.[28] in Malaysia stated that all *A. baumannii* isolates in their study were colistin and tigecycline sensitive.

Inadequate implementation of infection control guidelines and inappropriate use of antibiotics may be the cause of this high level tigecycline resistance.

This study revealed that statistically significant association occurs between (increasing of patients age (P=0.002), prior antibiotics administration (P=0.036) including penicillin, flouroquinolones, aminoglycosides and carbapenems, increasing length of hospital stay (P=0.018), and patients on mechanical ventilation (P=0.033) and isolation of tigecycline resistant strains.

Jiang et al.[29] reported that exposure to penicillin, fluoroquinolones and aminoglycosides were predictors for acquiring tigecycline resistance. Also valuable statistical association was found in cases with tigecycline resistance and patients' characteristics including sex (male), age (elderly), some underlying diseases (diabetes and hypertension) and mechanical ventilation.

Deng et al.[27] found no valuable statistical association between tigecycline resistance and sex, age of patient, duration of hospital stay and antibiotic therapy during the last month.

Selective pressure caused by other antibiotics may result in tigecycline non-susceptibility. The use of carbapenems might stimulate resistance to antibiotics other than carbapenems, including tigecycline, so carbapenems might be a powerful inducer of MDR *A. baumannii*, thus it should be used in a more reasonable way to lessen the spread of these strains[27].

Mechanical ventilation is an important risk factor; thus more attention is required by nursing staff to perform the proper disinfection procedures to ventilator to prevent respiratory tract infections.

Participation of RND efflux pumps in tigecycline resistant *A. baumannii* has been reported in several studies[27, 30-31].

The current study showed adeB gene in (13/18) 72.2% of tigecycline resistant strains and (7/12) 58.3% of tigecycline sensitive isolates. AdeJ was detected with statistical significant association (P= 0.023) in (12/18)

66.7% of tigecycline resistant strains and in (2/12) 16.7% of tigecycline sensitive isolates. On comparing adeJ and adeB gene expression, a significant difference was found between tigecycline resistant and tigecycline sensitive isolates with their transcription level increased significantly and correlated with tigecycline resistance. This indicated that over-expression of adeJ and adeB efflux pumps is a crucial mechanism in *A. baumannii* leading to diminished tigecycline susceptibility.

Deng et al.[27], detected adeB in 54 (84.4%) tigecycline resistant isolates and 8 (80%) tigecycline sensitive isolates. The adeIJK, adeFGH efflux pumps Genes were exhibited in 54 (84.4%) and 57 (89.1%) of tigecycline resistant strains respectively. They reported that adeB, adeJ and adeG genes expression was (means ± standard deviation [SD], 66.26 ± 82.53, 2.97 ± 4.57 and 2.85 ± 10.11) respectively in tigecycline resistant strains while it was (2.32 ± 1.18, 3.64 ± 8.25, 0.89 ± 0.82) respectively in tigecycline sensitive isolates. With no statistically important differences were found in the mean levels of adeJ and adeG expression between tigecycline resistant and tigecycline sensitive isolates. Thus other mechanisms of resistance were proposed to be involved.

Ruzin et al.[33] showed that, increased expression of adeABC is a prevalent mechanism for lowering tigecycline susceptibility among the 106 *A. baumannii* collected isolates.

Yang et al.[34] declared that the adeABC efflux pump had a major role in tigecycline resistance through Acinetobacter species.

Ardehali et al.[35] detected adeJ gene in 76/78 (98%) tigecycline resistant *A. baumannii* isolates. Quantitative analysis indicated that its expression increased from 32 to more than 256 times compared to ATCC strain.

Our results were dissimilar to a previous observation that adeABC genes were not expressed in tigecycline sensitive *A. baumannii* isolates[32].

In the present work, 9 (50%) tigecycline resistant isolates were identified as co-carrying adeJ and adeB genes simultaneously (P=0.018), while only one (8.3%) tigecycline sensitive strain carried both of them.

It was found that adeIJK acts with adeABC in a synergistic manner to expel compounds such as tigecycline and that tigecycline MIC decreases with a 3 and 8 fold after inactivation of adeIJK or adeABC, respectively, while inactivation of both pumps results in an 85 fold decrease[36].

CONCLUSION

We have documented emergence and wide spreading of tigecycline resistant *A. baumannii* strains in Benha University Hospital. This study indicates a significant role of RND type efflux pumps (adeB and adeJ) in tigecycline resistance of *A. baumannii*. Efforts should be done to develop efflux inhibitors as a possible way to control antibiotics resistance in nosocomial pathogens, appropriate antibiotic stewardship programs and surveillance are needful to control dissemination of tigecycline resistance among high-risk patients.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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