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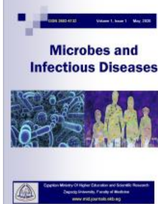

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Original article

Genetic Expression of *AdeR* and *AdeS* Genes in Multidrug-Resistant *Acinetobacter spp.,* Isolated from Patients in Menoufia University Hospitals

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ABSTRACT

Background: *Acinetobacter* is a serious nosocomial pathogen causing critical morbidity and mortality. The drug resistance of this organism is alarmingly high leaving few options for treatment. Numerous mechanisms are involved in its resistance to drug therapy. The active efflux mechanism is an important factor for the development of multidrug-resistant *Acinetobacter* which is regulated by the AdeRS operon. **Objective:** The aim of this study was to determine the expression of *AdeR* and *AdeS* genes in multidrug-resistant strains of *Acinetobacter* isolated from patients in Menoufia University Hospitals. **Methods:** This study included 100 strains of drug-resistant *Acinetobacter* isolated from patients in Menoufia University Hospitals. They were collected from different clinical samples. *Acinetobacter* strains were identified and their antibiotic susceptibilities were determined. Real-time PCR was performed to detect the expression of *AdeR* and *AdeS* genes. **Results:** The resistance of *Acinetobacter* isolates to tested antibiotics were (94%) to piperacillin-tazobactam, (90%) to ampicillin-sulbactam, (80%) to ceftazidime, (55%) to levofloxacin, (50%) to amikacin, and (~52%) to carbapenems. The *AdeR* and *AdeS* genes were expressed in (84%) and (88%) of isolates, respectively. The *AdeR* and *AdeS* genes were expressed in (88.4%) and (90.7%) of multidrug-resistant strains, respectively. **Conclusion:** The majority of *Acinetobacter* isolates are highly resistant to the most commonly used antibiotics. Also, high expression of adeRS genes may be responsible for the observed resistance among *Acinetobacter* isolates that demonstrate the possible role of efflux pump regulator genes in multidrug-resistant *Acinetobacter*.

Introduction

Acinetobacter is an aerobic, non-motile, nonfermenting, oxidase negative, catalase-positive, Gram-negative, opportunistic pathogen that plays an important role in nosocomial infections of immunocompromised patients [1]. This opportunistic bacterium is resistant to several types of antibiotics and responsible for many infections, including surgical wound infection, meningitis, ventilator-associated pneumonia, urinary tract infection, and bacteriemia [2]. It is considered one of the six most important multidrug-resistant micro-organisms in hospitals, especially in intensive care units (ICUs). Infections with this pathogen are often associated with high rates of morbidity and mortality [1]. *Acinetobacter* is widely distributed in nature and hospital environments because it can survive on both moist and dry surfaces and colonize human skin and respiratory tract [3]. *Acinetobacter* does not have fastidious growth requirements. It can

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grow at various temperatures and pH conditions [4]. *Acinetobacter* has attained resistance to most classes of known antibiotics. The remarkable ability of *Acinetobacter spp.,* to upregulate or acquires resistance determinants, makes it one of the organisms threatening the current antibiotic era [5].

The major mechanisms of resistance generally include producing antimicrobial-inactivating enzymes, modifying targets, reducing the membrane permeability, forming a biofilm, and overexpression of the membrane active efflux system [6]. The efflux pumps play a great role in the multidrug resistance of Gram-negative bacteria. There are five families of efflux pumps, including the multidrug and toxic compound extrusion (MATE) family, the resistance–nodulation–cell division (RND) family, the adenosine triphosphate (ATP)-binding cassette (ABC) family, the major facilitator superfamily (MFS) and the small multidrug resistance (SMR) family. Among those families of pumps, the RND systems are the most prevalent in MDR *Acinetobacter* [7]. The major clinically relevant in the RND efflux system is AdeABC efflux pump. Its overexpression is responsible for pumping out most antimicrobials [8].

The AdeABC consists of three structures; a transmembrane AdeB, an inner membrane fusion protein AdeA and an outer membrane protein AdeC which are chromosomally regulated by adeS (sensor kinase) and adeR (response regulator). The adeRS operon is located upstream of adeABC operon and is transcribed in the opposite direction [9]. The two-component regulatory system in signal transduction pathways. The AdeR protein (regulator), is a typical transcriptional regulator and the AdeS protein (sensor kinase) is shorter than the AdeR protein and has bacterial histidine kinase activity. The two proteins work together to regulate efflux pump gene expression in response to environmental stimuli [10]. Although the search for novel antimicrobials remains an important concern, it is a more urgent priority to investigate novel mechanisms of resistance in *Acinetobacter* to minimize hospital-acquired infections [11]. This work aims to study *AdeR* and *AdeS* genes expression in multidrug resistance *Acinetobacter*.

Material and Methods

Bacterial isolation and identification

This study was carried out by using 100 non-repetitive drug-resistant *Acinetobacter* isolates collected from different clinical specimens including sputum, endotracheal aspirate, bronchoalveolar aspirate, urine, blood, and wound swabs from patients in Menoufia University Hospitals. All the selected patients were subjected to complete history (personal, clinical, associated comorbidities, history of drug intake, and length of hospital stay before sampling).

The samples were obtained from patients by using standard microbiological sample collection methods. They were cultured on blood agar and MacConkey agar (Bio-Rad, USA) at 37°C for 18-24h. Each non-lactose fermenting colony on MacConkey agar media was picked up, further identified by microscopic examination using Gram stain, culture characteristics, and standard biochemical reactions (Triple Sugar Iron, citrate, oxidase, catalase, indole, urease, motility testing, and ornithine decarboxylase).

Antimicrobial susceptibility testing

Susceptibility tests were performed by disk diffusion method on Mueller Hinton agar plates (Bio-Rad, USA) according to CLSI 2018. The following antibiotics were used: ceftazidime (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), amikacin (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), piperacillin/tazobactam (100/10 µg), doxycycline (30 µg), ampicillin-sulbactam (10/10 µg), levofloxacin (5 µg), tobramycin (10 µg), imipenem (10 µg), meropenem (10 µg), tetracycline (30 µg), and gentamicin (10 µg). Plates were incubated at 35 ± 2°C in ambient air for 20–24 h.

RNA extraction and PCR analysis of *AdeR* and *AdeS* genes

All clinical isolates of *Acinetobacter spp.,* were tested for the expression of *AdeR* and *AdeS* genes by real time PCR. Total RNA extraction was performed by Gene JET RNA Purification Kit (Thermo Scientific, USA). Fresh pure colonies were obtained after overnight growth on MacConkey plates. RNA extraction was performed according to the instructions of the manufacturer's protocol [12,13].

Total extracted RNA from all isolates were reverse transcripted into complementary DNA (cDNA) using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) [14].

The expression of *AdeR* and *AdeS* genes was determined using 7500 a Real-Time Fast PCR instrument (Applied Biosystem). The RT-PCR reaction mixture was prepared in a volume of 20 µL comprised of 10 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, USA), 1 µl of forward primer for each gene, 1 µl of reverse primer for each gene as shown in **table (1)** (Thermo Scientific, USA) and 8 µl of cDNA and nuclease-free water. The PCR reaction was carried out under the following conditions: 1 cycle for 10 min at 95 °C for the initial denaturation, 40 cycles (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s) [15]. For each sample, the threshold cycle (Ct) of the target genes (*AdeR* and *AdeS*) was determined and normalized to the Ct value of the *rpoB* housekeeping gene.

Table 1. Primers' sequences of *AdeR*, *AdeS* and *rpoB* genes**.**

Gene	Primer sequence
AdeR	Forward:5′- AAAACGTGAAGGCATGAGTG -3′ Reverse :5′- CTTCCCAACCGTTTAATTCG -3′
AdeS	Forward: 5′- ACCGAGTTCCAAGACGAT -3′ Reverse :5′-CCTTTCAGTGCCACAATA -3′
rpoB	Forward: 5-′GGTCCTGGTGGTTTAACACG -3′ Reverse :5′- CGAATAACGATACGAGAAGCA -3′

Designed based on *adeR* and *adeS* gene region of *Acinetobacter* strains in the NCBI database.

This study was approved by the Research Ethics Committee. Written informed consent was obtained from all participants or their relatives

Statistical analysis

Data were collected, tabulated, and statistically analyzed by an IBM-compatible personal computer with SPSS statistical package version 23 (SPSS Inc. Released 2015. IBM SPSS statistics for windows, version 23.0, Armnok, NY: IBM Corp.)

Results

Bacterial isolates

As shown in **table (2)**, 85% of patients with *acinetobacter* infections had associated co-morbidity, and 87% of patients infected with *Acinetobacter spp.,* were exposed to an invasive procedure and exposed to previous antibiotic therapy. The highest isolation of *Acinetobacter* was from respiratory samples (sputum, endotracheal aspirate, and bronchial aspirate) (67%), followed by blood (23%), wound swab (6%), and urine (4%).

Antibiotic susceptibility (%)

Regarding antimicrobial susceptibility, *Acinetobacter* isolates were resistant to piperacillin-tazobactam (94%), ampicillin –sulbactam (90%), tobramycin (81%), doxycycline (80%), tetracycline (78%), gentamycin (72%), trimethoprim- sulfamethoxazole (69%), cefotaxime (67%), ceftazidime (66%) ,ciprofloxacin (64%), levofloxacin (55%), meropenem (53%), Imipenem (52%) and amikacin (50%), as shown in **table (3)**.

Regarding antibiotic susceptibility, the group I isolates represented (14%), while group II and III represented (28%), (58%), respectively, as shown in **table (4)**.

Distribution and analysis of *AdeR* and *AdeS* genes

The PCR results demonstrated a wide distribution of *AdeR* and *AdeS* genes among *Acinetobacter spp.,* tested in this study. The distribution of *AdeR* and *AdeS* gene expression differed significantly from group I to group II and III with *p* values (*0.009*) and (*0.04*), respectively as shown in **table (5)**.

CT of the expressed *AdeR* and *AdeS* genes differed significantly from group I to group II and III with *p* value (*<0.001*) for each gene as shown in **table (6)**.

There was a highly significant positive correlation between the two expressed genes in *Acinetobacter* isolates expressing both *AdeR* and *AdeS* genes (84) with *p*-value (*<0.001*) and r_s (Spearman correlation coefficient) (0.81) as shown in **figure (1)**.

Table 2. Demographic and clinical data of patients with *Acinetobacter* infections.

Variables	*Acinetobacter* isolates (n =100)
Age:	
Mean ± SD	50.24±20.76
Median	53.5
Range (Min.-Max.)	1-80
Sex:	
Male	55%
Female	45%
Invasive procedure:	
Yes	87%
No	13%
Previous Antibiotic intake:	
Yes	87%
No	13%
Associated co-morbidity:	
Yes	85%
No	15%
Type of sample:	
-Total respiratory samples	**(67) %**
Endotracheal aspirate	10%
Sputum	46%
Pleural effusion	2%
Bronchoalveolar lavage (BAL)	9%
- Blood culture	**23%**
- wound swab	**6%**
-Urine	**4%**

Table 3. Antibiotic susceptibility (%) of *Acinetobacter spp.,* isolates.

Antibiotic	Antibiotic disc concentration (μg)	*Acinetobacter* isolates (n =100)	
		S	R
Ampicillin sulbactam (SAM)	10/10	10%	90%
Piperacillin-tazobactam (TZP)	100/10	6%	94%
Cefotaxime (CAZ)	30	33%	67%
Ceftazidime (FEP)	30	34%	66%
Imipenem (IPM)	10	48%	52
Meropenem (MEM)	10	47%	53%
Tetracycline (TE)	30	22%	78%
Doxycycline (DO)	30	20%	80%
Ciprofloxacin (CIP)	5	36%	64%
Levofloxacin (LEV)	5	45%	55%
Gentamycin (CN)	10	28%	72%
Tobramycin (TOB)	10	19%	81%
Amikacin (AK)	30	50%	50%
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	31%	69%

S: Sensitive, R: Resistant

Table 4. Drug resistance patterns of *Acinetobacter* isolates.

Group	Pattern of resistance	Percentage
Group I	(isolates resistant to 1or 2 groups of antibiotics)	14%
Group II	MDR	28%
Group III	XDR	58%

MDR, multidrug resistant *Acinetobacter* isolates resistant to at least one agent in three or more antimicrobial categories. XDR, extensive drug resistant *Acinetobacter* isolates that are resistant to at least one agent in three or more antimicrobial categories (MDR) and also resistant to carbapenems.

Table 5. Comparison between the different groups of *Acinetobacter* isolates regarding the genetic expression of *AdeR* and *AdeS* genes.

Genes	*Acinetobacter* isolates				Total		Test of significance (FE)	P-value
	Group I n= (14)		Group II&III n = (86)					
	N	%	N	%	N	%		
***AdeR* gene:**								
Expressed	8	57.1	76	88.4	84	84	8.74	***0.009****
Not expressed	6	42.9	10	11.6	16	16		
***AdeS* gene:**								
Expressed	10	71.4	78	90.7	88	88	4.23	***0.04****
Not expressed	4	28.6	8	9.3	12	12		

FE: Fischer exact test

Table 6. Comparison between the different groups of *Acinetobacter spp.,* isolates regarding CT of expressed *AdeR* and *AdeS* genes.

Expressed Genes	*Acinetobacter isolates*		Test of Significance (t)	*P-value*
	GroupI (No = 8)	Group II&III (No = 76)		
CT of expressed *AdeR* gene				
Mean ± SD.	30.5±4.7	12.5±1.4	10.716	***<0.001****
Median	31.99	12.5		
Range	23.65-34.8	9.75-15.9		
	GroupI (No = 10)	**Group II&III (No = 78)**	**t**	***P-value***
CT of expressed *AdeS* gene				
Mean ± SD	39.6±5.02	14.7±1.8	15.533	***<0.001****
Median	39.2	14.6		
Range	32.84-45.4	11.9-19.14		

t: Student t-test, **highly significant

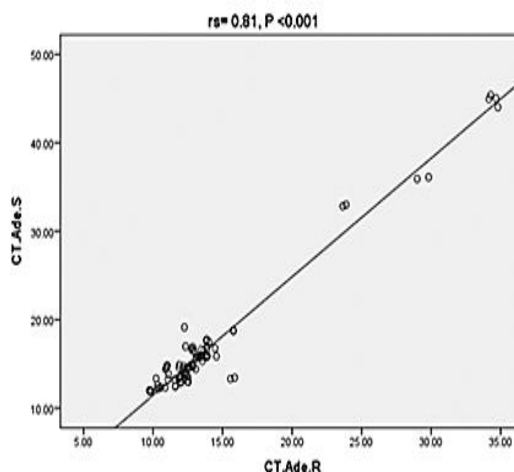

Figure 1. Correlation between expressed *AdeR* and *AdeS* (N=84).

Discussion

Acinetobacter is a common Gram-negative opportunistic pathogen. The infection of *Acinetobacter* is widespread, especially in intensive care units. The emergence of multidrug-resistant *Acinetobacter* and extensively drug-resistant *Acinetobacter* brings great challenges to global healthcare workers [16].

The goal of this study was to determine the distribution and expression of *AdeR* and *AdeS* genes in drug-resistant *Acinetobacter* isolates collected from patients in Menoufia University Hospitals.

In this study, it was documented that most *Acinetobacter* infected patients had previous antibiotics therapy (87%), were exposed to invasive procedures (87%), and had associated co-morbidities (85%) like diabetes, hypertension, chronic lung diseases, chronic liver diseases, chronic renal insufficiency, and malignancy. These results coincided with the results of other studies [17].

In this study, the *Acinetobacter spp.,* isolates were most commonly isolated from respiratory samples (67%) followed by blood (23%), wound swabs (6%), and urine samples (4%). This was by other studies in Egypt [18-20] and with a study in Lebanon who found that the majority of the isolates were recovered from respiratory samples [17] but these results did not go with the results of other studies [5,21,22].

The results of antimicrobial susceptibility testing for *Acinetobacter spp.,* isolates showed that they were highly resistant to piperacillin-tazobactam (94%), ampicillin-sulbactam (90%), tobramycin (81%), doxycycline (80%), tetracycline (78%), gentamycin (72%), trimethoprim-sulphamethoxazole (69%), cefotaxime (67%), ceftazidime (66%), ciprofloxacin (64%), levofloxacin (55%), meropenem (53%), imipenem (52%) and amikacin (50%). This was in agreement with a study in Taiwan [23] and a study in Egypt that documented higher resistance rates [20].

In this study, the resistance of *Acinetobacter* to carbapenems (imipenem and meropenem) was 52% and 53%, respectively. This was by other studies in Egypt [20,24,25]. In the Middle East and North Africa, there were different resistance rate of *Acinetobacter* to imipenem as following; 25.2% in a study in Kuwait [3], 47.9% in a study in Algeria [26], 80 % in a study in Turkey [27], 92.2% in a study in Saudi Arabia [1], 75.7% in a study in Morocco [28] and 87% in a study in Tunisia. [29].

This may be due to the resistance mechanisms have regional differences which may be caused by different phenotypes or genotypes of the clinically collected strains from different countries.

In this study 14% of *Acinetobacter* isolates were resistant to 1or 2 groups of antibiotics,28% were MDR and 58% were XDR. High multidrug resistance was also found in other studies [30,31]. The threatening ability of *Acinetobacter spp.,* to develop multidrug-resistance patterns was caused by either mutations or genetic elements (such as integrons, plasmids, transposons, or resistant islands) acquisition [32]. Inadequate usage of antibiotics in the community and hospitals, the lack of adequate infection control measures together with the lack of reporting, all contribute to the rise in resistance and promote the organism to acquire and express novel resistance mechanisms [33].

This study revealed that *AdeR* and *AdeS* genes were expressed in (84%) and (88%) of isolates, respectively. This went with **Atasoy et al.** where 88% of all *Acinetobacter* isolates carried *AdeR* and *AdeS* genes [10]. This also coincided with **Noori et al.** who documented that the distribution of *AdeS* and *AdeR* genes among *Acinetobacter spp.,* strains were 91%, and 77%, respectively [34] while **Lari et al.** documented that 36% of 50 clinical *Acinetobacter* isolates carried *AdeR* and *Ades* genes, simultaneously [35].

In this study, group I isolates harbored *AdeR* and *AdeS* genes by 57% and 71%, respectively. These results were higher than the results of **Atasoy et al.** who found that 33% of *Acinetobacter* strains sensitive to imipenem, meropenem and gentamycin carried *AdeR* and *AdeS* [10].

Strains resistant to 3 or more groups of antibiotics harbored *AdeR* and *AdeS* genes by 88.4% and 90.7%, respectively. This went with **Hou et al.** study in china which showed that 80% of Acinetobacter isolates resistant to imipenem, carried *AdeR* and *AdeS* genes [36]. This also was in accordance with the **Asadolah-Malayeri et al.** study in Iran where the prevalence of *AdeR* and *AdeS* genes among the *Acinetobacter* isolates were 98.3% and 60%, respectively in multidrug-resistant *Acinetobacter* isolates [8]. **Hassan et al.** found that *AdeR* and *AdeS* genes were positive in 96.8%, 63.4% of MDR isolates, respectively [9]. **A study by Noori et al.** from Iran found that the frequency of the *AdeR* and *AdeS* genes among *Acinetobacter* isolates that were 98% resistant to imipenem were 77% and 99%, respectively, in agreement with our results [34].

In this study, the expression of *AdeR* and *AdeS* genes significantly increased in Group II and III than in group I. These results went with **Dou et al.** who noticed that the mRNA expression of *AdeR* and *AdeS* genes increased 2.45-9.44 times in drug-induced resistant strains than the parental sensitive strains [37] but did not go with **Chen et al.** who found that the expression of *AdeR* gene in CRAB was decreased (3.3fold) compared by CSAB with no significant difference of the relative expression of *AdeS* [38]. **Atasoy et al.** found that strains of *Acinetobacter* that were sensitive to imipenem, meropenem, and gentamycin antibiotics and resistant strains to the same antibiotics showed similar levels of *AdeR* and *AdeS* genes expression [10]. **Kuo et al.** found that the level of *AdeR* expression decreased significantly in mutant resistant strains of *Acinetobacter* than from parental sensitive strains upon exposure to imipenem [7].

Based on *the* AdeRS finding from this study, the expression of *AdeR* and *AdeS* genes may be responsible for the resistance among *Acinetobacter* isolates to several antibiotics. The antibiotic policy in hospitals should be continuously evaluated to avoid irrational prescription of antibiotics and to treat infections according to their antibiotic susceptibility patterns.

Conclusion

Upon these results expression of *AdeR* and *AdeS* genes could be one of the controlling factors of AdeABC efflux pumps which play an important role in multidrug resistance of *Acinetobacter*. Further studies should be carried out to understand the other possible mechanisms of *Acinetobacter spp.,* drug resistance and for the development of novel therapeutic strategies.

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