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Detection of Acra and Acrb Genes in Locally Isolated Carbapenems Sensitive Enterobacter Aerogenes

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Abstract
Urinary Tract Infections (UTIs) are serious bacterial infections. In this study, which was extended from July/2018 to February/2019, 50 urine samples of confirmed urinary tract infection cases were collected. Of which, 24 cases were caused by *Enterobacter aerogenes*. The results show that 24 samples of them were caused by *Enterobacter aerogenes*, while the rest were caused by *E.coli*, *Acinetobacter* spp. The identification of the isolated bacteria was achieved using Vitek system, which was also used to do antibiotic sensitivity test. The results showed that the isolated *Enterobacter aerogenes* isolates were sensitive to carbapenems antibiotics, e.g imipenem and meropenem. Polymerase chain reaction (PCR) was used to detect some of the genes that are known to contribute in carbapenems resistance mechanism such as acriflavine (Acr) Efflux System. Surprisingly, the results showed that isolated *Enterobacter aerogenes* have some of acriflavine efflux genes, AcrA and AcrB, and these genes are important architectural genes of acriflavine (Acr) Efflux System. The obtained results clearly indicated that these sensitive strains have the capabilities to shift to resistance ones when these genes start expressing themselves under certain environmental conditions. The results are also prime importance and can explain the increasing frequency of the UTIs that can resist carbapenems treatment.

Keywords: carbapenems-resistance, acriflavine (Acr) Efflux System and Enterobacter aerogenes.

INTRODUCTION

The inability to treat common urinary tract infections effectively has the risk to be become a global health crisis and international organisations such as WHO and CDC, are regularly warning the international community about the emerging of resistant bacterial strains for multiple antibiotics types (MDR). Therefore, the investment in the search for new therapeutic agents are instantly needed (1). The extensive use of antibiotics and antimicrobial substances has caused emerging multiple-antibiotic resistant bacteria in various environments. These environmental stresses are leading to develop new defence strategies such as enzymatic inactivation or decreasing the cell permeability for the therapeutic agents. Also, Bacteria have an innate ability of bacteria to use mechanism to extrude a wide range of substrates through expression-overexpression of efflux pumps, which has recently been intensively researched (2). Of interest, efflux pumps have drawn much attention in the recent years. Generally, efflux pumps are classified into five different family: the ATP Binding Cassette (ABC) superfamily; the major facilitator superfamily (MFS); the Multidrug And Toxic Compound Extrusion (MATE) family; the small multidrug resistance (SMR) protein family and the

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Resistance-Nodulation-Division (RND) family. The pumps can either be specific or nonspecific for multiple substrates such as (3)(4)(5).

Enterobacter aerogenes uses pumps to eliminate toxic effects of many external substances such as antibiotics but also can be used to eliminate toxic metabolites or extrusion these metabolites into the external environments. Usually, Acriflavine (Acr) efflux system contributes to extrude toxic secondary metabolites and compounds that have a signaling role (6).

Carbapenems are unique group of β-lactams derived antibiotics with broad spectrum activity against gram-positive and gram-negative bacteria, which are resistant to hydrolysis by most β-lactamases. Carbapenems is thought to be extruded out the bacterial cells using efflux pumps such as acriflavine efflux pump Therefore, this class of antibiotics are currently used to treat multidrug resistance infections and are though to be "last-line agents" or "antibiotics of last resort" (7). The recent emergence of multidrug-resistant pathogens in Iraq and worldwide for carbapenems has threaten this class of lifesaving drugs (8). To overcome this problem, substantial efforts are given to better understand the fundamentals of antibiotics efflux mechanisms. Exploring the distribution of RND pump genes among bacterial that are sensitive to carbapenems is an important attempt to predict the development of bacterial resistance to the carbapenems and would help to rationalize the prescription of this group of antibiotics.

The aim of this study is to isolate carbapenems-sensitive *Enterobacter aerogenes* in patients with confirmed UTIs and detect the existence of some of Acr efflux pump genes within these sensitive isolates. Materials and Methods

Samples collection

50 urine samples from patients with confirmed UTIs, who admitted to Al-Husseini teaching Hospital, Karbala, Iraq, were collected. Urine samples were collected of midstream in sterilised cap. The samples were initially cultured on blood agar and incubated at 37 ℃, which showed a growth of around 10^5 cfu/ml and then sub-cultured on nutrient agar.

Bacterial Identification

Pure colonies of the isolated bacteria were selected using Nutrient agar and 5% sheep blood agar. Preliminary, the isolates were stained with gram stain. Subsequently, Eosin methylene blue agar (EMB) was used where *Enterobacter aerogenes* colonies are pinkish colonies, mucoid and larger colonies than *E. coli*, wherease *E.coli* have a dark center and greenish metallic sheen.

Confirmation of Identification was performed with the Vitek 2 compact (bioMerieux Inc. USA) system using GN ID REF21341 (identification-Gram-negative bacteria) card. Procedures were conducted following the manufacturer's instructions. AST-N291 (Gram-negative bacilli) cards were used to determine antibiotic susceptibility and the results were interpret using Vitek 2 compact software version 07.02.

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PCR Protocol

The DNA was extracted by using GeNet Bio kit (Korea, Cat. no. K-3000) and used as a template for polymerase chain reaction (PCR). Table 1 shows the used primers for the acrA, and acrB genes.

Table 1 List of PCR primers

Gene	Primer sequence (5′-3′)	Length of amplicon (bp)	Reference
acrA	F: TTGAAATTACGCTTCAGGAT R: CTTAGCCCTAACAGGATGTG	189	(9)
acrB	F: CGTACACAGAAAGTGCTCAA R: CGCTTCAACTTTGTTTTCTT	183	(9)

RESULTS AND DISCUSSION

Isolation and Identification

The results show that 48 % of the admitted cases of urinary tract infections were mainly caused by *Enterobacter aerogenes* and the same percentage was caused by *E.coli*. This percentage is globally reported for the causative agents of UTIs (10). Urinary tract infections can be caused by mono-bacterial species or mix of bacterial species; however, the highest percentage of the uncomplicated UTIs causes by mono-bacterial species. *E.coli* has shown to be a common UTIs pathogen, while other bacteria can cause the infection as well as other pathogens such as *Proteus mirabilis and Enterobacter aerogenes* (10), but this was not the case in the current study (Table 2). *Enterobacter aerogenes* caused 48% of the confirmed UTIs infection and share the same percentage with *E.coli.* Therefore, *Enterobacter aerogenes* is an important causative agent for UTIs locally and this can be explained by living habits of the residents as well as their lifestyle.

According to statistics from the Center of Disease Control (11), women are likely to experience at least one symptomatic UTI during their lifetime and of them, young and sexually active women have the highest incidence of UTIs. On the other hand, the prevalence of UTIs is significantly lower in men than in women and it can primarily happen in males with abnormalities in their urological structure or elders (Table 3). This could be due to the anatomy of women where they have short distance from the urethra to the anus and the urethral opening to the bladder. In addition, sexual intercourse can increase the possibility of cystitis (bladder infection).

Table 2: Bacteria distribution and their prospective frequency

Causative bacteria	***Frequency (%)***
Enterobacter aerogenes	(48%)

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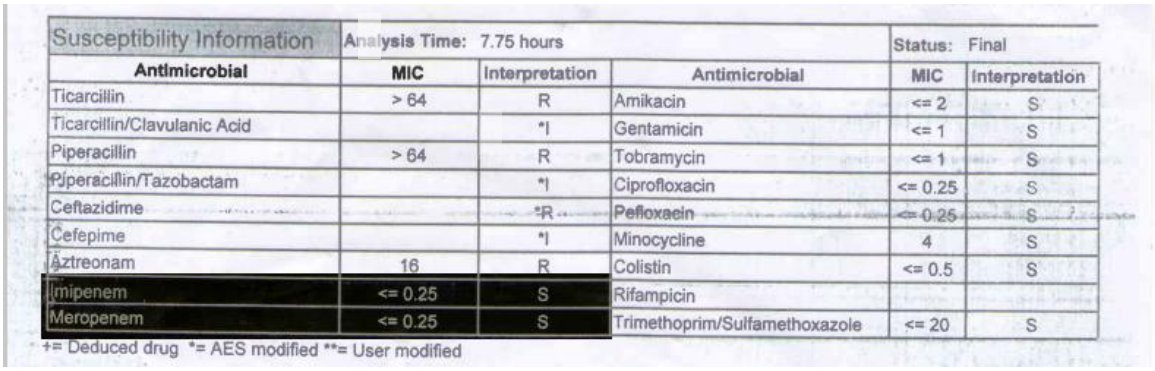
Escherichia coli	48%
Acinetobacter spp	2%

Table 3: Enterobacter aerogenes distribution according to gender

UTIs infections caused by *Enterobacter aerogenes*	
Gender	**Percent**
Female	(85%)
Male	(15%)

Vitek 2 compact was used to identify bacterial species as well as screen the susceptible of the isolated bacteria for the commonly used antibiotics in Iraqi hospitals and private clinics. The tested antibiotics ranges from Beta-lactams, macrolides, aminoglycosides and Extended-spectrum beta lactam, carbapenems. Figure 2 shows the microbiological report of the Vitek2 compact for the covered antibiotics and their obtained interpretation, R for resistance and S for sensitive.



Susceptibility Information — Analysis Time: 7.75 hours — Status: Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	> 64	R	Amikacin	<= 2	S
Ticarcillin/Clavulanic Acid		*I	Gentamicin	<= 1	S
Piperacillin	> 64	R	Tobramycin	<= 1	S
Piperacillin/Tazobactam		*I	Ciprofloxacin	<= 0.25	S
Ceftazidime		*R	Pefloxacin	<= 0.25	S
Cefepime		*I	Minocycline	4	S
Aztreonam	16	R	Colistin	<= 0.5	S
Imipenem	<= 0.25	S	Rifampicin		
Meropenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	<= 20	S

+= Deduced drug *= AES modified **= User modified

Figure 2: Vitek 2 compact report for carbapnems-sensitive *Enterobacter aerogenes*

Figure 2 shows that *Enterobacter aerogenes* is sensitive for both Imipenem and Meropenem, carbapenems antibiotics. The sensitivity was also confirmed using disc diffusion method, using 10 mcg concentration for both antibiotics. As this bacteria show sensitive to these antibiotics, It would be thought that it lacks the resistance mechanism for the these antibiotics. The other question addressed in this study, is the detection of the Acr efflux pump genes. Acriflavine efflux pumps can provide resistance to a broad range of antimicrobial such as antibiotics, biocides and heavy metals by facilitating the extrusion of these compounds out the bacterial cells and carbapenems have been targeted in this study (7).

Detection of AcrA and AcrB genes using Polymerase Chain Reaction

Bacterial use acriflavine efflux pump to extrude antibiotics out of the cells and carbapenems are among these antibiotics (11). Acr A and Acr B are main structural genes of the Acr efflux pump and are crucial to its function. Therefore, as a part of the current study, acrA and acr B, are detected, which are important to understand the potential mechanism that can be developed to resist carbapenems (7). Structurally, this pump is composed of AcrA and AcrB

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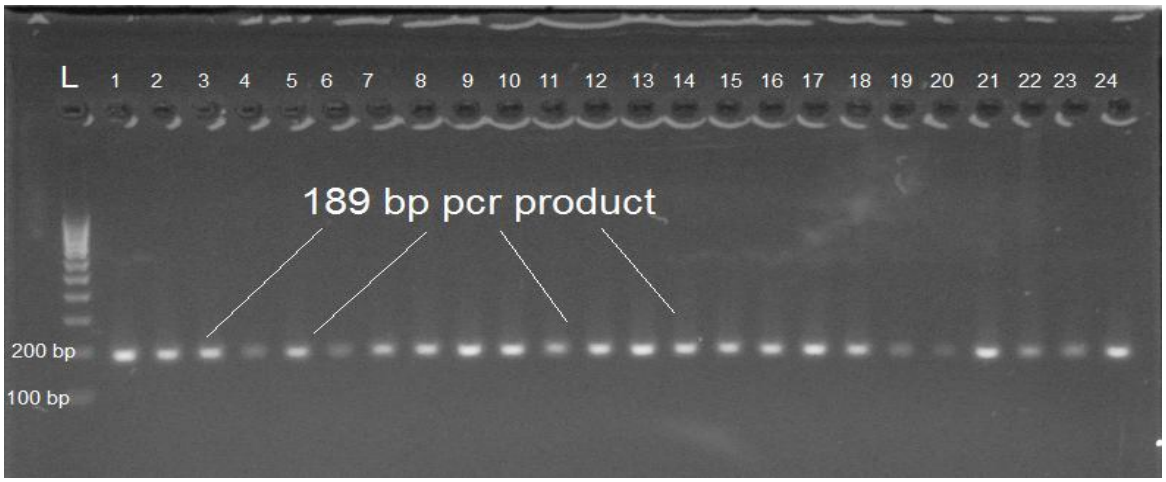
and TolC as functional genes, while AcrR, Acr S are used to regulate expression of acrAB (12). The isolated *Enterobacter aerogenes* are found to have both AcrA and AcrB and they are sensitive to imipenem and meropenem at the same time, which would suggest the probability to become resistance once the suitable conditions are available. Sensitive bacteria can naturally become resistant due to certain genetic chromosomal changes (in 10-20% of the cases) or extrachromosomal (less than 80% of the resistance cases) (12).

The exposure to antibiotics is not the cause in itself for the manifestation of the bacterial resistance to drugs. Expression of this efflux system is strongly regulated on transcription levels to facilitate the adaptation of bacteria to external stimulation (13). AcrR and AcrS are local transcription regulation factors that regulates AcrAB-TolC system, and lack of any of its constitute would loss its function as a consequence (14).

The importance of the current study is to follow the evolution of antibiotic resistance in the bacterial population in order to prevent and repress the emergence of multidrug-resistant strains of *Enterobacter aerogenes* that can still be treated with antibiotics.

(A)



(B)

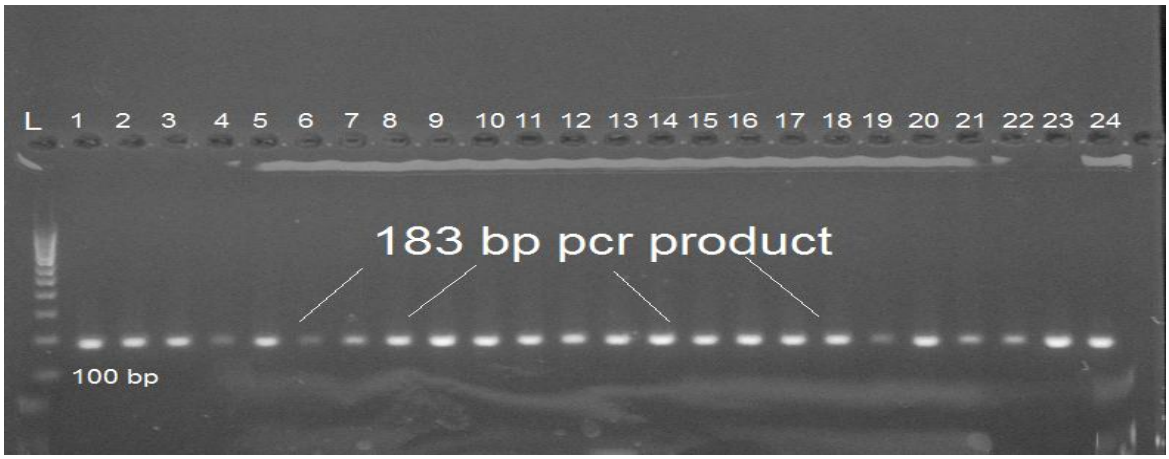


Figure 3: PCR products. (A) acrA gene in the isolated bacteria. (B) acrB gene. First lane shows the ladder and other lanes show PCR products

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CONCLUSIONS

The increased pressure from antibiotic usage that takes place in various environments, has led to evolve bacterial strains with high resistance profile. Uncomplicated UTIs are commonly caused by gram negative bacteria such as *E.coli*, however the results of the current study show that *Enterobacter aerogenes* is also share the same importance as uropathogenic bacterium with 48% causing rate .

Resistant profile in bacteria is not only due to the intensive use of antimicrobial but also because of the innate ability of bacteria to use various mechanisms to extrude toxic substances out of the microbial cells. One of these mechanisms are efflux pumps. The results of the current study show that even when the bacteria are sensitive, they still may have acriflavine efflux pumps genes that can give the potential to develop resistant profile. Therefore, understanding the mechanisms by which these pumps function and the range of substances that can be extruded by these pumps, would provide a very useful information about how to disturb them and prevent the bacteria from developing resistance.

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