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The expression of efflux pump AcrAB in MDR *Klebsiella pneumoniae* isolated from Iraqi patients

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Abstract

The mortality ratio was increased due to multidrug resistance (MDR) of pathogens, including *Kllebsilla pneumonia* (KP) which found to be widespread in hospitals with high resistance to all antibiotics that available in Iraq. Efflux pumps AcrAB-Tolc are belonging to RND (resistance nondulation division) family and it is intrinsic resistant chromosomal mechanism in KP bacteria. The results of gene expression using q(RT-PCR) that the main resistance of selective isolates of KP to Azithromycin is via efflux pump AcrAB and a significant difference was found before and after exposure to antibiotics. In addition, a positive correlation was observed between the gene expression of AcrA and the chloramphenicol concentration. The gene expression of the same gene was increased after the exposure to Imipenem with significant differences, while there was a decrease in expression and/or no significant difference in the gene expression of the same gene after the exposure to Amikacin and Ciprofloxacin, indicating that these bacteria are dependent on another source of resistance.

INTRODUCTION

Klebsiella pneumoniae (KP) the most important causes Nosocomial infaction belonging to enterobateriacecae gram-negative family [1], KP considered as opportunistic pathogens causes pneumoniae, urinary tract infections (UTIs), Bacteremia and liver abscess [2]; KP bacteria have many virulence factors that shared with other gram-negative pathogenic bacteria such as resistance to antibiotic especially that these acquired from hospitals with resistant to more than 80% of antibiotics [3].

Kllebsilla pneumonia have been developed multiple mechanisms to resist the antibiotics. These mechanisms include the expulsion of the antibiotic by the efflux pump, a protein-based structure, that eliminates undesirable substances in order to reduce the concentration of these substances within the bacterial cell and acts synergistically with decrease the membrane permeability [4]. The pumps can be divided into groups or families; (RND) family is the most important family including Gram-negative bacteria. Efflux pump AcrAB-Tolc is an example of the pumps containing the internal protein AcrB and the diffusion protein in preplasmic space AcrA and the channel that excrete the materials abroad TolC [5, 6]. The resistance mechanism by efflux pumps are the most important antibiotic resistance types because the efflux pump is able to remove more than one antibiotics such as B-lactam, floroquinolones, erythromycin and chloramphenicol [7].

The aim of this research is to collect many isolates of multidrug resistant *Klebsiella pneumoniae* bacteria as possible to detect and identification it by several identification methods. Also, this study aimed to detect the presence of efflux pump AcrA gene and measure the gene expression of it before and after the exposure to antibiotics to determine the correlation of AcrAB-TolC efflux pump with antibiotics.

MATERIALS AND METHODS

More than 100 isolates of urine, sputum, blood, wounds and burns were collected from the hospitals of the Medical City of Baghdad for the period from 1/10/2017 to 1/20/2018; all the samples were cultured on MaCconky agar and Blood agar and incubated at 37° C for 24 hours except the blood samples were incubated more than 48 hours; the growth isolated were examined by biochemical tests including oxidase, Catalase, IMVIC, KLI and also by using Vitek-2 (BioMérieux France) system, and also identification by identical gene 16S-23S rDNA internal transcribed spacer (ITS) using pneumoniae-specific primers [8].

Table 1: the specific primers used in the study

No.	Gene	Primer sequence	Primer	Product size	reference
1	16S–23S ITSD	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	F R	130	[8]
2	acrA	ATGAACAAAAACAGAGG TTTCAACGGCAGTTTTCG	F R	495	[9]
	Real Time PCR				
3	acrA in RT	GACTTGGTTTGTTCTGATGGCG CCGTCTGGAAGAAGGGATTAACC	F R		[10]
4	23s In RT	GGTAGGGGAGCGTTCTGTAA TCAGCATTCGCACTTCTGAT	F R		[12]

The antibiotic sensitivity test

The antibiotic sensitivity test was performed using two methods, the first one including minimal inhibitory concentration (MIC) method by using AST-GN76 vitek-2 kit which contains 17 antibiotics specific to the Gram-negative bacteria with different concentrations, while the second method is disk diffusion method; the two methods were performed according to Clinical and Laboratory Standards Institute (CLSI) [11] instruments; the tests were performed for 60 bacterial isolates and 26 isolates were selected for study AcrAB-TolC efflux pump.

Detection of the efflux pump AcrA using conventional PCR technique

This test was performed using specific primer of efflux pump AcrA gene (Table 1). Reaction conditions were initial denaturation step at 94° C for 5 min followed by 30 cycles consisting of denaturation (94° C for 1 min), annealing (52° C for 1 min) and extension (72° C for 1 min), followed by a final extension step at 72° C for 5 min**.**

The Extraction of the bacterial genome was done using Genaid (Thailand) and with AccuPower PCR PreMix Bioneer (Korea) amplification kit, then the product was loaded onto 2% agarose gel with ethidium bromide (5%).

Quantitative Real time- polymerase chain reaction (qRT-PCR)

The gene expression of efflux pump AcrA was performed by using quantitative RT-PCR using specific mRNA primers (Table 1); 23s internal gene was used as a house keeping gene to normalise mRNA levels. Reactions conditions as following: 40 cycles of denaturation (95° C, 15s), annealing (55° C, 30s), and extension (72°C, 45s) [12] using GoTag qPCR Master Mix GoTaq® 1-Step RT-qPCR System, MgCL2, Smart Cycler (Real time PCR) MIC (Australia). TRIzol™ Reagent Thermo Scientific (USA) kit was used for extraction of total RNA and its concentration measured by Quantus Florometer Promega (USA) kit. The threshold cycle (Ct) value was defined as the cycle number at which the fluorescence generated within a reaction crossed the threshold value, and the relative Ct value of target gene was compared with control by using Livak equation [13] as following:-

Folding $=2^{-\Delta\Delta ct}$

$\Delta\Delta Ct = \Delta$ Ct treated $- \Delta$ Ct control.

ΔCt = Ct gene – Ct Housekeeping gene.

Statistical analysis

The results were statistically analyzed using SPPS program using Student's T-test test. The results presented as Mean±S.E., and a differences of $p<0.05$ were considered statistically significant.

RESULTS

Collection and diagnosis of isolation

A 60 isolates of *K.pnumoniae* were collected, and these bacteria formed large colonies and mucosal strength on MaCconky and was non-heamolysis on blood agar; it was negative for oxidase test, indole test, red-methyl test and movement test, while it was positive for catalase test, Voges-proskauer test, citrate test, urease production, glucose and lactose fermentation and Co_2 gas production. Depending on the diagnosis by vitek-2 system that included 47 tests, the results revealed that 99% of isolated was belonging to KP, and the percentage of KP in the samples was 45% in urine, 20% in wounds 15% in sputum and blood and 5% in burns, while for molecular identification using 16-23s, the results found that all isolates were belonged to type *K. pneumonia*.

The antibiotic sensitivity test

This test was performed for 60 identification isolates (Fig. 1), the results showed that the percentage of extended spectrum B-lactamase (ESBL) enzymes by 53.3%. Additionally, the percentage of resistant isolates to antibiotics was 100% to Ampicillin, 45% to Piperallin/Tazobactam, 46% to Cefoxitin, 85% to Cefazoli, 80% to Cefotazidim, 81% to Ceftriaxon, 76.6% to Cefipim, 35% to Ertapenem, 30% to Imipenem, 35% to Amikacin, 26% to Ciprofloxacin, 18%.3 to Levofloxacin, 45% to Gentamicin, 5% to Tigecycline, 93.3% to Nitrofurantoin and 66.6% to Trimethoprim/Sulfamethoxazole. While the sensitivity test using disk diffusion method, the results was similar to those found using the previous method in addition to Chloramphenicol with resistant percentage was23.3% and was 58.3% to Azithromycin. 26 isolates were selected to detect the gene of efflux pump AcrA, We choose two isolates one is sensitive and the other one is resistant to above antibiotics to estimate the gene expression of the efflux pump.

The detection of the efflux pump AcrA gene

The presence of efflux pump AcrA gene was detected for 26 isolates and all of these isolated were positive for this assay as shown in (Fig. 2) that showed the presence of the gene in all selected isolates.

Gene expression of AcrAB-Tolc efflux pump using q(RT-PCR)

A single sensitive and resistant isolate to (Ciprofloxacin, Amikacin, Imipenem, Azithromycim , Chloramphenicol) with using MBC for each isolate in the estimation of the gene expression in addition another medium concentration in the resistant isolate (Table 2).

The gene expression of AcrAB-Tolc gene was measured by studying the AcrA gene in the sensitive and resistant isolates. The results to these two isolates were seen in (Fig.3 and 4). The gene expression significantly increased ($p<0.005$) in the sensitive isolates after exposure with 5 antibiotics in comparison to control group. The gene expression increased in resistant isolates after exposure with Impenem, Chloramphenicol and Azithromycin in both concentrations with significant differences with control group, while Amikacin at 128 µg/ml concentration showed that there is no significant effect in the expression of the efflux pump. Interestingly, the exposure with Amikacin at 8 µg/ml concentration decreased efflux pump gene expression with significant difference ($p<0.05$) compared to control group. In addition, there was a significant decreasing in the gene expression of the efflux pump gene after the exposure with Ciprofloxacin at 512 µg/ml and 16 µg/ml in comparison to control group, while no significant difference was seen found in the gene expression between two concentrations of Ciprofloxacin.

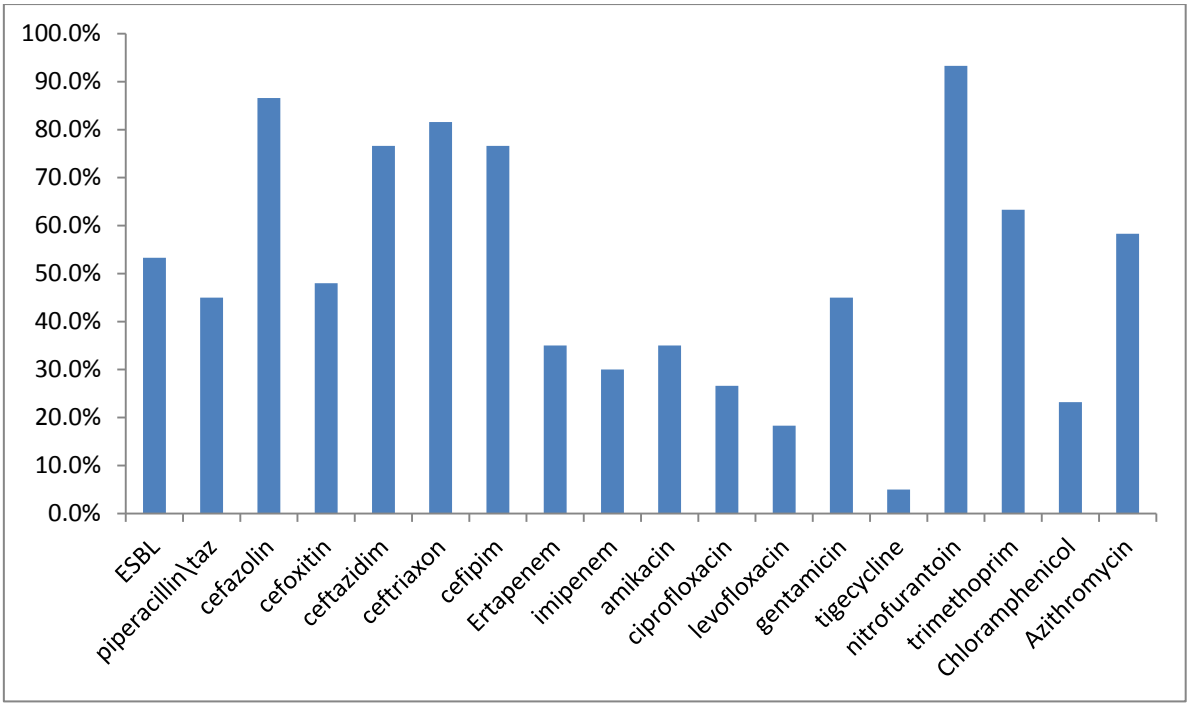

Figure 1: The antibiotics sensitive test for 60 isolate of KP bacteria collected from different sources.

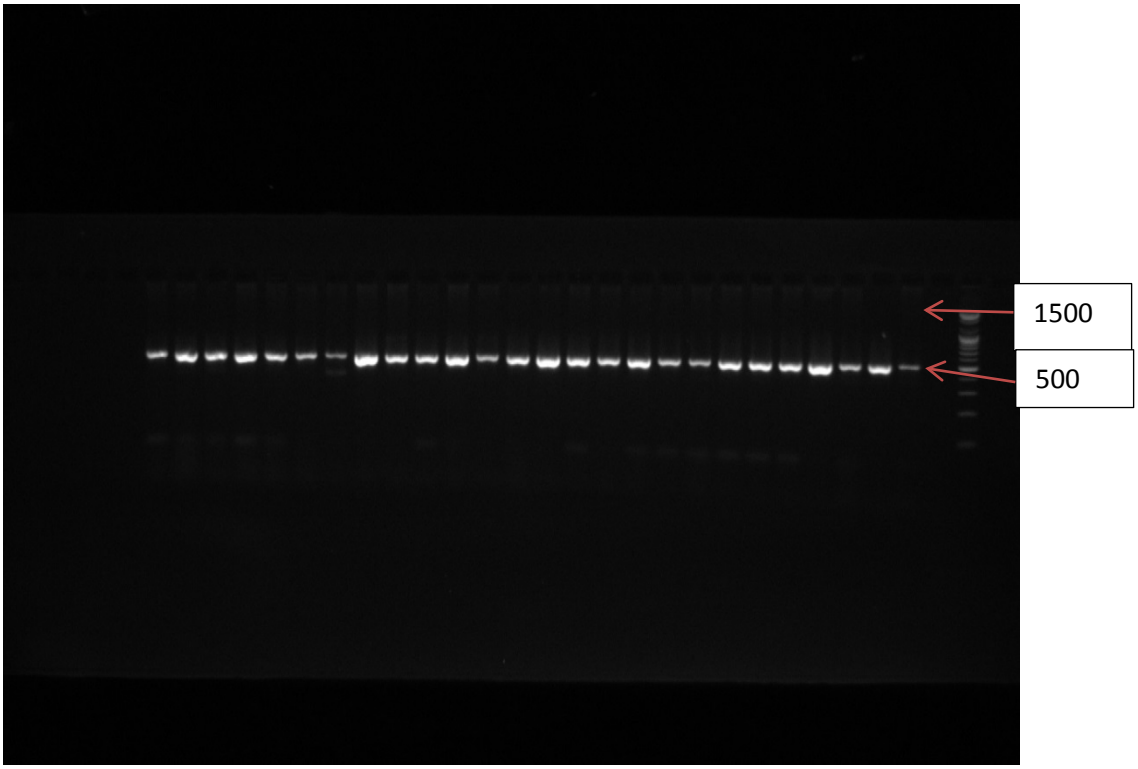

Figure 2: Electrophoresis of the product of efflux pump AcrA gene in *K.pneumoniae* with size (495 pb). The product loade on 2% agaros gel with ethidium bromide (5%) with laddere 100 bp.

Table 2: The results of MIC for the sensitive and resistant isolates.

Sample	Ciprofolxacin (µg/ml)	Amikacin (µg/ml)	Imipenem (µg/ml)	Chloramphenicol (µg/ml)	Azithomycin (µg/ml)
Sensitive	1	8	0.5	4	8
Resistant	1024	256	256	512	128

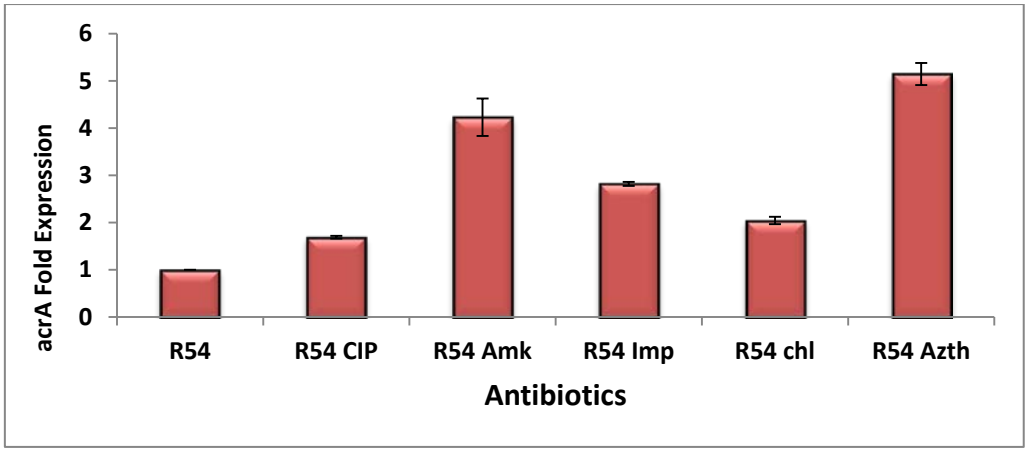

Figure 3: The gene expression of efflux pump acrA in the sensitive isolate after and befor the exposure with Ciprofloxacin, Imipenem, Amikacin, Chloramphenicol, Azithromycin.

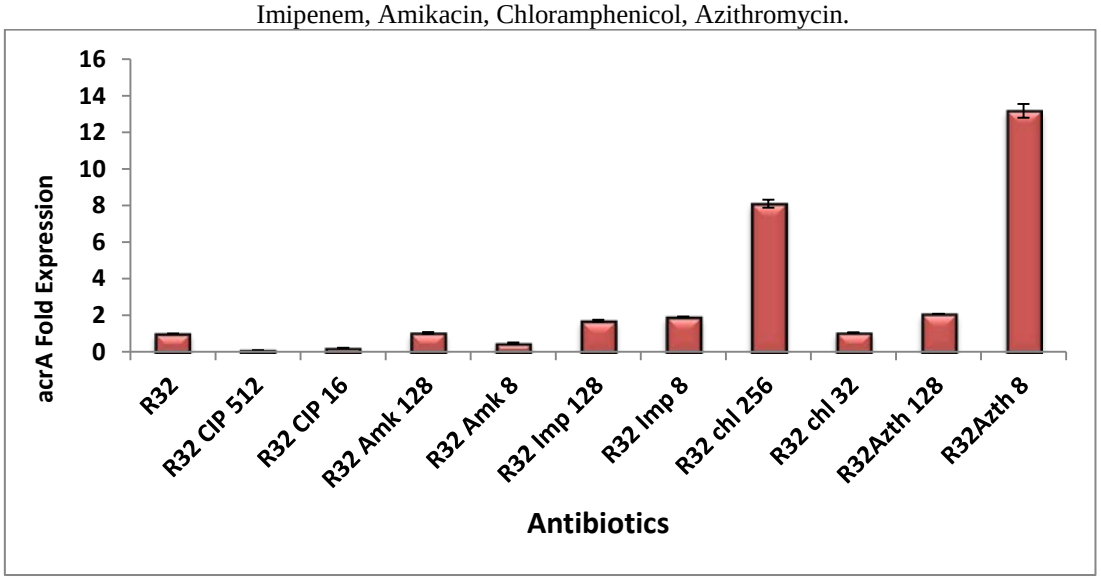

Figure 4: The gene expression of efflux pump acrA in the resistant isolate after and befor the exposure with Ciprofloxacin, Imipenem, Amikacin, Chloramphenicol, Azithromycin.

DISCUSSION

The results of morphological and biochemical tests showed that the collected isolates are belonging to *K. pneumoniae* and this in agreement results of [14]. The study of [15] described new strains of *K. pneumoniae* bacteria isolated from the patients in the United States and these isolates were being resistant to all antibiotics and similar to those isolated in this study; also the current study described that this Pan-Resistant isolates is due to two reasons either that acquired a mobile elements or due to accumulated through chromosomal mutations.

Interestingly, the study of [16], which examined the antibiotics sensitivity test using MIC method for 10 antibiotics in AST-GN30 system, revealed that the number isolates resistant to Ceftazidime, Amoxicillin, Ceftriaxone, Cefepime, Imipenem, Ciprofloxacin, Levofloxacin, and Gentamicin were less than to those that resistance isolates found in the current study. Furthermore, the rate of detection of ESBL enzymes was 26% depending on the manual method, while the ratio of detection of the same enzymes was about double the previous proportion. Moreover, the resistance ratio of the previous antibiotics in the study of [17] was similar to those obtained observed in the present study what except Imipenem and Amikacin whis was much higher than found in the previous study.

Interestingly, [18] found that the percentages of resistant *K. pneumoniae* isolates were agreement with the percentages were consistent to those obtained in this study for Ampicillin, Ceftriaxon and Trimethoprim / Sulfamethoxazole, however the percentages of other antibiotic are disagreement with the same study. Additionally, research of [19] that estimated the resistant percentages of *K. pneumoniae* isolates were agreement with current study except Gentamicin, Ciprofloxacin and Chloramphenicol. The study of [20] also referred to the numbers and percentages of *K. pneumoniae* that resistant to antibiotics in addition to Azithromycin and the percentages were similar to the percentage obtained by the current study.

Several studies have shown that one of the resistance methods of bacteria to carbapenem antibiotics group is by inhibitory enzymes [21]. The study of [12] revealed that after the exposure of bacteria to these antibiotics, the gene expression of efflux pump AcrAB was increased in *K.*

pneumoniae isolates that resistance to Cabapenem group, so these results is in agreement with our results in the current study.

The studies of [22] and [6] and mentioned the role of the efflux pumps mechanisms in the resistance to Chloramphenicol antibiotics, especially efflux pump AcrAB-Tolc in *K. pneumoniae*. They also noted that most of the resistant isolates to Chloramphenicol were produced ESBL enzymes, result in to have multiple resistance mechanism to antibiotics; these findings were consistent with the current study demonstrating the resistance to Chloramphenicol antibiotic by efflux pump AcrAB-Tolc.

The current study found that the main resistance of bacteria to Azithromycin was via efflux pump AcrAB through testing the sensitive and resistance isolates where a significant increasing were observed in the gene expression of this pump after the treatment with different concentrations of target antibiotic in comparison to control group; however no previous studies related to this results were found because this result may a novel observation.

The present study was used the exposure with Amikacin for the first time exposure and determined its effect on the gene expression of the efflux pump AcrAB. The gene expression of AcrAB was significantly elevated in the sensitive isolate after the exposure with Amikacin, while a significant decrease in the gene expression of AcrA was detected in the resistant isolates after the exposure with high concentration of target antibiotic, and this may due to the presence of bacterial inhibitory enzymes to antibiotics belonging to Aminoglycosid group, where these enzymes able to modify the effective group of these antibiotics, making them with less infinity to bind with the target region [23], there are many inhibitory enzymes are presences in Gram-negative bacteria, or the resistance is via other mechanisms [24].

Furthermore, [25] determined the gene expression of efflux pump AcrAB in *E. coli* including AcrA gene and also the study the effect of fluoroquinolone antibiotics on the gene expression of the studied gene. The results of the study in the sensitive isolates to Norfloxacin were similar to the results of the sensitive isolation obtained in the current study where the gene expression of efflux pump AcrA gene was increased, while this result was not consistent with those in the resistant isolates. Although the different concentrations were used with resistant isolates; MIC for isolates had a maximum of 256 µg/ml in the previous study, while MIC in this study was 1024 µg/ml. Furthermore, [26] found that the gene expression of efflux pump AcrA was increased in *E. coli* which treated with Ciprofloxacin antibiotic at 1 µg/ml of concentration, this is in agreement with the observation of the current study in the sensitive isolation. These findings indicated that the resistant isolate takes a different pathway to resist Ciprofloxacin such as other efflux pumps or changing the target location or changing membrane permeability [27].

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