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Gene expression of the efflux system acrAB, oqxAB, and marA operons of *klebsiella pneumoniae* isolated from patients that urinary tract infection

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ABSTRACT

Approximately 150 million individuals globally suffer from urinary tract infections (UTIs), which are among the most prevalent bacterial illnesses. *Klebsiella pneumoniae* is a common opportunistic pathogen that causes nosocomial infections. The aim of this study is to determine the efflux pump genes (*AcrAB*, *OqxAB*, and *MarA*) that were isolated from a variety of UTI patients under the supervision of a specialist physician at two healthcare facilities, AL-Diwaniyah General Teaching Hospital and Maternity and Pediatrics Teaching Hospital in the AL-Diwaniyah city, Iraq. The study investigated the prevalence of *K. pneumonia*, which causes UTIs. Thirty-six isolates were identified as K. pneumonia according to the manual culture characteristics and confirmed by the Vitek-2 system. The efflux pump genes (AcrAB, OqxAB, and MarA) use the real-time PCR technique. It was found that there was a high rate of gene expression of AcrAB (17.3%), but the ratio of OqxAB and MarA genes was (8.90%) and (6.95%), respectively.

Keywords: *K.pnuemoniae, UTIs, Real-time PCR, AcrAB*

التعبير الجيني لنظام التدفق acrAB و oqxABوmarAلبكتيريا klebsiella pneumoniae المعزولة من المرضى الذين يعانون من عدوى المسالك البولية

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خلاصة

يعاني ما يقرب من 150 مليون شخص على مستوى العالم من التهابات المسالك البولية(*UTIs*) ، والتي تعد من أكثر الأمراض البكتيرية انتشارًا. الكلبسيلة الرئوية هي مسببات الأمراض الانتهازية الشائعة التي تسبب عدوى المستشفيات. الهدف من هذه الدراسة هو تحديد جينات مضخة التدفق (*AcrAB*، *OqxAB*، و *MarA*)التي تم عزلها من مجموعة متنوعة من مرضى التهاب المسالك البولية تحت إشراف طبيب متخصص في اثنين من مرافق الرعاية الصحية، مستشفى الديوانية التعليمي العام ومستشفى الولادة والأطفال. المستشفى التعليمي لطب الأطفال في مدينة الديوانية، العراق. بحثت الدراسة في مدى انتشار الالتهاب الرئوي.*K* ، الذي يسبب عدوى المسالك البولية. تم تشخيص 36 عزلة على أنها *K. pneumonia* طبقاً لخصائص الاستزراع اليدوي وتأكيدها بواسطة نظام *Vitek-2*. تستخدم جينات مضخة التدفق(*AcrAB* ، و*OqxAB*، و *MarA*)تقنية *PCR* في

الوقت الحقيقي. وقد وجد أن هناك نسبة عالية من التعبير الجيني لـ*AcrAB (17.3%)* ، لكن نسبة جينات *OqxAB* و *MarA*كانت (8.90%) و(6.95%) على التوالي.

الكلمات المفتاحية: *K.pnuemoniae*، عدوى المسالك البولية، *PCR*في الوقت الحقيقي، *AcrAB*

1. INTRODUCTION

One of the essential types of bacteria that causes infections such as UTIs is *Klebsiella pneumoniae*. It is a Gram-negative, opportunistic, non-motile, facultatively anaerobic, rod-shaped bacterium, singly arranged in pairs or short chains; the range is from 0.3 to 1.0 µm in width and around 0.6 to 6.0 µm in length, non-spore forming[1, 2, 3]. *K. pneumoniae* is oxidase-negative and lactose-fermenting. It has a thick polysaccharide capsule that gives its colonies on agar plates a mucous appearance [4, 5]. *K. pneumoniae* is normal flora of human skin, oropharynx, or gastrointestinal tract [6]. It causes more infections in humans, such as respiratory tract infections, UTIs, bloodstream infections, bacteremia, suppurative infections, cholangitis, and rarely osteomyelitis or meningitis, especially in the immune-compromised or in those suffering from underlying disease conditions such as diabetes mellitus[7, 8]. Hospital-acquired pneumonia has a higher percentage of contributing factors. Hospital-acquired pneumonia is the fourth most prevalent infection in hospitals, primarily affecting hospitalized patients. This type of pneumonia is commonplace among newborns, premature infants, and individuals who rely on mechanical ventilation [9,10]. UTIs are a common problem that can be difficult to treat due to bacterial resistance, and can affect people of all ages and sexes [11]. *AcrAB* and *OqxAB* are the two efflux system genes in *K. pneumoniae* that have been examined most of antibiotic resistance [12, 13, 14,15]. The inherent resistance of *K. pneumoniae* isolates to fluoroquinolones, particularly ciprofloxacin, is mostly attributed to the AcrAB efflux pump. Furthermore, this pump is resistant to beta-lactams, macrolides, trimethoprim, tetracycline, and chloramphenicol. [16,17]. In *K. pneumoniae*, the OqxAB resistance determinant is frequently detected chromosomally, while in other *Enterobacteriaceae* species, it is usually found on plasmids. [18]. Numerous factors, including antibiotics, environmental stresses, and genetic alterations, can control the expression of these efflux pumps' genes [19,20]. K. Pneumoniae possessed some mechanisms of (MDR) such as Beta-lactamase production , porin loss and efflux pumps. The aim of the current study is to isolate and diagnose *K.Pneumoniae* that causes UTIs, conduct an antibiotics resistance test using the Vitek-2 system, identify the operons AcrAB, OqxAB, and MarA gene expression.

2. MATERIAL & METHOD

2.1. Thirty-six K.pnuemoniae isolates were collected from patients with UTIs under the supervision of a specialist physician of different age groups and

genders at two healthcare facilities, AL-Diwaniyah General Teaching Hospital and Maternity and Paediatrics Teachings Hospital in AL-Diwaniyah City, from October 20, 2023, to January 15, 2024. All samples collected were examined by Gram staining and inoculated on a primary media, including blood agar and MacConkey agar, which were used for pathogen isolation using a sterile loop. After that, the agar plates undergo a 24-hour aerobic incubation at 37°C. After that, the bacterial identified by laboratory test and then the automated approach Vitek®2 AST-N222 (BioMeneux, Turkey) was used to make the diagnosis, and a facilitation document was issued by the College of Medical Biotechnology, Al-Qadisiyah University, Iraq—document number 1576,2023/9/17.

2.1. Quantitative Reveres Transcription Real-Time PCR (RT-qPCR).

Gene	Sequence(5'-3')	Product size	NCBI Reference code
16S rRNA gene	F: TTCGATGCAACGCGAAGAAC	123 bp	LC764401.1
	R: TTTCACAACACGAGCTGACG		
AcrAB gene	F: AAACGGCAAAGCGAAAGTGG	108 bp	OQ808798.1
	R: ATTGAGCCGGTGGTCTGATC		
OqxAB gene	F: AAAGTGACCGCCCCTATTGAC	119 bp	MN273774.1
	R: TACACCGTCTTCTGCGAGAC		
MarA gene	F: TAAGAAAGAGACCGGCCATTCC	109 bp	NC_016845.1:c2572399-2572025
	R: TTCCGCCAGGTACAGGATTG		

Quantitative Real-Time PCR was utilized to quantify efflux pump gene expression detection in multidrug-resistant K. pneumoniae isolates and normalize the data using the housekeeping gene. The procedure was followed in accordance with [21][22] and included the following steps:

2.1.1. Total RNA extraction

Using a (Total RNA Extraction Kit), total RNA was extracted from MDR *K. pneumonia* isolates by the following procedures, which were followed as directed by the manufacturer: After inoculating bacterial isolates on Luria Bertani broth with a two µg/ml concentration of inducible ethidium bromide and incubating at 37ºC to produce bacterial cells (OD600:0.8-1.0), the bacterial cells were harvested by centrifuging them for 1 minute at 10,000 rpm, and the supernatant was then removed after adding 1 ml of Trizol reagent and aggressively vortexing the bacterial pellets for 10 sec at room temperature. Each tube was filled with 200 µl of chloroform and then shaken ferociously for a minute. The mixture was incubated on ice for five minutes and then centrifuged for 15 minutes at 4C at 13,000 rpm. After transferring the supernatant into a fresh 1.5 ml microcentrifuge tube, 500µl of isopropanol was added. Subsequently, the blend is mixed by rotating the tube four or five times, then set at 4C for ten minutes and centrifuged at 4C° and 13,000 rpm for 10 minutes. After discarding the supernatant again, 1 ml of 80% ethanol was added and vortexed—next, centrifuge at 4C° for 15 minutes at 13,000 rpm. The RNA pellet was allowed to air dry while the supernatant was disposed of. After dissolving the RNA pellet in each sample with 100 µl of free nuclease water, the extracted RNA sample was stored at -80 degrees Celsius.

2.2. The Real-Time PCR primers:

in this work, Primer3 plus and the (NCBI-Genebank) sequence are used to detect and quantify the efflux pump genes in *K. pneumoniae* isolates. Co. Ltd Scientific Research provided these primers from Iraq, as shown in the following table (1).

Table 1: The qPCR detection gene primers for *K.pneumoniae* with their nucleotide sequence:

3. RESULTS

The present study enrolled 36 samples from patients with urinary tract infection (UTI) under the supervision of a specialist physician at two healthcare facilities, AL-Diwaniyah General Teaching Hospital and Maternity and Paediatrics Teachings Hospital in AL-Diwaniyah City, from October 20, 2023, to January 15, 2024, with age range (5-62) years. The aim is to investigate bacterial infection using bacteriological culture of urine samples. The *K. pneumoniae*

isolates were subjected to a sensitivity test using the VITEK-2 system to identify (MDR) isolates. The results are displayed in Table (2). The current findings demonstrate that 25 (69.4%) of the *K. pneumoniae* isolates were multi-drug resistant isolates, meaning they were resistant to multiple drugs.

Table 2: Sensitivity test results by VITEK-2 system.

VITEK-2 system	**Bacterial Isolates**	***p*-value**
	Klebsiella Pneumoniae ***n* (%)**	
Positive, *n* (%)	25 (69.4 %)	0.071 ¥ NS
Negative, *n* (%)	11 (30.6%)	

Table 3: The MDR *K. Pneumoniae* according to mean age, gender distribution:

Characteristic	***MDR K. Pneumoniae*** ***n* (%)**	***P***
Age (years)		
Mean ±SE	24.60 ±2 72	0.134 † NS
Range	7-55	
< 20 year, *n* (%)	11 (44.0%)	0.682 ¥ NS
20-29 year, *n* (%)	5 (20.0%)	
> 30 year, *n* (%)	9 (36.0%)	
Sex		
Male, *n* (%)	11 (44.0 %)	0.382 ¥ NS
Female, *n* (%)	14 (56.0 %)	

Table 4: The comparison between gene expression in *K. Pneumoniae* isolates:

Characteristic	*AcrAB*	*OqxAB*	*MarA*	*P*
Gene expression				
Mean± SD	**17.31 ± 7.80^{A}**	**8.90± 3.29^{B}**	**6.95 ± 3.3^{B}**	**0.018 †S**
Range	1.95-83.86	1.62-35.26	1.74-36.00	
Different letters denote the significant differences at p< 0.05				

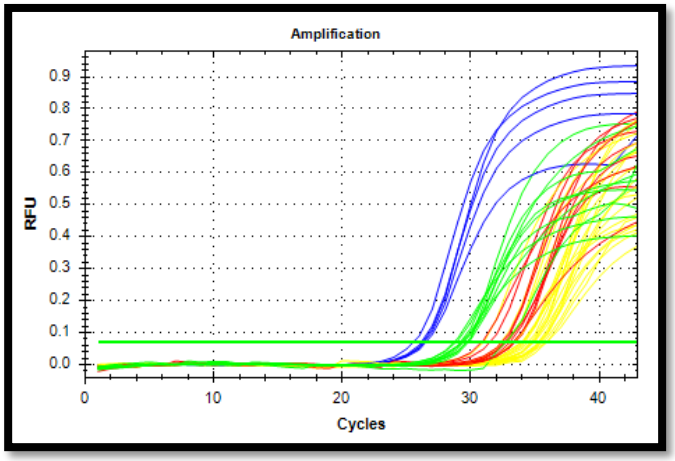

Figure 1: The Real-Time PCR amplification plots of efflux pump genes MDR *K.pneumoniae* isolate. The blue plots (*AcrAB*), the green plots (*OqxAB*), the yellow plots (*MarA*), and the red plots (16SrRNA).

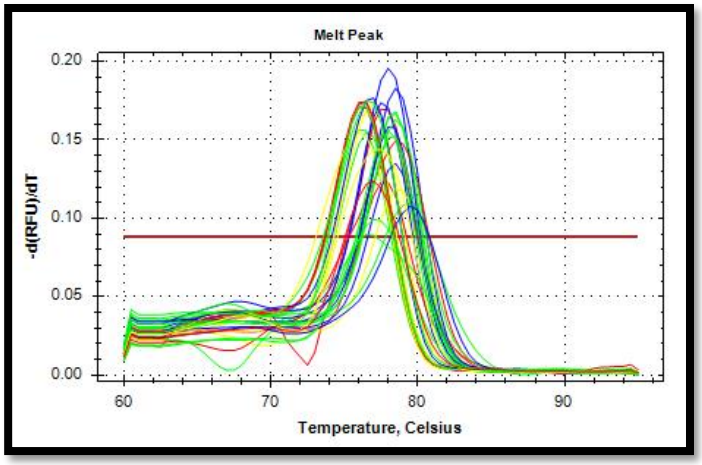

Figure 2: The Real-Time PCR melting of efflux pump genes MDR K.pneumoniae isolate. The blue plots (AcrAB), the green plots (OqxAB), the yellow plots (MarA), and the red plots (16SrRNA)

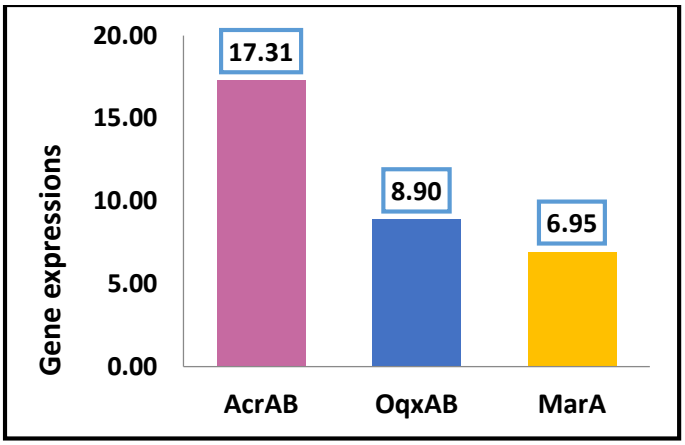

Figure 3: The comparison between gene expression in *K. Pneumoniae*

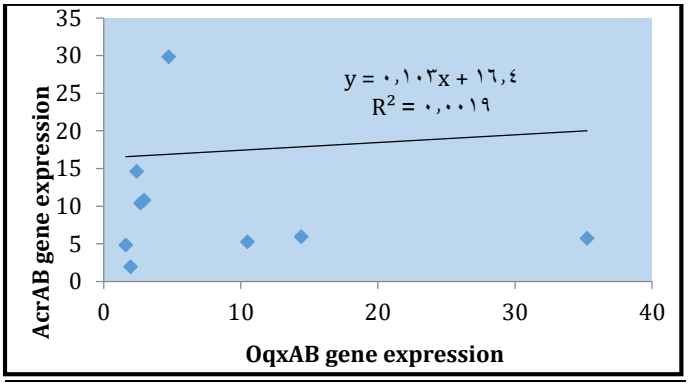

Figure 4: The Logestic scatter blot AcrAB gene and OqxAB gene expression among K. Pneumoniae isolates.

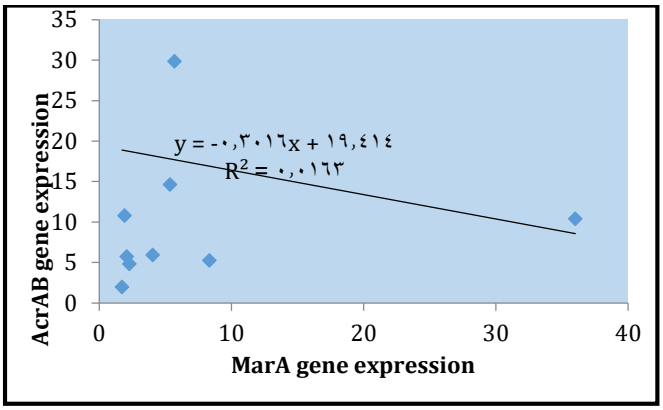

Figure 5: The Logestic scatter blot *AcrAB* gene and *MarA* gene expression among *K. Pneumoniae* isolates.

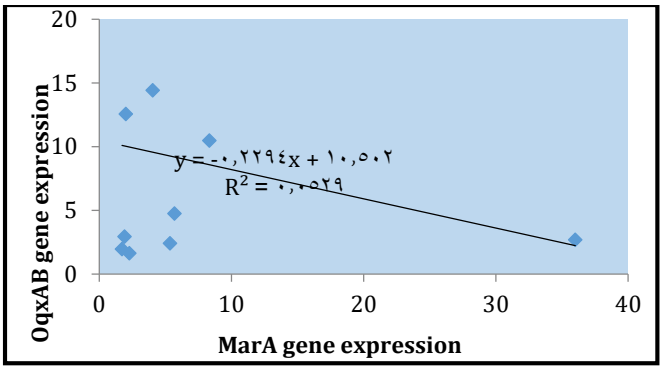

Figure 6: The Logestic scatter blot *OqxAB* gene and *MarA* gene expression among *K. Pneumoniae* isolates.

4. Discussion

Thirty-six isolates (28.6%) belonging to the type K.pneumoniae were isolated and identified from 200 urine samples from patients with UTIs. When comparing the percentage of the current study with previous studies, we find it identical to study [25] in Iraq, where the percentage of infection with this bacteria was (29.2%).These percentages are due to the approximation of the conditions of the geographical area in which the samples were collected, which is considered an average percentage in the conditions of sample collection. K. pneumoniae is regarded as one of the major bacterial causes of UTIs for people of all ages and races [1,3], *K. Pneumoniae* isolates were submitted to the sensitivity test to detect (MDR) isolates by VITEK-2 system. The results shown in (table-2) in which 25 isolates (69.4%) of *K. Pneumoniae* isolates was resistance to several drug and considered MDR isolates, and the MDR K. Pneumoniae according to mean age and sex distribution. Moreover, the results shown in (table-3) that the mean age of UTI patients with MDR *K. Pneumoniae* infection was 24.60 ±2.72 years and the range was 7-55 years. Furthermore, most of UTI patients with MDR *K. Pneumoniae* infection enrolled in the present study were less than 20 years of age, 11 (44.0%), as shown in(table -3).

Regarding sex, UTI patients with MDR *K. Pneumoniae* infection included 11 (44.0 %) and 14 (56.0 %) with male and female, respectively. The current study aim to measure the gene expression rate of the resistance genes, namely the efflux pump genes *AcrAB*, *OqxAB* and *MarA*, of the bacteria k.pneumoniae in patients suffering from UTIs. The modern technique Real_time PCR was used, as all bacterial isolates possessed the genes by 100%. However, the gene expression rate varied from one isolate to another due to the sample concentration, the conditions of sample collection, and the severity of inflammation in the patient. *AcrAB* gene expression in *K. Pneumoniae* isolates were 17.31±7.80, shown in (table-4), where *K.penumoniae* isolates were all

possessed *AcrAB* gene (100%). While it was found in a previous study conducted by researchers [23] in "Iran", that *AcrAB* gene was more prevalent in *K.penumoniae* strains due to the *AcrAB* efflux pump, which was thought to be one of the primary pumps causing *K.pneumoniae* isolates' intrinsic resistance to ciprofloxacin and tetracycline. The percentage of the *AcrAB* gene's genetic expression in *K.penumoniae* was approximately (41%), while the *OqxAB* gene expression in K. *Pneumoniae* isolates were 8.90±3.29, as shown in (table-4). Also, researchers found in their study in China [25] that the *OqxAB* efflux pump gene is found in all isolates of *K.pnuemoniae* bacterium, and they was noted that the mechanisms that mediate the efflux, including high expression of the efflux pump OqxAB play a significant role in tigecycline resistance. Moreover, *MarA* gene expression in *K. Pneumoniae* isolates were 6.95±3.3, as shown in(table-3). In a study conducted by researchers [15] indicated that (93%) of MDR *K. pneumoniae* isolates possessed the *MarA* efflux pump gene, as its gene expression rate was approximately (37%). The expression of the genes in *K. Pneumoniae* isolates has been compared, and the results are demonstrated in(table -3) and Figure (3). The mean of gene expression was 17.31±7.80, 8.90± 3.29, and 6.95± 3.3 for *AcrAB*, *OqxAB*, and *MarA* genes, respectively. The mean expression was higher in AcrAB in comparison with other genes, and there was a significant difference ($P<0.05$). However, the expression was a non-significant difference between the *OqxAB* and *MarA* genes themselves ($P < 0.05$).

This difference in the rates of the gene expression for these genes may be due to the functional importance of the efflux pump gene *AcrAB* in withstanding environmental stress and resistance to antibiotics, which may lead to an increase in its gene expression in *K.pnuemoniae* isolates. In a previously conducted local study, it was shown that *K.pnuemoniae* may respond faster and more effectively to chemicals or environmental conditions that stimulate the expression of the *AcrAB* gene to a greater extent than the *OqxAB* and *MarA* genes [26]. In a previous study, it was shown that the gene expression rate of the *AcrAB* gene was higher than the gene expression rate of the *OqxAB* and *MarA* genes. The reason was due to the difference in the population distribution of the strains that express the *AcrAB* efflux pump gene more compared to the other two genes. Also, the physical and environmental conditions may be the reason for these differences. Environmental conditions such as temperature and humidity may affect gene expression rates. The study also indicated that a genetic fusion may occur between the *AcrAB* gene and another gene, which leads to an increase in its gene expression rate in *K.pnuemoniae* isolates [27]. As we previously stated," the modern technology Real-time PCR was used in the current study to obtain the required result, as shown in (figure 1 and 2).

It is clear to us that the gene expression rate of the efflux pump gene *AcrAB* is the highest compared" to the other genes, *OqxAB* and *MarA* genes in the *K. pneumonia* isolates. Other previous studies that used PCR technology considered less accurate than real-time PCR technology. They did not accurately determine the gene expression rate of efflux pump genes [28,29]. In other previous studies, real-time PCR technology was used to express the resistance genes *AcrAB*, *OqxAB*, and *MarA* [15,30,31]. The Logestic regression model shows that the correlation of gene expression such as *AcrAB* gene expression in which have direct correlate with *OqxAB*, genes among *K. Pneumoniae* as in (figure-4). The results shown that *K. Pneumoniae* enhance expression of *OqxAB*, in relation to the expression of AcrAB gene. But the *MarA* gene expression in which have indirectly correlate with both *AcrAB* and *OqxAB* genes among *K. Pneumoniae* as in (figure-5 and 6). This result shows that *K. Pneumoniae* enhances expression of *AcrAB* and *OqxAB* genes, in suppression the expression of *MarA* gene.

5.CONCLUSION

1. Most of the isolates of *K.pnuemoniae* bacteria under study were taken from patient with UTIs with multiple drug resistance (MDR).
2. All *K.pnuemoniae* isolates possessed efflux pump genes, *AcrAB*, *OqxAB*, and *MarA* which encode efflux pump proteins.
3. The current study showed that the expression rate of the efflux pump gene *AcrAB* was the highest rate compared to the expression rate of *OqxAB* and *MarA* genes, reaching (17.31%).

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