



Research Article

The Relationship Between OqxAB Efflux Pump and Drug Resistance in *Klebsiella pneumoniae* Isolated from Clinical Sources

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Abstract

Background: Because *Klebsiella pneumoniae* resistance to the majority of antibiotics is developing quickly, it is important to study the efflux pump system that bacteria carry and the genes that encode them in order to find effective ways to stop or limit this resistance. **Objective:** To find out how common OqxAB-efflux pump genes are and if there is a link between these genes and antibiotic resistance in *Klebsiella pneumoniae* that has been isolated from different clinical sources. **Method:** In this investigation, 174 various clinical specimens were collected from Baghdad hospitals. Based on morphological characteristics, culture media, biochemical testing, the Vitek-2 system, and molecular diagnosis by the *16S rRNA* gene, only 97 isolates were recognized as *Klebsiella pneumoniae*. The antibiotic susceptibility of the isolates to 21 antibiotics was also determined. The presence of the active efflux pump was determined phenotypically (ethidium-bromide cartwheel method) and genotypically by multiplex PCR. **Results:** All isolates were resistant to ampicillin and amoxicillin-clavulanate (100%). On the other hand, the percentage of resistance to cefotaxime, ceftazidime, and cefixime was 95.87%, 94.84%, and 95.87%, respectively. While tigecycline had the lowest resistance rate (11.43%), In the genotype detection assay for efflux pump genes, the results show that the percentages of *oqx A* and *oqx B* are 65% and 83.6%, respectively. **Conclusion:** The *oqxA* and *oqxB* genes have a direct relationship with antibiotic resistance in *Klebsiella pneumoniae*.

Keywords: Antibiotic resistance, Efflux pump, *Klebsiella spp*, *OqxAB* gene.

العلاقة بين جينات مضخة التدفق OqxAB ومقاومة المضادات الحيوية لبكتيريا الكلبسيلا الرئوية المعزولة من المصادر السريرية

الخلاصة

الخلفية: يعد التطور السريع لمقاومة الكلبسيلا الرئوية لجميع المضادات الحيوية تقريباً يجعل هذه البكتيريا مشكلة صحية مهمة. إن دراسة نظام مضخة التدفق التي تحملها البكتيريا والجينات المسؤولة عنها مهمة لإيجاد الطرق المناسبة للحد من هذه المقاومة. **الهدف:** الكشف عن انتشار جينات مضخة التدفق والعلاقة بينها وبين مقاومة المضادات الحيوية في الكلبسيلا الرئوية المعزولة من مصادر سريرية مختلفة. **الطريقة:** تم جمع 174 عينة سريرية مختلفة من مستشفيات بغداد. بناءً على الخصائص المورفولوجية، والوسائط الزرع، والاختبار البيوكيميائية، ونظام Vitek-2، والتشخيص الجزيئي بواسطة جين *16S rRNA*، تم التعرف على 97 عزلة فقط على أنها كلبسيلا رئوية. كما تم تحديد حساسية المضادات الحيوية للعزلات لواحد وعشرين من المضادات الحيوية. تم تحديد وجود مضخة التدفق النشط باستخدام النمط الظاهري (طريقة عجلة الإيثيديوم-بروميد) و النمط الجيني باستخدام تقنية Multiplex PCR. أظهرت النتائج أن جميع العزلات كانت مقاومة للأمبيسيلين والأموكسيسيلين-كلافولانات (100%). من ناحية أخرى، كانت النسبة المئوية لمقاومة السيفوتاكسيم والسيفتازيديم والسيفيكسيم 95.87% و 94.84% و 95.87% على التوالي. بينما كان للتيجيكليين أقل معدل مقاومة (11.43%). في اختبار الكشف عن التركيب الوراثي لجينات مضخة التدفق، أظهرت النتائج أن نسب *oqxA* و *oqxB* هي 65% و 83.6% على التوالي.

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INTRODUCTION

Klebsiella pneumoniae is one of the most common nosocomial pathogens, causing infections in the urinary tract, bloodstream, and lungs. This bacterium can develop resistance to cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides [1,2]. Because there are fewer therapeutic options for treating such resistant bacteria, multidrug resistance is common among *K. pneumoniae* isolates and is causing severe problems in hospitals and healthcare facilities [3]. *K. pneumoniae* isolates can develop several mechanisms that result in antibiotic resistance to several types of antibiotics [4]. The presence of multidrug efflux pumps is one of the mechanisms providing an increase in bacterial resistance to a variety of antimicrobial compounds, including antibiotics, dyes, antiseptics, and detergents [5]. A lot of research has been done on the *OqxAB* and *AcrAB* genes in *K. pneumoniae* to find out how they are linked to antibiotic resistance [6]. *OqxAB* has been realized chromosomally in *K. pneumoniae* and is usually found as a plasmid in other *Enterobacteriaceae* species. This resistance characteristic is responsible for decreased susceptibility to olaquinox and other fluoroquinolone drugs such as ciprofloxacin, norfloxacin, and flumequine [7]. This efflux pump has only been observed in clinical isolates of *K. pneumoniae* in various investigations, and it is predicted that this efflux pump is frequently distributed among ESBL (extended-spectrum beta-lactamase)-producing and carbapenemase-producing isolates [8,9]. The aims of this investigation were to detect efflux pump *oqxAB* genes by multiplex PCR among *Klebsiella pneumoniae* isolated from different clinical sources and evaluate the role of these genes in antibiotic resistance.

METHODS

Isolation and identification of bacterial isolates

The Baghdad College of Science's ethics committee (CSEC/0522/0062) gave its approval to the project. In this investigation, 97 *K. pneumoniae* isolates were isolated from 174 different specimens (urine, wound smears, sputum samples, blood, and burn smears) collected from several hospitals in Baghdad from April to July 2022. All isolates were identified using selective and differential medium culture, biochemical testing, and confirmation with a Vitek-2 system. In addition, the *16SrRNA* gene was used for molecular detection.

Antibiotics susceptibility of Klebsiella pneumoniae

The disk diffusion method was used to test the sensitivity of twenty-one different antibiotic discs, including: ampicillin AMP (10 µg), amoxicillin-clavulanate AMC (10/20 µg), cefixime CFM (5µg),

cefotaxime CTX (30 µg), ceftazidime CAZ (30 µg), vancomycin VA (30µg), trimethoprim TMP (5µg), trimethoprim/sulfamethoxazole STX/ST (10/20 µg), aztreonam ATM (30 µg), ciprofloxacin CIP (5 µg), nitrofurantoin NIT (300 µg), amikacin AK (10 µg), tigecycline TGC (15 µg), tetracycline TET (30 µg), imipenem IMP (10 µg), chloramphenicol CHL (30 µg), tobramycin TOB (10 µg), Nalidixic acid NAL (30 µg), ertapenem ERT (10 µg). While colistin and polymixin B were examined using the minimum inhibitory concentration (MIC) by the broth microdilution technique. The results were interpreted in accordance with the Clinical Laboratory Standards Institute (CLSI) 2022 [10], with the exception of tigecycline, which is interpreted in accordance with the FDA guidelines because there is no CLSI with Enterobacteriaceae for tigecycline [11].

Phenotypic identification of efflux pumps system

According to Martins *et al.* [12], the ethidium bromide (EB) agar cartwheel method was used to determine the phenotypic identification of efflux pumps. Highly resistant *K. pneumoniae* isolates were grown in BHI broth for 24 hours at 37 °C. After incubation, the bacterial suspension was diluted in 5 ml of phosphate-buffered saline (PBS), and their concentrations were set at 0.5 McFarland standard. Then, Muller Hinton agar (MHA) plates containing various concentrations of ethidium bromide (0, 0.5, 1.0, 1.5, and 2.0 mg/l) were radially divided into 8 sections. (Cartwheel pattern). On the day of the experiment, the plates were made and protected from light. EB-MHA plates were cultured with *K. pneumoniae* isolates. After incubation for 16–18 hours at 37 °C in the dark, After the incubation period, an ultraviolet (UV) trans-illuminator was used to evaluate the isolates that have efflux pumps.

DNA Extraction

The genomic DNA of *K. pneumoniae* isolates was extracted using the Monarch® Genomic DNA Purification Kit (NEB, England).

DNA concentration estimation

The concentration of the extracted DNA was examined using Qubit 4.0 (Invitrogen/USA).

Primers Design

A software called Geneious Prime bioinformatics gave us the basic information we needed to make target primers for *K. pneumoniae* and check the binding site and right annealing temperature for each set of primers.

Additionally, the primers were examined using a variety of internet tools, especially OligoAnalyzer by IDT (Integrated DNA Technology). The primer sequence and amplicon size are described in Table 1.

Table 1: The oligonucleotide primer sequences used in PCR amplification

Primer	Sequence (5'-3')	Amplicon size (bp)
<i>16Sr</i> RNA	F: 5'-CGGTCTGTCAAGTCGGATGT-3' R: 5'-AGCGTCAGTCTTTGTCCAGG-3'	172bp
<i>OqxA</i>	F 5'-CGTCTCGGGATACATTGATAA-3' R 5'-TTTGATAGTGGAGGTAGGTTG-3'	473 bp
<i>OqxB</i>	F 5'-AGATCCGTCCACTCAATATTC-3' R 5'-GCTCGTCTTATGTCAATGACT -3'	328 bp

Polymerase chain reaction (PCR)

The PCR technique was performed to confirm *K. pneumoniae* identification using the *16S rRNA* gene. A specific primer was designed for the *16S rRNA* gene. The conventional PCR procedure started with a denaturation step at 95 °C for 5 min, just one cycle, and was then followed by 30 cycles of denaturation at 95 °C. The reaction mixture then undergoes 35 cycles of annealing at 59–60 °C for 45 seconds. The reaction mixtures then undergo 35 cycles of the extension stage for one minute. After seven minutes of the final extension step at 72 °C, the amplification finally stops. The agarose gel (2%) containing RedSafe dye was used to find out the PCR products, and a UV light was used to observe the gel.

Multiplex PCR for *OqxAB* genes detection

The whole reaction takes 25 µl and has 12.5 µl of OneTaq (NEB®) Mastermix, 3 µl of DNA sample, 1 µl of 10 pmol/l of each primer, and 5.5 µl of free-nuclease water. The reaction was carried out under the ideal PCR conditions for both genes.

Statistical analysis

The Statistical Analysis System SAS program [13] was used to detect the effect of different factors on study parameters. The Least Significant Difference (LSD) test (Analysis of Variation, ANOVA) was used to significantly compare between means. In this study, the Chi-square test was used to compare percentages with $p < 0.05$.

RESULTS AND DISCUSSION

In this study, biochemical tests and Chromagar medium were used to identify 174 samples of bacteria. Only 97 were confirmed to be *K. pneumoniae* using Vitek 2 systems and molecular diagnosis using the *16S rRNA* gene. Using a 100–200 bp DNA ladder to look at the

molecular size of the bands from the PCR test showed that the *16S rRNA* gene has a volume of 172 base pairs (Figure 1).

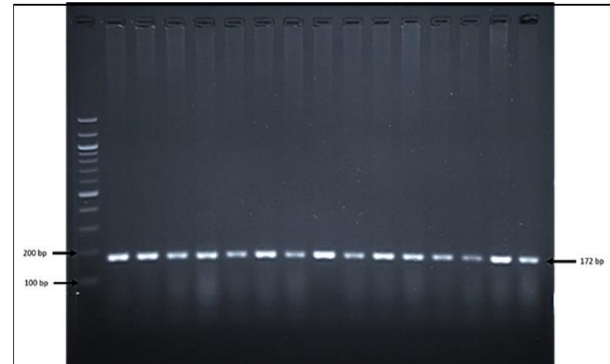


Figure 1: PCR product evaluation for the *16SrRNA* gene of *K. pneumoniae* used agarose gel electrophoresis Lane L: 100-200 bp DNA ladder, Lane (1-15) positive for PCR with a 172 bp PCR product size.

Our results agree with those of Ghaima and Tamara [14], who collected 260 clinical samples from urinary tract infections. Researchers demonstrated that only 76 of the 260 bacterial isolates belonged to *Klebsiella pneumoniae* by utilizing both conventional methods and a molecular diagnostic method based on the *16S rRNA* gene (159 bp). *K. pneumoniae* was distributed as shown in Figure 2, demonstrating that it is most frequently observed in UTIs (42.26%; 41/97), followed by sputum (26.70%; 20/97).



Figure 2: The colonies of *K. pneumoniae* on A, Blood Agar B, Chromagar C, and MacConkey Aga after 24 hours of incubation at 37 C.

K. pneumoniae was isolated from burns, wounds, and blood at frequencies of 14.43% (14/97), 13.40% (13/97), and 9.27% (9/97), respectively. This result was consistent with the findings of [15], who collected clinical specimens from two Tehran hospitals and found that *K. pneumoniae* was isolated from urine, sputum, and blood with percentages of 61.7%, 11.7%, and 8.5%, respectively (Figure 3).

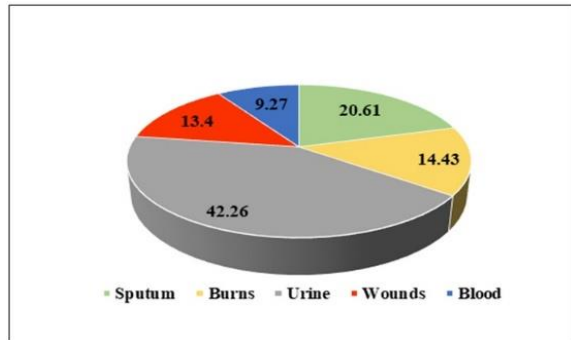


Figure 3: The frequency of *K. pneumoniae* among different clinical sources (%) based on the source of infection.

It was found that 97 different types of *K. pneumoniae* bacteria were resistant to ampicillin, amoxicillin-clavulanate, vancomycin (100%), cefotaxime (95.87%), ceftazidime (94.84%), cefixime (95.87%), trimethoprim/sulfamethoxazole (89.69%), trimethoprim (91.75%), Aztreonam (84.53%), nitrofurantoin (64.94%), polymixin B (62.8%), tobramycin and tetracycline (55.67%), ciprofloxacin (59.79%), amikacin (50.51%), Nalidixic acid (43.29%), chloramphenicol (41.23%), and colistin (38%) (Figure 4).

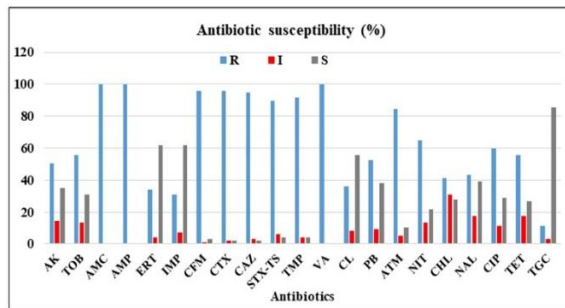


Figure 4: Antibiotic resistance pattern of *Klebsiella pneumoniae* (%).

Drug inactivation, target transformation, decreased permeability, and increased efflux activity are just a few

Table 2: the prevalence of MDR and XDR-*K. pneumoniae* based on source of infection

Sample type	Isolates number	Frequency (%)*	Frequency (%)**	Frequency (%)***	Frequency (%)****	p-value
Sputum	20	18(90)	18(18.55)	2(10)	2 (2.06)	0.0001
Burns	14	0(0.00)	0(0.00)	14(100)	14(14.43)	0.0019
Urine	41	31(75.60)	31(31.95)	2(4.87)	2(2.06)	0.0001
Wounds	13	9(69.23)	9(9.27)	0(0.0)	0(0.0)	0.0025
Blood	9	3(33.33)	3(3.09)	2(22.2)	2(2.06)	0.667
p-value	---	0.0001	0.0001	0.0001	0.0001	---

Percentage (%*) of MDR-*K. pneumoniae* relative to the source, percentage (%)** of MDR-*K. pneumoniae* relative to total no. of *K. pneumoniae* isolate, percentage (%)*** of XDR-*K. pneumoniae* relative to the source, percentage (%)**** of XDR-*K. pneumoniae* relative to total no. of *K. pneumoniae* isolate.

of the mechanisms that contribute to antibiotic resistance. The efflux pump is an important MDR mechanism. The efflux pump can get rid of a variety of antibiotics because it has a multi-substrate property. It can also add to resistance mechanisms that lower the amount of antibiotics inside cells and boost mutation accumulation [16]. MDR, or multidrug resistance, is the ability of a bacterial pathogen to withstand lethal doses of structurally different antibiotics (three to five) that can eradicate non-resistant strains. The World Health Organization has recognized MDR as a serious threat to human health [16]. A bacterial species is said to be drug-resistant (PDR) if it is not susceptible to any antimicrobial agent, excluding two or fewer antimicrobial groups (Figure 5).

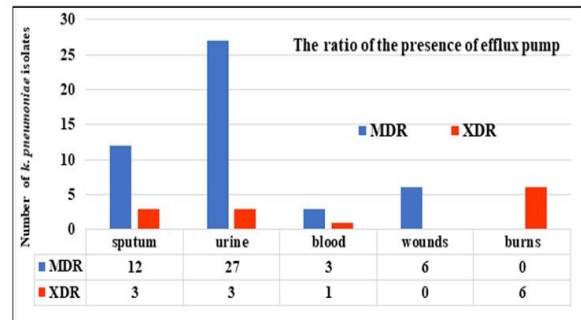


Figure 5: The distribution of efflux pump genes among MDR and XDR *K. pneumoniae* strains isolated from various clinical sources

Extensive drug resistance (XDR) means that the bacteria is not susceptible to any antimicrobial agent [17]. According to the current study, 61 (62.88%) *K. pneumoniae* isolates were classified as MDR isolates because they were shown to be resistant to at least three, four, and five antibiotic classes. While 20.61% of *K. pneumoniae* isolates were XDR because they were resistant to six antibiotic classes, the PDR pattern was not observed due to the use of a limited number of antibiotic classes in this investigation. On the other hand, the source of the sample was compared to the percentage of antibiotic resistance (Table 2).

It was found that all *K. pneumoniae* strains found in burns were not susceptible to any of the 19 antibiotic classes used in this study. The percentage of multidrug resistance to strains that were isolated from sputum, urine, wounds, and blood is as follows: 90%, 75.60%, 69.23%, and 33.33%, respectively. In the present study, a statistically significant difference between MDR, XDR, and the source of infection was observed. MDR and XDR-*K. pneumoniae* isolates that were isolated from sputum and urine had a higher significant value ($p=0.0001$) than *K. pneumoniae* isolates isolated from burns ($p=0.0019$) and wounds ($p=0.0025$). While there is a non-significant difference in blood *K. pneumoniae* isolates. The reason for the high resistance of burn isolates was that those burn patients underwent operations and had their burns contaminated by *K. pneumoniae* from hospitals, which has a high level of resistance. For those patients with septicemia, the reason for the high resistance of bacteria is that they acquire bacteria from catheters during dialysis. The detection of efflux pump activity was carried out in 85 bacterial isolates of *Klebsiella pneumoniae* (more resistant strains) using the cartwheel method, using ethidium bromide as an indicator for phenotypic detection. The result showed that 75.30% (61/81) of the isolates had active efflux pumps at 0.5, 1 $\mu\text{g/mL}$, and 1.5 $\mu\text{g/mL}$ of EtBr, as shown in Figures 5 and 6, depending on the lowest concentration of ethidium bromide at which the isolates did not show fluorescence under the ultraviolet light source [18]. This result is closely related to the result obtained by [19], who found 75% of *K. pneumoniae* isolates have an active efflux pump. The results disagreed with those reported by Akinpelu *et al.* [20], who found all 18 *Klebsiella* isolates had an active efflux pump. The distribution rate of efflux pumps among highly resistant *K. pneumoniae* strains isolated from various clinical sources was also indicated in our study. Results show that the *K. pneumoniae* that possess an active efflux pump were mainly isolated from sputum, with a percentage of 75%. While isolates from urine, wounds, blood and burns had 73.17%, 46.15%, 44.44 and 42.85%, respectively. The *oqxAB* gene is found in clinical isolates of *Enterobacteriaceae* and *Klebsiella pneumoniae* on chromosomes and/or plasmids flanked by IS26-like elements, conferring resistance to quinoxalines, quinolones, nitrofurantoin, and several detergents and disinfectants (benzalkonium chloride, triclosan, and SDS). It could propagate alongside other antimicrobial resistance genes [9]. In order to detect the presence of the *oqxA* (473 bp) and *oqxB* (328 bp) efflux pump genes and determine the prevalence of each gene among more resistant *K. pneumoniae* clinical isolates, multiplex polymerase chain reaction (PCR) was used for each DNA-extracted sample and confirmed by the evaluation of bands using gel electrophoresis as well as by comparing the molecular sizes of bands with DNA ladders of 300 and

500 base pairs. The results of multiplex PCR reactions for *oqx* AB genes are shown in Figure 6 and Table 3.

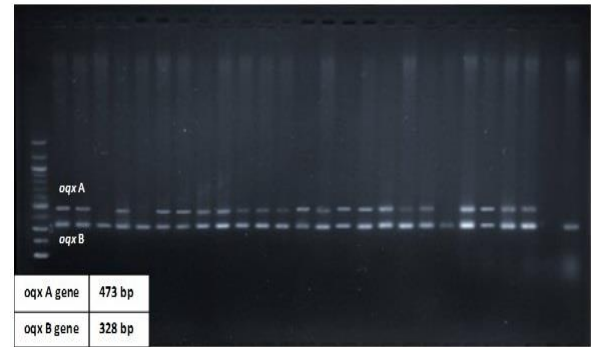


Figure 6: Agarose gel electrophoresis for *oqxAB* genes of *K. pneumoniae* isolates. lane L: 300-500 bp DNA ladder, lanes 1-15 results of PCR products of *oqxAB* genes with 328-473 bp amplicon.

Table 3: *OqxAB* efflux pump genes in *K. pneumoniae* isolates

Gene name	Highly resistant <i>K. pneumoniae</i> isolates		
	No. of +ve isolates	No. of -ve isolates	<i>p</i> -value
<i>oqxA</i>	39(63.9)	22(36.06)	0.0074
<i>oqxB</i>	51(83.60)	10(16.39)	0.0001

The prevalence of *oqx* AB efflux pump genes was detected among 61 *K. pneumoniae* clinical isolates (all positive isolates in the phenotypic test), and results show that the percentages of *oqx A* and *oqx B* are 65% and 83.6%, respectively. The results disagreed with those of Dehnamaki *et al.* [21], who found a total of 100 *K. pneumoniae* isolates from UTI patients. Molecular distribution tests revealed that 57% and 56% of isolates possessed the *oqxA* and *oqxB* genes, respectively. Besides, the article by Amereh *et al.* [22] indicates that among one hundred *K. pneumoniae* isolates, the frequency of *oqxA* and *oqxB* was 95% and 98%, respectively. The efflux pumps are physiologically involved in the extrusion of various metabolites, dyes, and chemicals that are toxic to the cells. In addition, the efflux pumps play an important role in the emergence of multiple drug resistances among bacteria [5]. The efflux pumps can decrease the intracellular concentration of antibiotics, which is a critical factor in bacterial survival [23]. In our study of the correlation between efflux pumps and antibiotic resistance, the results show that all *Klebsiella pneumoniae* isolates that have efflux pumps are resistant to ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, cefixime, aztreonam, trimethoprim/sulfamethoxazole, trimethoprim, and vancomycin with a percentage of 100%. In addition, the percentage of resistance for ciprofloxacin (85.24%), nitrofurantoin (81.96%), tetracycline (78.68%), amikacin (73.77%), and tobramycin (68.85%) Ho *et al.* [24], Wyres and Holt [25], and Li *et al.* [21], the role of the *oqxAB* efflux systems in antibiotic resistance was shown, where the researchers noticed that the presence

of the pump produces resistance to antibiotics such as quinolones, nitrofurantoin, and chloramphenicol, as well as detergents and disinfectants. One study in Iran comprised 100 *K. pneumoniae* isolates from UTI patients, which agrees with our findings. Molecular distribution tests revealed that 57% and 56% of isolates, respectively, possessed the *oqxA* and *oqxB* genes. Fluoroquinolone and beta-lactam resistance phenotypes were substantially related to the existence of *oqxA/oqxB* and *qepA* efflux genes [21]. Another study comprised 110 clinical strains of *K. pneumoniae* acquired from patients referred to hospitals in Tehran. The PCR approach revealed that the predominance of *oqxA/oqxB* genes is 52 (47.27%). The results showed that the OqxAB efflux pumps are important part of how

antibiotic-resistant *K. pneumoniae* isolates are. Because antimicrobial resistance genes can be passed on pretty easily, it is very important to use molecular methods to accurately identify resistance genes in order to stop the spread of resistant strains [26]. This study also shows how the efflux pump genes are spread among highly resistant *K. pneumoniae* strains that were taken from different clinical sources (Table 4). A statistically significant difference was observed in the present study between the source of infection and the prevalence of efflux pump genes ($p < 0.01$).

Table 4: The distribution of efflux pump genes among highly resistant *K. pneumoniae* strains isolated from various clinical sources

Sample type	Urine (32)	Sputum (13)	Wound (6)	Burns (6)	Blood (4)	Total= 61	<i>p</i> -value
<i>oqx A n</i> (%)	20(62.5)	8(61.53)	2(33.3)	6(100)	3(75)	39	0.0001
<i>oqx B n</i> (%)	27(84.37)	11(84.61)	5(83.3)	6(100)	2(50)	51	0.0001

Conclusion

All isolates of *K. pneumoniae* were 100% resistant to amoxicillin-clavulanate, ampicillin, and vancomycin, while the lowest resistance was against tigecycline with a percentage of 11.43%. The distribution of the *oqxB* gene was more frequent than that of the *oqxA* gene. All *Klebsiella pneumoniae* isolates that have OqxAB genes are resistant to most antibiotics.

Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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