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Molecular Characterization of Efflux Pump and Porin Related Genes in Multidrug Resistance *Klebsiella pneumoniae* Isolates Recovered from Erbil Hospitals

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ABSTRACT

Background: Multidrug-resistant efflux pumps are one of the most significant methods by which bacteria can evade the effects of numerous antimicrobials. The aim of this study was to determine the prevalence of multidrug-resistant MDR, extremely drug-resistant XDR, and pan-drug-resistant PDR *K. pneumoniae* phenotypes in clinical isolates, as well as to examine the detection and prevalence of efflux pump genes *ompK35*, *ompK36*, *tolC*, and *acrAB* in multidrug-resistant *K. pneumoniae* strains.

Materials and Methods: This study was conducted on 60 isolates of *K. pneumoniae* collected from different clinical samples in Erbil hospitals. The frequency of MDR, XDR, and PDR *K. pneumoniae* phenotypes was characterized through an antibiotic susceptibility profile, and genes associated with efflux pumps and porins were detected by polymerase chain reaction (PCR) assay.

Results: All 60 *K. pneumoniae* isolates were resistance to one or more classes of antibiotics tested in this study. The maximum resistance was observed with ampicillin (95%), followed by piperacillin acid (83%). Among all isolates the percentage of MDR, XDR, and PDR were (38%), (37%), and (25%) respectively. Furthermore, the distribution of efflux pump genes was *acrAB* (95%) and *tolC* (93%), while the prevalence of porin-coding genes *ompK35* and *ompK36* were (80%) and (82%) respectively.

Conclusion: Prevalence of MDR, XDR, and PDR *K. pneumoniae* strains in Erbil city is worrisome in hospital wards. Furthermore, Genes encoding efflux pump and porins such as *acrAB*, *tolC*, *ompk35*, and *ompK36* were highly distributed among all isolates.

Key words:

K. pneumoniae, efflux pump, *acrAB*, *tolC*, *ompK35*, *ompK36*, multidrug resistance.

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الخلاصة

مقدمة: تعد مضخات التدفق المقاومة للأدوية المتعددة واحدة من أهم الطرق التي يمكن للبكتيريا من خلالها تجنب تأثيرات العديد من مضادات الميكروبات. هدفت هذه الدراسة إلى تحديد مدى انتشار MDR المقاوم للأدوية المتعددة ، و XDR شديد المقاومة للأدوية ، والأنماط الظاهرية PDR الرئوية المقاومة للأدوية في العزلات السريرية *K. pneumoniae* ، وكذلك لفحص اكتشاف وانتشار جينات مضخة التدفق *ompK35*، *ompK36*، *tolC* ، و *acrAB* في سلالات *K. pneumoniae* المعزولة المقاومة للأدوية المتعددة.

طرق العمل: أجريت هذه الدراسة على 60 عزلة من بكتيريا *K. pneumoniae* جمعت من عينات سريرية مختلفة في مستشفيات أربيل. تم تمييز تواتر الأنماط الظاهرية لـ MDR و XDR و PDR تم تمييز الأنماط الظاهرية لـ *K. pneumoniae* من خلال ملف تعريف حساسية المضادات الحيوية ، وتم اكتشاف الجينات المرتبطة بمضخة التدفق و البورين عن طريق مقايسة تفاعل البلمرة المتسلسل (PCR).

الاستنتاجات: ان انتشار سلالات MDR و XDR وPDR *K. pneumoniae* في مدينة أربيل أمر مقلق في أجنحة المستشفيات. علاوة على ذلك ، تم توزيع الجينات المشفرة لمضخة التدفق و porins مثل *acrAB* ، *tolC* ، *ompk35*، و *ompK36* بشكل كبير بين جميع العزلات.

الكلمات المفتاحية:

K. pneumoniae ، مضخة التدفق ، *acrAB*، *tolC*، *ompK35*، *ompK36* ، مقاومة الأدوية المتعددة

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INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is a rod-shaped, Gram-negative, non-motile, encapsulated, facultatively anaerobic, bacterium [1 and 2]. It ferments lactose and exhibits a mucoid phenotype on culture media [3]. It is associated with extra-intestinal infections and life-threatening illnesses, including septicemia and endocarditis [4]. Continual and unrestricted use of antimicrobial medications for treatment of bacterial infections, in addition to horizontal gene transfer among *K. pneumoniae* correlates with the spread of MDR bacteria in clinical settings [5 and 6]. The efflux pump system is one of the main reasons of the appearance of MDR bacteria in clinical isolates [7].

MDR is described as resistance to at least one agent across three or more antimicrobial classes. Whereas XDR is described as resistance to at least one antimicrobial agent in all antimicrobial categories other than two or hardly any antimicrobial classes [8]. Efflux pumps are transport proteins responsible for innate or induced resistance to various antibiotics, based on the chromosomal or plasmid origins of efflux genes, accordingly. Such proteins are responsible for the expulsion of hazardous substrates, including antibiotics, from cells into the surrounding environment [9]. Consequently, reducing antibiotic concentrations within the cell [10]. Gram-negative bacteria have acquired multiple methods of tolerance to currently used antimicrobials [11].

Genes encoding for the MDR efflux pump system *AcrAB-TolC* and *MdtK*, as well as major outer membrane porin (*OmpK35* and *OmpK36*), have significant clinical aspects on the incidence of bacterial drug resistance in *K. pneumoniae* isolates [12]. The AcrAB and mdtK systems belong to the *K. pneumoniae* resistant nodule (RND) and multi-antimicrobial extrusion (MATE) efflux pump families [13]. AcrAB-TolC pumps is accountable for tetracycline, quinolone, and chloramphenicol resistance in several Strains isolated [14]. Some of these antimicrobials are transported by MATE pumps, namely the mdtK system [15]. Membrane proteins like OmpK35 and OmpK36 are essential for antibiotic perforation inside cells and sensitivity to carbapenems and cephalosporins [11]. Data on the prevalence of efflux pump-related genes of *K. pneumoniae* strains are limited in the Kurdistan region. Accordingly, the present study has been conducted to find out the multi-drug resistance profiles, and frequency of MDR, XDR, and PDR phenotypes as well as to investigate the existence and prevalence rate of efflux pump-related genes in *K. pneumoniae* strains recovered from different types of nosocomial infections.

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Materials and Methods

Specimen's collection and sources

Between July 2021 and February 2022, 60 unique isolates of *K. pneumoniae* were isolated from different hospitals in Erbil city, Kurdistan, Iraq. The purified colonies were recognized as *K. pneumoniae* by different biochemical and standard diagnostic tests such as urease and catalase test, as previously mentioned [16]. To affirm the identification of all isolates, Vitek II automated system (bioMérieux Marcy l''Etoile, France) (Vitek Systems Version: 06.01) was applied. The purified isolates were stored in brain heart infusion broth (BHI) (Oxoid) supplemented with 15% glycerol at − 20 °C for further study. This research project was allowed in all likelihood by the Scientific and Research Ethics Committee at the College of Health Sciences, Hawler Medical University/ Iraq, ethic approval number 69 which was accepted in 26/11/2022. All persons had given their acceptance to share in the study.

Antibiotic susceptibility pattern

To assess the sensitivity profile, the identified isolates were exposed to a group of antimicrobials comprising Imipenem (IMI), Meropenem (MEM), Ertapenem (ETP), Piperacillin (PIP), Piperacillin-tazobactam (PTZ), Ampicillin (AMP), Amoxicillin Clavulanic Acid (AMC), Ciprofloxacin (CIP), Ceftriaxone (CTX), Ceftazidime (CAZ), trimethoprim-sulfamethoxazole (SXT), Gentamicin (GN), Amikacin (AK), by Vitek II automated system *in agreement* with the manufacturer's instructions [17]. Clinical and Laboratory Standards Institute (CLSI) breakpoints were used to analyze data, and results were recorded as susceptible (S) or resistant (R) [18].

Detection of efflux pump and porin genes using PCR

Genomic DNA extraction

Using the Beta Byern Biotech DNA isolation kit (Beta Byern, Germany) in agreement with the manufacturer's protocol, whole genomic DNA was extracted from the LB-grown colonies of every isolate. To evaluate the extracted DNA's purity, the absorbance ratio A260/A280 was measured with a Nanodrop spectrophotometer. Until further usage, the crude DNA extracts were refrigerated at -20°C.

Molecular methods

Primer sequences for *K. pneumoniae* efflux pumps (*acrAB* and *tolC*) and porin genes (*ompK35* and *ompK36*) were used as depicted previously in (Table 1). Each PCR tube consisted of a total volume of 20 µL of a reaction mixture as mentioned previously

[19]. Conventional PCR for amplification of targeted genes (*acrAB* and *tolC)* were performed under the conditions prescribed in [14], regarding *ompK35* and *ompK36* genes the condition mentioned in [19] were followed using the BioRad thermal cycler (MJ Mini, BioRad, USA). The amplified products were separated on a 2.0% agarose gel and electrophoresed at 80 V, 30 mA for 1 hour. DNA fragments were visualized under an ultraviolet (UV) transilluminator. A PCR DNA ladders with 100 bp augmentation were used as a DNA standards.

Table 1. List of primers, amplicon sizes, and annealing temperatures.

Target Genes	Primer sequence (5′-----3′)	Amplicon size (bp)	Tm °C	Reference
AcrAB	F: ATCAGCGGCCGGATTGGTAAA R: CGGGTTCGGGAAAATAGCGCG	312	53	[14]
Ompk35	F:GCAATATTCTGGCAGTGGTGATC R: ACCATTTTTCCATAGAAGTCCAGT	109	57	[19]
Ompk36	F:TTAAAGTACTGTCCCTCCTGG R:TCAGAGAAGTAGTGCAGACCGTCA	130	58	[19]
TolC	F: ATCAGCAACCCCGATCTGCGT R: CCGGTGACTTGACGCAGTCCT	527	51	[14]

Results and Discussion

A total of sixty (40%) non-repetitive *K. pneumoniae* strains were isolated from 150 different clinical specimens, isolates were identified based on bacterial colony morphology on agar media, isolates which showed positive catalase and urease test were confirmed by VITEK 2 Compact. Urine samples (25, 41.6%) were the most common source of *K. pneumoniae*, the second largest source was sputum samples (22, 36.6%), followed by blood samples (11, 18.3%), then wound swabs (2, 3.3%) as shown in (Figure 1). [12] observed that the total incidence of *K. pneumoniae* in healthcare illnesses in Iran was (44%). They determined that the frequency of *K. pneumoniae* in urine, blood, sputum, wound and rectal swab samples was (64 %), (38%), (35%), and

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(33.33%) respectively. [20] observed that 20% of healthcare infections in Iran health facilities were caused by *K. pneumoniae* strains. They identified urinary tract infections (UTIs) among the most predominant infections caused by *K. pneumoniae* isolates (63%). Previously, comparable outcomes discovered in Pakistan and India respectively [21 and 22]. In addition, a greater proportion of *K. pneumoniae* variants in UTIs have been documented in the United States [22].

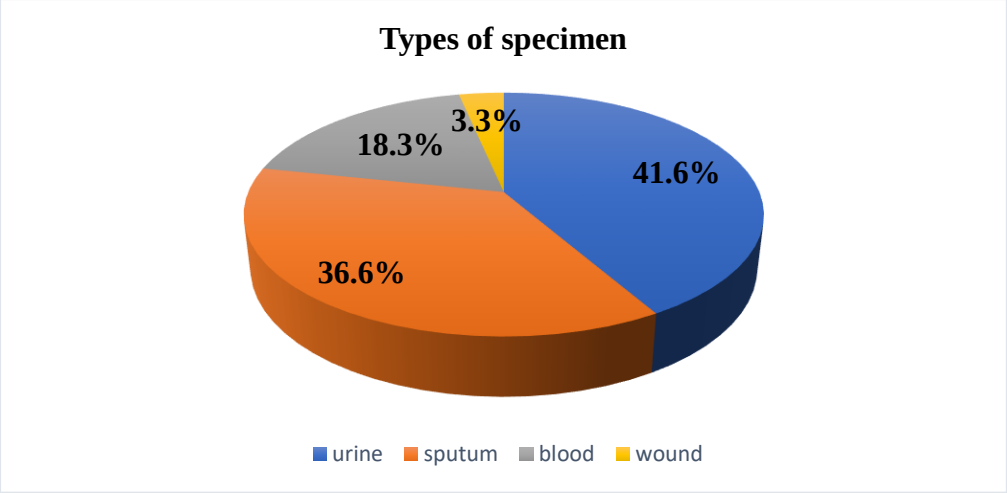

Figure 1. Distribution of *K. pneumoniae* recovered from clinical specimens.

The antimicrobial sensitivity profile of all *K. pneumoniae* isolates upon 13 antimicrobial drugs from 8 antimicrobial classes is shown in (Table 2). According to the results of the present study, all isolates were resistant with varied rates to tested antimicrobials. It has been observed that *K. pneumoniae* strains possess the highest frequency of resistance against ampicillin (95%), piperacillin (83.3%), ceftazidime (81.7%), piperacillin-tazobactam (75%), amoxicillin-clavulanic acid (73.3%), ceftriaxone (68.3%), ciprofloxacin (66.7%), amikacin (63.3%), gentamicin (61.7%) and trimethoprim-sulphamethoxazole (61.7%). However, *K. pneumoniae* isolates possess the lowest frequency of resistance against imipenem (41.7%), meropenem (38.3%), and ertapenem (36.7%). All *Klebsiella pneumoniae* isolates have undergone antibiotic susceptibility testing to multiple classes of antibiotics, including antipseudomonas, folate pathway inhibitors, penicillins, beta-lactamase inhibitors, aminoglycosides, carbapenems, fluoroquinolones, and cephalosporins extended spectrum. As determined, it was resistant to at least three classes of antibiotics.

The current study found that (38%) of the isolates were multidrug resistant (MDR) *K. pneumoniae*. It is characterized by resistance to at least one agent of three or

more antibiotic classes [8]. The high rate of antimicrobial resistance found in our study may be attributed to the lack of stringent policies governing antimicrobial use in the city of Erbil. Our study demonstrates that most isolates were resistant to Ampicillin (95%). Similar investigations have discovered a high ampicillin resistance in China [23]. Significantly higher rates of antimicrobial resistance have been identified in strains recovered from urine (26.7%) and sputum (26.6%) samples.

Table 2. Antibiotic resistance pattern of *K. pneumoniae* strains

Antibiotic category	Antimicrobial agent	Resistance %		Sensitive %		Total %
Aminoglycosides	Amikacin	38	63.3%	22 36.7%		60 100%
	Gentamicin	37	61.7%	23 38.3%		60
Carbapenems	Imipenem	25	41.7%	35 58.3%		60 100%
	Meropenem	23	38.3%	37 61.7%		60 100%
	Ertapenem	22	36.7%	38 63.3%		60 100%
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin-tazobactam	45	75%	15	25%	60 100%
Extended-spectrum Cephalosporins	Ceftriaxone	41	68.3%	19 31.7%		60 100%
	Ceftazidime	49	81.7%	11 18.3%		60 100%
Fluoroquinolones	Ciprofloxacin	40	66.7%	20 33.3%		60 100%

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Folate pathway inhibitors	Trimethoprim-sulphamethoxazole	37	61.7%	23 38.3%		60	100%
Penicillins	Ampicillin	57	95%	3	5%	60	100%
	Piperacillin	50	83.3%	10	16.7%		
Penicillins + β-lactamase inhibitors	Amoxicillin-clavulanic acid	44	73.3%	16	26.7%	60	100%

Furthermore, the results of this study demonstrated grouping of the *Klebsiella pneumoniae* phenotype according to the antimicrobial resistance profile, as shown in (Figure 2). The study found that 15 (25%) isolates were resistant to each of the eight antimicrobial categories identified as PDR *K. pneumoniae*. Twenty-two (37.0%) isolates identified as XDR *K. pneumoniae* were unsusceptible to any agent except for two or fewer antimicrobial categories. In addition, resistance to one or more of this agents of three or more antimicrobial categories was observed in 23 (38%) isolates classified as MDR *K. pneumoniae*.

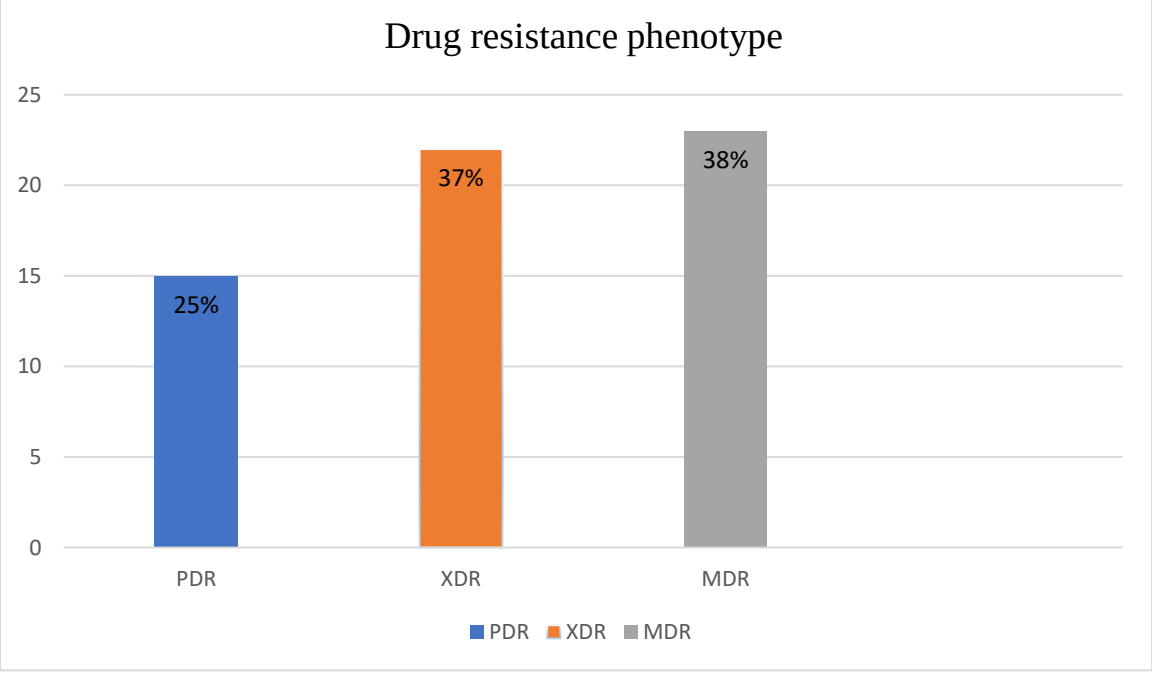

Figure 2. Drug resistance phenotype of *K. pneumoniae* recovered from clinical specimens.

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PCR assay using specific primers for efflux pump and porin genes demonstrated that the most prevalent observed efflux pump genes of the *K. pneumoniae* were *acrAB* (95%) followed by *tolC* (93%). Nevertheless, the frequency of porin-coding genes was less abundant regarding *ompK36* (82%) and *ompK36* (80%) as shown in (Figure 3) and (Table 3). Furthermore, It has been observed that *acrAB* gene coding for the efflux pump system was determined in (100%) of urine samples. Besides *tolC* gene was found in all samples recovered from sputum while it is not detected in any isolates recovered from the wound. All isolates obtained from wound samples had the *ompK35* and *ompK36* porin-coding genes.

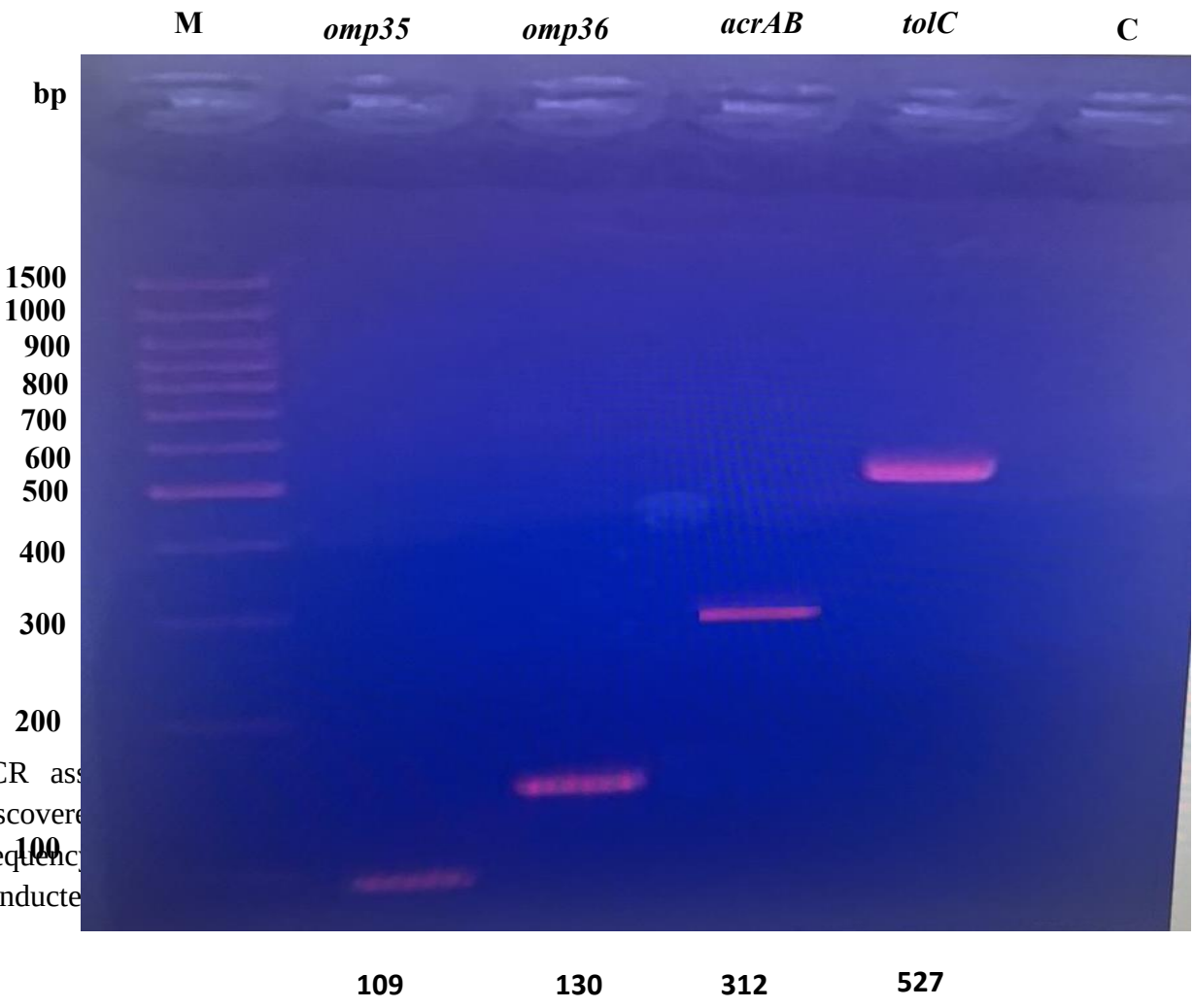

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Figure 3. PCR product for porin and efflux pump related genes *in K. pneumoniae*. M is DNA marker. The size of each PCR product is indicated, C is negative control.

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Table 3. Distribution of porin and efflux pump genes in *Klebsiella pneumoniae* strains isolated from different types of nosocomial infections

Type of samples	Genes coding for porins and efflux pump							
	Ompk35		***Ompk36***		***TolC***		***AcrAB***	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Urine (25)	24(96%)	1(4%)	21(84%)	4(16%)	24(96%)	1(4%)	25(100%)	0
Blood (11)	6(55%)	5(46%)	8(73%)	3(27%)	10(91%)	1(9%)	10(91%)	1(9%)
Wound (2)	2(100%)	0	2 (100%)	0	0	2(100%)	1(50%)	1(50%)
Sputum (22)	16(73%)	6(27%)	18(82%)	4(18%)	22(100%)	0	21(96%)	1(5%)
Total (60)	48(80%)	12(20%)	49(82%)	11(18%)	56(93%)	4(7%)	57(95%)	3(5%)

K. pneumoniae contributes significantly to the global incidence of antibiotic resistance [4]. Antibiotic efflux pumps are among the most important antibiotic resistance mechanisms utilized by clinical strains of *K. pneumoniae* [24]. The higher efflux of the antimicrobial drug reduces its intracellular levels, hence promoting microbial viability [25]. Both *ompK35* and *ompK36* contribute to the virulence and infection of *K. pneumoniae*. *ompK36* or *ompK*35/*ompK36* deletions can reduce the pathogenicity of hypervirulent bacteria and make them more vulnerable to neutrophil clearance [26].

In the current study, the prevalence of the AcrAB efflux pump system was 95%. The *acrAB* gene, encoding the efflux pump system, was found in 100% of urine samples. This was consistent with an Egyptian study [14]. Another study was then conducted in Saudi Arabia [27]. Several genes important for intracellular invasion and maintenance have been found to be deregulated in mutant strains lacking the AcrAB-tolC efflux pump [28]. Additionally, the *tolC* gene was identified in all sputum samples. This was the same as a study reported in Saudi Arabia [27].

The antibiotic efflux pump is one of the most important mechanisms of antibiotic resistance postulated by clinical isolates of *K. pneumoniae*. Accessory genomes are primarily responsible for carbapenem resistance and can also be associated with alterations in coding regions. Carbapenem resistance in *K. pneumonia* can be affected

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by up regulation of efflux pumps [29], mutations of outer membrane proteins in the nuclear genome [30], and overproduction of ESBL enzymes or AmpC-β-lactamase in accessory genomes [31].

It has been concluded from the present study that *K. pneumoniae* isolates express a high prevalence of resistance against ampicillin, piperacillin, ceftazidime, piperacillin-tazobactam, and amoxicillin-clavulanic acid antibiotics. Furthermore, observation of a high prevalence of MDR, XDR, and PDR *K. pneumoniae* strains in Erbil city is worrisome in hospital wards.

AcrAB efflux system is one of the primary antibiotic resistance pathways in multidrug-resistant *K. pneumoniae* strains. We detected high distribution of *acrAB*, *tolC*, *ompk35*, and *ompK36* genes in our isolates, which increase the survival of these pathogens when they are exposed to different antimicrobial agents.

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Conflict of interests.

There are non-conflicts of interest.

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