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Unveiling Efflux Pump-Mediated Antibiotic Resistance in *Klebsiella pneumoniae* Clinical Isolates: A Call for Strategic Intervention

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Abstract

Introduction: *Klebsiella pneumoniae* stands as a significant opportunistic pathogen, often implicated in hospital-acquired infections. The escalating levels of antibiotic resistance, propelled by novel bacterial survival mechanisms, present a dire challenge, leading to treatment failures and amplified mortality rates. Efflux pump systems which play a pivotal role in antibiotic resistance actively expel toxic compounds, thereby fostering bacterial survival and the emergence of resilient strains. This study endeavored to examine the frequency of efflux genes in clinical isolates of *K. pneumoniae*.

Methods: Ninety-six non-repetitive clinical isolates of *K. pneumoniae* were obtained from patients attending Khatam-Al-Anbeya hospital in Zahedan, Iran. Standard biochemical laboratory methods were employed to identify all bacterial isolates. Furthermore, genomic DNA extraction was performed using the boiling method, followed by multiplex polymerase chain reaction (PCR) targeting the *AcrAB*, *MdtK*, and *TolC* efflux pump genes.

Results: Our findings revealed high prevalence rates: 94 (97.91%) for *AcrAB*, 92 (95.83%) for *TolC*, and 43(44.79%) for *MdtK* genes, indicating the widespread presence of efflux pump coding genes in *K. pneumoniae* isolates. Moreover, 92 isolates contained multiple studied genes.

Conclusion: Efflux pump-mediated antibiotic resistance represents a significant challenge in the treatment of *K. pneumoniae* infections. This study highlights the urgent need for strategies to combat efflux pump-mediated resistance and preserve the efficacy of antibiotic therapies against this clinically important pathogen.

Keywords: *Klebsiella pneumoniae,* Efflux genes, Clinical isolates, Antibiotic resistance

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Introduction

Antibiotic resistance has emerged as a formidable global health crisis, undermining the effectiveness of conventional treatment strategies and posing significant challenges to infectious disease management.[1,2] Among the myriad of bacterial pathogens implicated in antibiotic resistance, *Klebsiella pneumoniae* stands out as a particularly concerning opportunistic pathogen, capable of inducing a diverse range of infections, ranging from urinary tract infections to potentially life-threatening bloodstream infections, particularly in hospitalized patients.[3] Infections caused by *K. pneumoniae* are associated with elevated rates of morbidity and mortality, especially when complicated by multidrug resistance (MDR), emphasizing the urgent need for effective therapeutic interventions.[4] The rise of antibiotic resistance in *K. pneumoniae* can be attributed to various factors, including the assimilation of resistance genes through lateral gene transfer, mutations in chromosomal genes encoding antibiotic targets, and the expression of efflux pump systems.[5,6] Efflux pumps, integral membrane proteins capable of actively transporting antibiotics and other antimicrobial agents out of bacterial cells, represent a major mechanism for MDR in Gram-negative bacteria, including *K. pneumoniae*.[7,8] Efflux pumps confer resistance to multiple classes of antibiotics by expelling these agents from the bacterial cytoplasm, thereby reducing intracellular drug concentrations below

the threshold required for bactericidal or bacteriostatic activity.[9] Moreover, efflux pump-mediated resistance can facilitate the emergence of MDR strains through the selection of mutations that further enhance pump activity or alter antibiotic targets.[10,11] Consequently, the widespread dissemination of efflux pump genes within clinical isolates of *K. pneumoniae* poses a remarkable challenge to antimicrobial therapy and infection control efforts in healthcare settings.[12]

Efflux pump systems in *K. pneumoniae* encompass a diverse array of proteins that belong to different families, including the resistance-nodulation-division superfamily, the major facilitator superfamily (MFS), and the small multidrug resistance family.[13,14] Among these, the AcrAB-TolC efflux pump stands as one of the most thoroughly researched and clinically significant efflux systems in Gram-negative bacteria, particularly in *K. pneumoniae*.[15] The *AcrAB-TolC* pump, comprised of three components (*AcrB*, *AcrA*, and *TolC*), functions as a tripartite complex spanning the inner and outer layers of the bacterial cell envelope, facilitating the efflux of a broad spectrum of antibiotics, detergents, and other toxic compounds.[16]

In addition to the *AcrAB-TolC* system, other efflux pumps such as *MdtK* have been implicated in conferring antibiotic resistance in *K. pneumoniae*.[17] *MdtK*, a member of the MFS family, is known to confer resistance to multiple antimicrobial agents, including fluoroquinolones, tetracyclines, and chloramphenicol, by actively extruding these compounds from the bacterial cytoplasm.[18] The prevalence and clinical significance of efflux pump-mediated antibiotic resistance in *K. pneumoniae* have garnered increasing attention in recent years, particularly in the context of nosocomial infections and the global dissemination of MDR strains.[19] Understanding the distribution and genetic determinants of efflux pump systems in clinical isolates of *K. pneumoniae* is crucial for devising effective therapeutic strategies to combat antibiotic resistance and mitigate the impact of nosocomial outbreaks.[20] As such, this study aimed to investigate the prevalence of efflux pump genes, including *AcrAB* and *MdtK*, in clinical isolates of *K. pneumoniae* obtained from hospitalized patients in Zahedan Hospital, Iran. By elucidating the molecular mechanisms underlying antibiotic resistance in *K. pneumoniae*, this study sought to explore the development of targeted therapies and disease prevention strategies to combat the rising tide of MDR infections caused by this clinically significant pathogen.

Materials and Methods

Sample Preparation

Ninety-six clinical isolates of *K. pneumoniae* were obtained from patients, consisting of 58 women and 38 men, who were hospitalized at Khatam Al-Anbiya hospital, affiliated with Zahedan University of Medical Science, Zahedan, Iran. All clinical samples were initially preserved in Cary-Blair transport media (Laboratorios Conda, S.A., Spain) and expeditiously transferred to the laboratory while maintained on ice. Upon reaching the laboratory, a loopful from each sample was promptly streaked onto MacConkey agar (Oxoid, UK) and EMB agar (HiMedia Laboratories) within a time frame of 4 hours from collection. The plates were subsequently incubated at 37 ˚C for 18–24 hours. Following incubation, up to three colonies displaying characteristic *K. pneumoniae* morphology were identified and selected for additional biochemical analysis. These analyses comprised oxidase, indole, methyl red, Voges-Proskauer, nitrate reduction, urease production, Simmons' citrate agar (HiMedia Laboratories), and a range of sugar fermentation assays.[21]

DNA Extraction

DNA extraction was performed using the boiling method.[22] A single colony from each clinical isolate was inoculated into five milliliters of Brain Heart Infusion (BHI) broth (Oxoid, UK) and incubated at 37 ˚C for 18-24 hours. Subsequently, the bacterial culture was centrifugated at 3000 rpm for 5 minutes. After discarding the supernatant, the bacterial pellets were resuspended in 200 µL of nuclease-free distilled water and then exposed to 95 ˚C for 10 minutes. The boiled lysates were then centrifuged at 14 000 rpm for 30 minutes, and the supernatant was collected to obtain DNA templates, which were stored at -80 ˚C until further use.

Polymerase Chain Reaction Methods

The prevalence of *AcrAB, MdtK*, and *TolC* efflux pump genes was ascertained in one polymerase chain reaction (PCR) using three forward and reverse primers through a multiplex PCR method. Primers targeting the *AcrAB*, *MdtK,* and *TolC* efflux pump genes were designed based on known sequences Table 1.

Table 1 displays the primer sequences utilized for the PCR identification of the *AcrAB*, *MdtK*, and *TolC* genes.

A total volume of 20 µL was prepared by adding 2 µL of DNA template, 10 µL of PCR 2 × RedMasterMix (Amiqon), and 1 µL of each primer (10 µM) and nuclease-free water.

Table 1. The Primer Sequences for the PCR Identification of the *AcrAB*, *MdtK*, and *TolC* Genes

Gene Name		Primer Sequence	Size (bp)
AcrAB	Forward	ATCAGCGGCCGGATTGGTAAA	311
	Reverse	GGGTTCGGGAAAATAGCGCG	
TolC	Forward	ATCAGCAACCCCGATCTGCGT	527
	Reverse	CCGGTGACTTGACGCAGTCCT	
MdtK	Forward	GCGCTTAACTTCAGCTCA	453
	Reverse	GATGATAAATCCACACCAGAA	

Note. PCR: Polymerase chain reaction.

PCR conditions comprised an initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of denaturation at 95 ˚C for 30 seconds, annealing at 56 ˚C for 30 seconds, extension at 72 ˚C for 1 minute, and a final extension at 72 ˚C for 5 minutes. The analysis of PCR products was conducted by gel electrophoresis in a 1.5% agarose gel, ethidium bromide staining, and image analysis using a Gel Doc 1000 (Vilber Lourmat, France) to determine the presence of specific amplicons indicative of the *AcrAB*, *MdtK*, and *TolC* genes. Then, a molecular weight marker (100 bp ladder, Fermentas) with increments of 100 bp was used (Figure 1).

Results

The analysis of the PCR results revealed a high prevalence of efflux pump genes among the clinical isolates of *K. pneumoniae*. Specifically, the presence of the *AcrAB* efflux pump gene was observed in 94 (97.91%) of isolates, whereas the *TolC* gene was detected in 92 (95.83%) of isolates. Furthermore, the *MdtK* gene was identified in 43 (44.79%) of isolates (Table 2). Notably, all isolates (100%) harbored at least one efflux pump coding gene (Table 3). These findings underscore the widespread presence of efflux pump-mediated antibiotic resistance mechanisms in *K. pneumoniae*.

Furthermore, the genetic presence of *AcrAB*, *MdtK*, and *TolC* genes was observed in 41 out of 70% of isolates, while a significant majority of 52.08% showed the co-occurrence of *AcrAB* and *TolC*. It is worth noting that *AcrAB* and *MdtK* had lower incidences, as depicted in Table 3.

Discussion

The increasing prevalence and dissemination of antimicrobial resistance pose a substantial risk to public health worldwide, resulting in increased morbidity, mortality, and healthcare costs.[23] Efflux pump-mediated antibiotic resistance is a mechanism used by bacteria to avoid the effects of antimicrobial agents.[17] In this investigation, the prevalence of efflux pump genes was assessed in clinical isolates of *K. pneumoniae* collected from hospitalized patients at Zahedan Hospital. The obtained results showed a high occurrence of efflux pump genes in the *K. pneumoniae* isolates, with the *AcrAB* efflux pump gene being the most common, found in 97.91% of isolates. This finding aligns with previous studies indicating the widespread presence of *AcrAB* efflux pumps in clinical isolates of different Enterobacteriaceae species, including *K. pneumoniae*.[24] The *AcrAB* efflux pump functions as a multidrug efflux system, conferring resistance against a broad spectrum of antibiotics such as β-lactams, fluoroquinolones, tetracyclines, and chloramphenicol.[25] Additionally, the findings demonstrated the presence of the *TolC* gene that specifies an outer membrane protein crucial for the function of *AcrAB-TolC* efflux pump complexes in 95.83% of *K. pneumoniae* isolates. The co-occurrence of *TolC* with *AcrAB* efflux pump genes emphasizes the significance of this tripartite efflux pump system in mediating antibiotic resistance in clinical isolates of *K. pneumoniae* (Table 3). Similar findings have been reported in previous studies, highlighting the connection between the presence of *TolC* and *AcrAB* efflux pump genes in MDR Gram-negative bacteria.[26] Furthermore, in addition to *AcrAB* and *TolC*, we examined the frequency of the *MdtK* efflux pump gene, which was present in 44.79% of *K. pneumoniae* isolates. The *MdtK* efflux pump is part of the MFS and contributes to resistance against diverse antimicrobial agents, including fluoroquinolones and tetracyclines.[27] Although the prevalence of the *MdtK* gene was lower

Table 2. The Frequency of *AcrAB*, *MdtK*, and *TolC* Genes

Gene Name	*AcrAB*	*MdtK*	*TolC*
Number	94	43	92
Frequency %	97.91	44.79	95.83

Table 3. Pattern Distribution of *AcrAB, MdtK,* and *TolC* Genes between *Klebsiella pneumoniae* Isolates

Pattern	*AcrAB*	*MdtK*	*TolC*	Number
KP1	+	+	+	41
KP2	+	-	+	50
KP3	+	+	-	1
KP4	+	-	-	2
KP5	-	-	+	1
KP6	-	+	-	1
Total				96

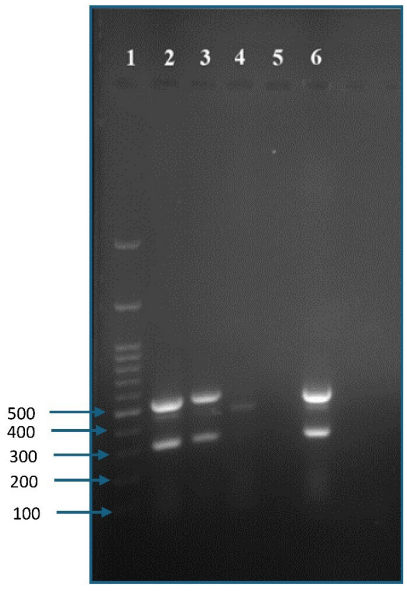

Figure 1. The Gel Electrophoresis Results of the PCR Products Obtained from *AcrAB*, *MdtK*, and *TolC* Genes. *Note.* PCR: Polymerase chain reaction; Lane1 shows a 100 bp ladder marker, Lane 2 exhibits the PCR products for *TolC* (523 bp), *MdtK* (453 bp), and *AcrAB* (311 bp), Lane 4 displays the PCR product for *MdtK* (453), Lane 5 is the negative control, and Lane 6 is the positive control

than that of *AcrAB* and *TolC*, its identification highlights the diversity of efflux pump-mediated resistance mechanisms utilized by *K. pneumoniae*. The result of the current study offers valuable insights into the prevalence of efflux pump genes in clinical isolates of *K. pneumoniae*, underscoring the pressing need for effective strategies to address antimicrobial resistance in healthcare settings. Efflux pump inhibitors present a promising approach to combat efflux pump-mediated resistance by increasing the effectiveness of antibiotics that are targeted by these pumps.[28] However, the development of clinically effective efflux pump inhibitors encounters various challenges, including identifying compounds with suitable adequate pharmacokinetics and mitigating potential toxicity.[29] Comparison with other similar studies consistently reveals findings regarding the prevalence of efflux pump genes in clinical isolates of *K. pneumoniae*. For example, Wasfi et al investigated the prevalence of efflux pump genes in 36 MDR clinical isolates and found a high prevalence of *AcrAB-TolC* efflux pump genes, with 82% of isolates carrying at least one efflux pump gene.[30] Similarly, Pakzad et al detected the presence of *AcrAB* efflux pump genes in 100% of *K. pneumoniae* ciprofloxacin-resistant isolates obtained from clinical samples.[31]

Furthermore, our findings align with global surveillance data that show the widespread presence of efflux pump genes within clinical isolates of *K. pneumoniae*. Moreover, the World Health Organization (WHO) Global Antimicrobial Surveillance System (GLASS) has documented a significant occurrence of MDR *K. pneumoniae* isolates worldwide, where efflux pump-mediated resistance significantly contributed to the dissemination of resistance mechanisms.[32]

The identification of *MdtK* alongside *AcrAB* and *TolC* genes in 44.709% of isolates suggests a potential synergy or cooperative interaction between these efflux systems in conferring MDR. Although the role of *MdtK* in antimicrobial resistance is less well-characterized compared to *AcrAB-TolC*, its co-occurrence with these established efflux pump components indicates its possible involvement in augmenting the efflux capabilities of bacterial cells. The *AcrAB-TolC* efflux pump system is well-known for its role in conferring resistance to a broad spectrum of antimicrobial determinants, functioning as a key mechanism for expelling various toxic compounds from bacterial cells.[28] The presence of both *AcrAB* and *TolC* genes in a significant proportion of isolates (52.08%) underscores the significance of this efflux pump system in mediating antimicrobial resistance. Furthermore, the lower frequencies of *AcrAB* and *MdtK* co-detection, as indicated in Table 3, could signify various factors influencing the prevalence and expression of these genes within the bacterial population. It is plausible that genetic variations, selective pressures from antimicrobial usage, or environmental factors might contribute to the differential distribution of these genes within the isolates.

Conclusion

In conclusion, our study highlights the high prevalence of efflux pump genes in clinical isolates of *K. pneumoniae* obtained from hospitalized patients. The detection of *AcrAB*, *TolC*, and *MdtK* efflux pump genes underscores the importance of efflux pump-mediated resistance mechanism in conferring MDR in *K. pneumoniae*. Efforts to develop novel therapeutic strategies, including efflux pump inhibitors, are warranted to combat the spread of antimicrobial resistance and preserve the efficacy of existing antibiotics. However, some scientific limitations hinder the study's potential to provide conclusive evidence regarding gene function and the role of efflux pumps in antibiotic resistance. Addressing these limitations through rigorous validation of gene expression, antibiotic susceptibility testing, and determination of minimum inhibitory concentration would enhance the robustness and reliability of the study's findings.

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Conceptualization: Ahmad Rashki.
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Competing Interests

The authors have no conflict of interests to declare.

Ethical Approval

Ethical approval for this study was then obtained from the Iranian Ministry of Health and Medical Education, as well as the Research Council of the University of Zabol and the Research Ethics Committee (IR.UOZ.REC.1402.031). In addition, patient medical data and personal information were handled with utmost confidentiality.

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