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Assessment the prevalence and activity of efflux pump encoded system (*AcrAB-TolC*) and biofilm formation in MDR *Klebsiella pneumoniae* isolates

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Abstract:

Background: Multidrug-resistant (MDR) *Klebsiella pneumoniae* represents a serious public health risk because of its capacity to resist multiple antibiotics. Many virulence factors contribute to this resistance. One of the significant virulence factors is the overexpression of efflux pumps, such as the *AcrAB-TolC* system. **Aim:** The study aims to detect the *AcrAB-TolC* triplet efflux pump genes, as well as to assess efflux pump activity and the production of biofilm in *K. pneumoniae* isolates after testing their antibiotic susceptibility. **Methods:** The study included patients of various ages who seek five hospitals in Baghdad, Iraq, from which 150 clinical samples were collected (Urine, blood, sputum, wounds, and burns). Only 50 isolates exhibited characteristics of *K. pneumoniae* were diagnosed by conventional methods Such as cultures and biochemical tests. **Results:** The study identified that 80% of *K. pneumoniae* isolates were multidrug-resistant (MDR), showing complete resistance to ampicillin (100%) and significant resistance to cefotaxime (76%) and ceftazidime (70%), while only 10% resistance was noted for chloramphenicol. The ethidium bromide (EtBr)-agar Cartwheel method confirmed that all isolates exhibited high efflux pump activity (100%). All isolates were biofilm producers to varying extents, as detected via the microtiter plate method. Molecular detection confirmed the presence of the 16S-23S internal transcribed spacer unit and the efflux pump genes *AcrAB-TolC* in all ten selected isolates (100%). **Conclusion:** The high prevalence of efflux pump activity and the ubiquitous of efflux genes shows the significant role of these virulence factors for antibiotic resistance across MDR *K. pneumoniae* isolates

Keywords: *Klebsiella pneumoniae*, multidrug resistance, Efflux pump, Biofilm, Cartwheel

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Introduction

K. pneumoniae is a Gram-negative bacterium belonging to *Enterobacteriaceae* family. It is characterised by its opportunistic nature, exhibiting an increased tendency to cause various infections (1). The World Health Organization (WHO) classified a list of the six most hazardous antibiotic-resistant bacteria. This list is named the ESKAPE family, and *K. peumoniae* belongs to this family (2). Multidrug resistance (MDR) *K. pneumoniae* is a highly urgent and significant bacterial resistance problem. The spread of MDR *K. pneumoniae* isolates is a global concern, making antibiotic therapy more challenging (3). Multi-drug efflux pumps (EPS) represent a significant virulence factor in the MDR of *K. pneumoniae*, especially the resistance-nodulation-division (RND) superfamily, which plays a significant role in MDR. These pumps limit the accumulation of antibiotics within the cell, reducing the antibiotic concentration to subtoxic levels (4). *AcrAB-TolC* EP system is the most prevalent RND efflux in *K. pneumoniae* and is one of the major causes of the rise of MDR strains of *K. pneumoniae*. The *AcrAB-TolC* EP is vital to how MDR strains of *K. pneumoniae* become resistant to many antibiotics, such as quinolones, tetracycline, and chloramphenicol

(5). The tripartite RND EP *AcrAB-TolC* is composed of the periplasmic membrane fusion protein *AcrA*, the internal transporter *AcrB*, and the external membrane channel *TolC* (6). *AcrB* assembles with *AcrA* and *TolC* channels to form a drug-extruding efflux complex that directly surrounds the whole cell envelope in *K. pneumoniae* cells. Proton-motive force (PMF), that supplies the *AcrAB-TolC* system energy to actively export drug molecules, is essential for this drug efflux mechanism (7). This study aimed to identify EP genes *AcrAB-TolC* in *K. pneumoniae* isolates by PCR amplification. Different clinical sources and to evaluate the EP activity in various concentrations of ethidium bromide by cartwheel method.

Material and methods

Samples collection

One hundred fifty-eight samples were collected from different clinical sources (Urine, blood, sputum, burn, and wound) From November 2023 to April 2024 from patients visiting hospitals in Baghdad, including Al-Imamain Al-Kadhimain teaching hospital, Al-Kadhimiya Children's Hospital, Central Teaching Hospital of Pediatrics, Yarmouk Teaching Hospital, and Baghdad Medical City (Child Protection Teaching Hospital and Specialized Burns Hospital). Samples were collected from patients of both genders, aged 1 day – 61 years.

Isolation and identification

All isolates were cultured on various media, such as blood agar and MacConkey agar, overnight incubated at 37°C. Colony morphology was used to identify isolates. All isolates were primarily identified based on the primary cultural characteristics of the colonies, including colour, shape, texture, and size. Different methods are used to identify and classify bacterial isolates, such as culture on selective media (HiCrome *Klebsiella* Selective Agar Base). In addition, further identification was done using biochemical tests.

Antibiotic susceptibility test

The Antibiotic susceptibility of *K. pneumoniae* isolates to 15 antibiotics was performed using the Kirby Bauer disk diffusion method was carried out in accordance with Coudron *et al*., (8) and the antibiotics were selected based on recommendations from the Clinical Laboratory Standards Institute (CSLI, 2023) (9). The antibiotic discs used in this study as the following: Amikacin (AK), Gentamicin (GEN), Amoxyclav (Amoxycillin Clavulanic acid), Ampicillin (AMP), Azithromycin (AZM), Aztreonam (AZT), Cefotaxime, Ceftazidime (CAZ), Chloramphenicol (C), Ciprofloxacin (CIP), Levofloxacin (LE), Co-Trimoxazole (Sulpha/ Trimethoprim) (COT), Imipenem (IMP), Nitrofurantoin (N), Tetracycline (TE).

Quantitative detection of biofilm

The microtiter plate assay was used to detect the production of biofilm in all isolates of *K. pneumoniae*. Briefly, 10 µl of a 0.5 McFarland suspended bacterium was added to each well of a 96-well sterile polystyrene microtiter plate filled with 190 µl of Luria broth (LB) medium that had 1% glucose added to it. Incubation of the plate was done at 37°C for 24 hours. Following incubation, the cells were washed and removed from the well using sterile distilled water. After cell fixation with methanol, the cells were stained with 1% crystal violet and then incubated for 15 minutes. Glacial acetic acid (30%) was used to dissolve the stain attached to the cells that formed the biofilm. Subsequently, the absorbance of the cells was measured at a wavelength of 630 nm (10). The isolates were classified as follows:

- Strong biofilm (S) = $OD \geq 4 \times ODc$
- Moderate biofilm (M) = $ODc \leq OD \geq 2 \times ODc$
- Weak biofilm (W) = $ODc \leq OD \leq 2 \times ODc$
- Negative (N) = $OD < ODc$

Ethidium Bromide (Etbr)-Agar Cartwheel Method

The phenotypic and qualitative detection of the EP activity in *K. pneumoniae* isolates was carried out using the Cartwheel method, a method developed by Martins *et al.*

(11). This method is simple and it requires Trypticase Soy Agar (TSA) and a fluorescent compound known as EtBr. EtBr acts as a substrate for the majority of EPs, exhibiting efflux activity. The experiment was performed on bacterial isolates with antibiotic-resistance characteristics, including MDR and non-MDR isolates. In addition, ensure that the EtBr-TSA plates are freshly prepared and stored in a light-protected environment. For fluorescence analysis, it is necessary to have at least one reference strain on each plate to serve as a control for comparison. The procedure was carried out according to (11) but with different concentrations of ethidium bromide (0.125, 0.25, 0.5, 1, 1.5, 2.0, 2.5, and 5 mg/ml).

Molecular assay

Molecular identification and detection of gene

The molecular identification of *K. pneumoniae* and the detection of EP genes *AcrA*, *AcrB* and *Tol-C* were done by extracting DNA from isolates of *K. pneumoniae* using the protocol of the Presto™ Mini gDNA Bacteria Kit.

The primers used in this study table (1), were manufactured by Macrogen and provided in a lyophilised state. The lyophilised primers were reconstituted in 300 µl of nuclease-free water to achieve a final 100 pmol/µl concentration. To make a working primer solution, 10 µl of the reconstituted primer was mixed with 90 µl of nuclease-free water until the concentration reached 10 pmol/µl. The primers and the working solutions were stored frozen at -20 °C until used.

After primer preparation, PCR amplification was performed with a final volume of 25 µl, the component of the PCR amplification reaction. Specifically, 1 µl of each primer, 12.5 µl of the premix, and 2 µl of template DNA were added to the tube. The remaining volume was filled with sterile deionized water, and the mixture was vortexed. After adding the deionized water, the primers and DNA template were added.

A negative control containing all components except the DNA template was included, which was replaced with distilled water. The PCR reaction tubes were then placed in a thermocycler instrument (qTOWER^3/ Analytik Jena / Germany), and amplification was performed as described in the following table (2). The annealing temperature were used is the same for all the genes used in this study.

Gel Electrophoresis

The amplified PCR products were detected by agarose gel electrophoresis to investigate the presence of genes and determine the specificity of the PCR reaction. The protocol of agarose gel electrophoresis of the PCR product was conducted according to the protocol specified by Green and Sambrook (13). Agarose gel was prepared in a concentration of 2% mixed with the dye (ethidium bromide). The PCR product sample was mixed with loading dye in a 5:1 ratio and precisely loaded into solidified gel wells. 5 µl of a 100 bp DNA ladder was also put onto the gel for comparative analysis. After electrophoresis, the gel was placed under a UV light source in a dark room to view the DNA, and it was immediately photographed using a gel documentation system.

Statistical Analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of different factors on study parameters. The chi-square test was used to significantly compare this study's percentages (0.05 and 0.01 probability) (14).

Results

Isolation and Identification

All samples were streaked on MacConkey and blood agar, then incubated overnight at 37 °C. Only 134 (90.5%) show positive bacterial species growth. The percentage of isolates exhibiting positive growth of *K. pneumoniae* was 46% (n = 62). These isolates were distinguished based on their colonies' morphological characteristics, as shown in Figure 1 (A) on MacConkey agar. They were large and pink due to lactose fermentation and mucoid due to *Klebsiella* encapsulation. On blood agar, the colonies were non-hemolytic grey-white, indicating that they do not produce blood hemolysis.

HiCrome *Klebsiella* Selective Agar Base

The HiCrome *Klebsiella* Selective Agar Base is used for selective isolation and simple detection of Klebsiella species from various sources. All 62 isolates of *Klebsiella* were cultured in selective media. All isolates H2S production fermentation. Out of the *Klebsiella* isolates which is grown on chrome selective media, only 80.64% (n = 50) of exhibited a purple- magenta colony, which indicates a positive result, as shown in Figure 1(B). *Klebsiella* species specifically cleave the medium's chromogenic substrate and produce a purple-magenta-colored colony, thereby aiding in the simple detection of organisms (15).

biochemical is the Kligler Iron Agar test, which differentially identifies gram-negative enteric bacilli based on dextrose, lactose, and isolates carry positive characteristics of *K. pneumoniae*. In contrast, 19.35% (n = 12) of isolates exhibited another result related to other *Klebsiella* species

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Table (1): The study used primers, along with their oligonucleotide sequences, amplicon sizes, and references.

Primer name		Sequence (5'-3')	Amplicon sizes (bp)	References
K. pneumoniae 16S-23S	F	ATTTGAAGAGGTTGCAAACGAT	130	(12)
	R	TTCACTCTGAAGTTTTCTTGTGTTC		
AcrA	F	TACACCAAAGTCACCTCGCC	148	Designed
	R	TCATTGCTCGACTGGGTGAC		
AcrB	F	TGTCCTCAAATGGCGACTCC	154	Designed
	R	AACGCTAATCCCTTGCTGCT		
Tol-C	F	CATCCTGAATCCCTGCCGTT	103	Designed
	R	CTCTAACGTCACCAGCGGTT		

Table (2): PCR Thermo-Cycling program for *Klebsiella pneumoniae* internal transcribed spacer (16S-23S) and *AcrA, AcrB* and *Tol-C* efflux pump genes

Stage	**Temperature**	**Time**	**Cycle**
Initial denaturation	92	3 min	1
Denaturation	92	30 sec	35
Annealing	57	30 sec	
Extension	72	1 min	
Final extension	72	3 min	1

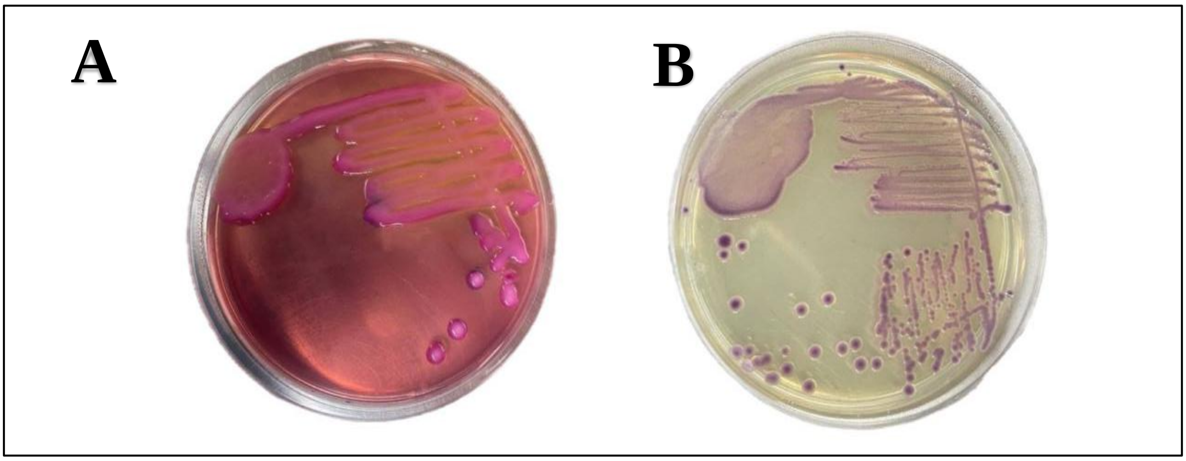

Figure (1): Phenotypic Characteristics of *K. pneumoniae* colonies on culture media. A: Growth of pink colonies of *K. pneumoniae* on the MacConcky agar plate and B: Growth of purple magenta colonies of *K. pneumoniae* on the HiCrome *Klebsiella* Selective Agar Base plate

Distribution of *Klebsiella pneumoniae* samples according to source, gender and age group

Out of 50 *K. pneumoniae* isolates, 33 (66%) were from males, which is higher than in females, only 17 (34%) followed by blood samples at 30% (n=15), the sputum samples rate at 6% (n=3), while the lowest rate was in wound samples at 2% (n=1) and burn samples at 2% (n=1). The study's age distribution was between newborns (a few days) and 61 years old for both genders, and the classification was based on biological and health-related criteria to reflect differences across life stages, as illustrated in Table (3). *K. pneumoniae* Infections are more prevalent, 32% in (newborn- infants), and the infection is found in males 13 (26%), significantly higher than in female infants. There was no significant gender difference in infections in children (2–12 years), adolescents (13–18 years), or young adults (19–30 years). Middle-aged adults (31-50 years) exhibited a similar infection rate between genders, with 10% of males and 8% of females being affected, and there were no notable gender-based differences. While the lowest percentage of *K. pneumoniae* infections among the age group (51-61) years with no significant gender differences.

Table (3): Distribution of *K. pneumoniae* according to age group and its relationship with sex

Resource	**Age group**							**P-value**
	1day - 1year Infants	2-12 Children	13-18 Adolescents	19-30 Young adult	31-50 Middle-aged Adults	51-61 Older Adults	Total	
Male	13 (26%)	5 (10%)	5 (10%)	3 (6%)	5 (10%)	2 (4%)	33	0.0098 **
Female	3 (6%)	5 (10%)	2 (4%)	2 (4%)	4 (8%)	1 (2%)	17	0.266 NS
Total	16	10	7	5	9	3	50	---
P-value	0.010 **	1.00 NS	0.256 NS	0.654 NS	0.738 NS	0.563 NS	--	---
**** (P≤0.01), NS: Non-Significant.**								

Antibiotic susceptibility test

This test was conducted on fifty *K. pneumoniae* isolates that had been diagnosed. The findings indicated that the *K. pneumoniae* isolates exhibited variability in their susceptibility and resistance to 15 antibiotics, as shown in Table (4). *K. pneumoniae* isolates showed 80% (n=40) MDR. The study's findings indicate that all isolates exhibited total resistance (100%) to ampicillin.

Most isolates resist cephalosporins such as, cefotaxime (76%) and ceftazidime (70%).

Co-Trimoxazole (Sulpha/Trimethoprim) resistance was found in 72% of isolates. In this study, 66% of isolates show moderate resistance to amoxyclav (β-lactamase inhibitors. The percentage of resistance to Azithromycin and Aztreonam was 68% and 64%, respectively. Various isolates exhibit aminoglycoside resistance, including gentamicin (58%) and amikacin (46%).. The isolates' resistance to nitrofurantoin was 56%. For Quinolones antibiotics, 54% and 16% of the isolates were resistant to ciprofloxacin and

Table 4: Antibiotic susceptibility of *Klebsiella pneumoniae* isolates to various antibiotics.

Antibiotic	**Sensitive**		**Resistance**		**Intermediate**		**P-value**
	No.	%	No	%	No.	%	
Amikacin	27	54	23	46	0	0	0.0001 **
Gentamicin	21	42	29	58	0	0	0.0001 **
Amoxiclav	17	34	33	66	0	0	0.0001 **
Ampicillin	0	0	50	100	0	0	0.0001 **
Azithromycin	16	32	34	68	0	0	0.0001 **
Aztreonam	17	34	32	64	1	2	0.0001 **
Cefotaxime	12	24	38	76	0	0	0.0001 **
Ceftazidime	12	24	35	70	3	6	0.0001 **
Chloramphenicol	45	90	5	10	0	0	0.0001 **
Ciprofloxacin	18	36	27	54	5	10	0.0001 **
Levofloxacin	29	58	8	16	13	26	0.0001 **
Co-Trimoxazole	14	28	36	72	0	0	0.0001 **
Imipenem	27	54	22	44	1	2	0.0001 **
Nitrofurantoin	11	22	28	56	11	22	0.0001 **
Tetracycline	26	52	24	48	0	0	0.0001 **
P-value	--	0.0001 **	--	0.0001 **	--	0.0042 **	---
**** (P≤0.01).**							

Levofloxacin, respectively. However, carbapenems remained very effective. The results indicated that carbapenems (imipenem) had more efficacy than penicillin and cephalosporins since only 44% of these isolates showed resistance to imipenem. For tetracycline antibiotics, only 48% of isolates were resistant to tetracycline. The highest sensitivity percentage was for chloramphenicol, which was only 10% resistant.

Quantitative detection of biofilm

The present study confirmed that all 50 isolates are capable of biofilm production, exhibiting significantly various degrees of thickness (strong, moderate, and weak). The results of the quantitative biofilm production test are shown in Table (5). Among all 50 clinical isolates, 31 (62%) show weak biofilm formation, 30 (26%) show moderate biofilm formation, and 6 (12%) show strong biofilm formation. There was a statistically significant biofilm formation level among isolates (P≤0.01).

Table 5: Number and percentage of biofilm formation of *Klebsiella pneumoniae* clinical isolates for Assessment of biofilm formation method

Total Number of Isolates 50	**Biofilm Formation Isolates**	
	Patterns	No. (%)
K. pneumoniae	Strong	6 (12%
	Moderate	13 (26%)
	Weak	31 (62%)
P-value		0.0001 **
**** (P≤0.01)**		

All 50 *K. pneumoniae* isolates, the MDR and non-MDR, were evaluated for EPs activity using the ethidium bromide cartwheel method. Reference strains were used as positive and negative controls for fluorescence analysis. The results indicated that both MDR and non-MDR isolates demonstrate phenotypically positive EP activity across all tested concentrations of EtBr, involving the high concentration of 5 mg/l, as shown in Figure (2), exhibited their inability to retain ethidium bromide and their strong ability to expel it from their cells using the EP.

Molecular Assay

Molecular identification of *K. pneumoniae*

The study looked at a total of n=10 the most resistant MDR isolates from 50 isolates of *K. pneumoniae*, and these isolates were selected from different clinical samples. Species identification was done by PCR amplifying the 16S–23S internal transcribed spacer unit of *K. pneumoniae*. The findings revealed that all (100%) ten bacterial isolates belonged to the species *K. pneumoniae*. The positive isolates identified by biochemical test gave positive results in detecting 16S–23S internal transcribed spacer unit by polymerase chain reaction amplification. The PCR products were confirmed by the analysis of bands with gel electrophoresis and by comparison of the bands' molecular size with a DNA Ladder of 100 bp are shown in Figure (3). Another study by (35) found 103 isolates were confirmed as *K. pneumoniae* by 16S–23S rDNA internal transcribed spacer.

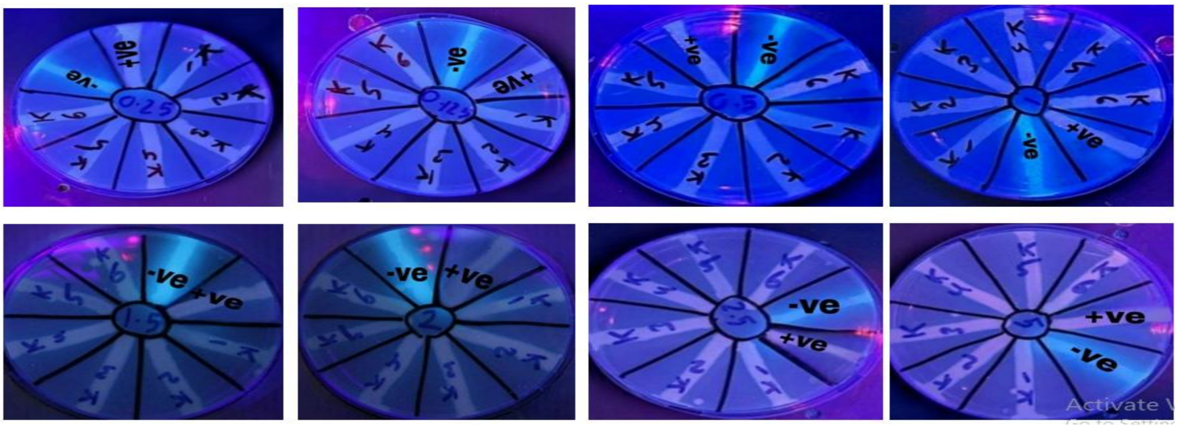

Figure 2: Phenotypic assessment of effluent pump activity in *K. pneumoniae* isolates using the Cart-Wheel method with various concentrations of ethidium bromide.

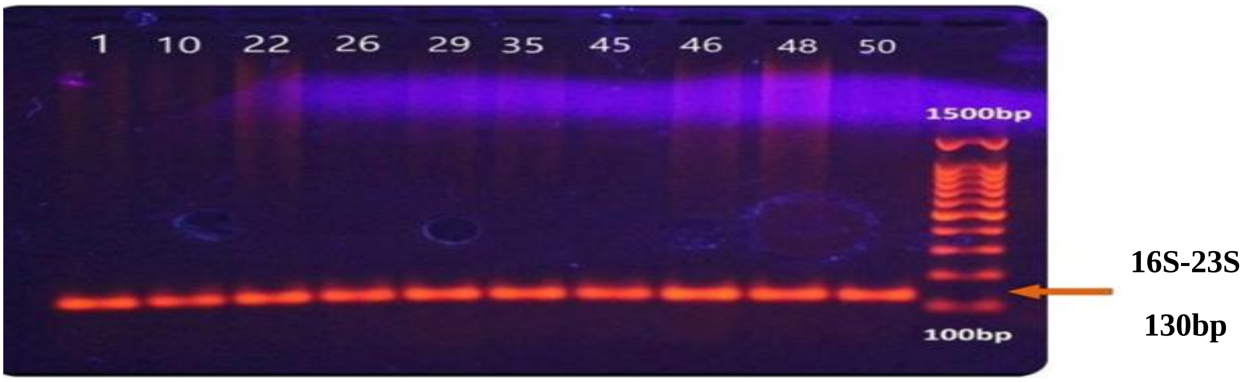

Figure 3: Agarose gel electrophoresis of PCR products reveals DNA bands of *Klebsiella pneumoniae*. PCR products of (16S–23S), size (130 bp). All the PCR products were electrophoresed on 2% Agarose and 150V / 30 mins with DNA ladder (100 bp).

Molecular detection of *AcrAB-TolC* efflux pump genes

AcrAB-TolC genes were detected in 10 MDR *K. pneumoniae* isolates by DNA amplification using the PCR technique and positively confirmed by agarose gel electrophoresis. The result revealed that the *AcrAB* genes were present in all 100% of isolates. The finding also revealed the presence of the *TolC* gene, which encodes an outer membrane protein essential for the functionality of *AcrAB-TolC* EP complexes in all *K. pneumoniae* isolates.

The gel electrophoresis results of *AcrAB-TolC* EP genes are amplified by PCR in Figure (4).

Discussion

The results of *Klebsiella* isolates which is grown on chrome selective media, aligns with a study conducted by (16) in the Kurdistan Region of Iraq, which revealed that only 61% (n = 50) of isolates had Positive *K. peumoniae* Characteristics. While, 38% (n = 31) had characteristics of other Klebsiella species. In the samples source distribution, the highest rate of positive isolates was in urine samples at 60% (n=30), which may be because *K. pneumoniae* is considered one of the predominant pathogens responsible for urinary tract infections (UTIs) among the *Enterobacteriaceae* family (17). The prevalent infection in infants' group may be due to their immature immunity system, especially the newborns often need urgent

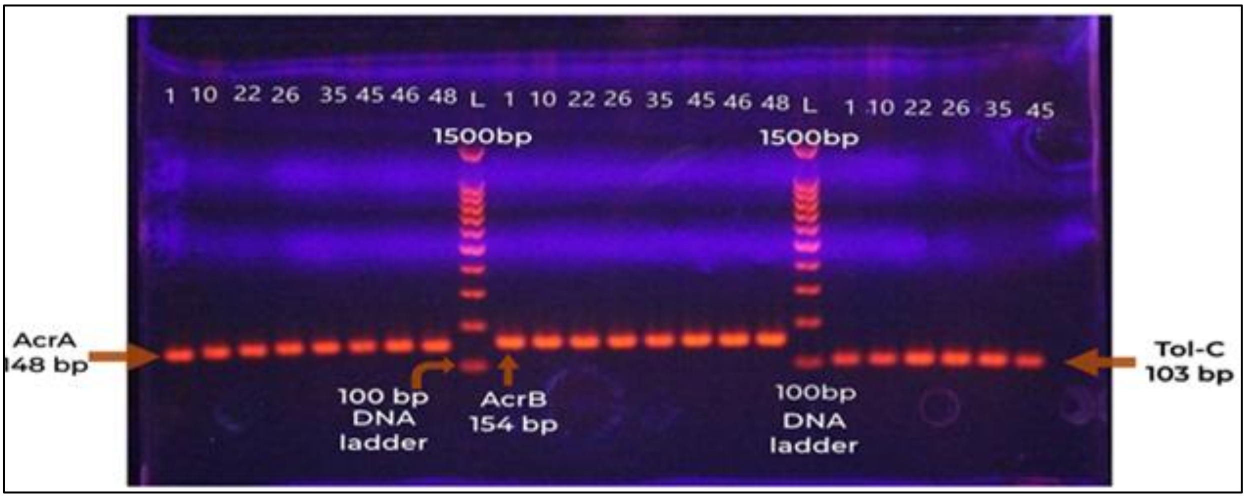

Figure 4: Agarose gel electrophoresis of PCR products reveals DNA bands of *Klebsiella pneumoniae*. PCR products of AcrA, AcrB, and TolC genes, size (148 bp, 154 bp, and 103 bp), respectively. All the PCR products were electrophoresed on 2% Agarose and 150V / 30 mins with DNA ladder (100 bp).

medical care, resulting in increased exposure to hospital-associated pathogens, especially *K. pneumoniae* is prevalent nosocomial pathogen in neonatal intensive care units, where infections can multiply due to the regular use of invasive procedures (18). The AST results revealed total resistance to ampicillin this finding aligns with many local studies, one of which was a study in Baghdad, Iraq (19), which reported 100% resistance to ampicillin. The results of resistance to cephalosporins are similar to the study by (20) in Al-Anbar, where *K. pneumoniae* had a resistance rate of 74% for cefotaxime and 69% for ceftazidime. Additionally, a study by (21) in Baghdad indicated that *K. pneumoniae* displays high resistance to cephalosporins. The prolific production of β-lactamase enzymes by *K. pneumoniae* may account for its significant resistance to ampicillin and third-generation cephalosporin. Co-Trimoxazole (Sulpha/Trimethoprim) resistance result is similar to (22) a recent study in Baghdad, which found that 72.13% of *K. peumoniae* isolates were resistant to COT, but a previous study (23) found a lower resistance (27.14%) to COT. Several studies have shown a highly significant resistance to amoxyclav (β-lactamase inhibitors), reported by (24) and (25), with 96% and 87.80% rates of resistance, respectively. In this study, isolates show moderate resistance, which agrees with findings from (26), where 68% displayed resistance to amoxyclav. The findings of aminoglycoside resistance align with research conducted in Baghdad by (27), which reported a moderate resistance level of 57.1% to gentamicin and 42.8% to amikacin. The isolates have more resistance to ciprofloxacin than levofloxacin. This result agrees with a study by (28) that found the resistance of *K. pneumoniae* isolates to ciprofloxacin was 52.5% higher than their resistance to levofloxacin was 36.7%. The isolates resistance to chloramphenicol agreed with the findings of a study by (29), which found that most isolates of *K. pneumoniae* exhibit the lowest resistance to chloramphenicol 1.29%. The correlation between *K. pneumoniae's* antibiotic susceptibility and resistance was statistically significant ($P \leq 0.01$).

Regarding biofilm formation, the variation in biofilm thickness may be due to differences in the isolates' ability to produce biofilm. This study's result agrees with (30) and (31) findings that weak biofilm former isolates had higher rates than strong biofilm former isolates. While the result by (32) found that 104 (80%) isolates exhibited a strong ability for biofilm formation, 16 (12.3%) showed a moderate ability, and 10(7.6%) exhibited a weak ability. Numerous variables affect biofilm formation, including environmental factors (composition of culture medium, osmotic pressure, biofilm culture plates, aerobic conditions, and pH) and genetic factors, such as the absence of critical genes or mutations.

The EtBr-Agar Cartwheel assay results revealed the non-MDR isolates exhibited high efflux activity, even though they lacked resistance to multiple antibiotics. This finding indicates that non-MDR isolates mainly depend on EPs as their principal defense mechanism against antibiotics but lack additional resistance mechanisms such as modification of drug target, enzymatic degradation or another mechanism typically present in MDR isolates. Conversely, MDR isolates probably utilize a combination of resistance mechanisms, including EP overexpression, to become more antibiotic-resistant. The results of this study are closely related to the results obtained by (33), which found that 100% of all *Klebsiella spp*. Isolates were positive for EP activity. In addition, a study by (5) reported that 91% of MDR strains of *K. pneumoniae* phenotypically exhibit EP activity, while 100% of non-MDR strains of *K. pneumoniae* exhibit efflux pump activity. In contrast, another study by (34) found that 38% of the *K. peumoniae* isolates exhibit positive EP activity. The high antibiotic resistance possessed by the isolates in this study may be due to the prevalent presence of efflux pump genes among the isolates, especially the *AcrAB-TolC* EP system, which is the most prevalent efflux system in *K. pneumoniae* and has resistance to many antibiotics (5). These findings of this study agree with (36) finding that all isolates 100% contain *AcrAB* genes, and another study by (37) found a high prevalence of *AcrAB* among the isolates of *K. pneumoniae* revealed that the *AcrAB* EP genes were detected in 94 (97.91%) of isolates. In contrast, the *TolC* gene was found in 92 (95.83%) of isolates.

Conclusion

These results conclude that the ubiquitous existence of EP genes *AcrAB-TolC* among the *K. pneumoniae* isolates and the higher activity of the EP in the presence of ethidium bromide contribute to the development of multidrug resistance and that all the isolates in this study may be dependent primarily on efflux pump to extrude the toxic compound.

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