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Correlation between Efflux Pump Genes and Biofilm Formation with Antibiotic Resistance of *Klebsiella pneumoniae*

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Abstract

Background: *Klebsiella pneumoniae* is a common pathogen that causes nosocomial infections. The efflux pump, antibiotic resistance and the capacity to form biofilm are significant virulence factors of *K. pneumoniae* that play an essential role in infection persistence. The aim of this study was to determine the distribution of acrAB-tolC efflux pump genes and whether there is a link between these genes with antibiotic resistance and biofilm formation in *K. pneumoniae* isolated from various clinical sources. **Methods:** In total, 174 different clinical samples were collected from Baghdad hospitals. Based on morphological characteristics, culture media, and biochemical tests, only 97 were identified as *K. pneumoniae*. Diagnostic results were confirmed using the Vitek-2 system and molecular diagnosis by the 16SrRNA gene. The sensitivity of the isolates to fourteen antibiotics was tested. The ability of the most resistant isolates to produce biofilms was also examined. Efflux pump genes (*acrA*, *acrB* and *tol*C) were investigated by polymerase chain reaction (PCR). **Results:** All isolates (100%) were found to be resistant to ampicillin and amoxicillin-clavulanate. On the other hand, the resistance percentage to cefotaxime was 95.87%, while tigecycline had the lowest resistance rate of 11.43%. In addition, the ability of bacteria to produce biofilms varied as 63.07% were found to be strong, 29.23% were moderate, 7.69% were weak, and 23.52% were non-biofilm-producing. According to the findings, the percentages of *acrA*, *acrB*, and *tolC* in the genotype detection assay for efflux pump genes were 100%, 95.08%, and 85.1% respectively. **Conclusion:** A close connection was detected between efflux pumps, biofilm formation and antibiotic resistance.

Keywords: *Klebsiella pneumoniae, acrAB-tolC* efflux pump, biofilm production

العلاقة بين جينات مضخة التدفق وتكوين الغشاء الحيوي ومقاومة المضادات الحيوية لبكتيريا الكلبسيلا الرئوية

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الخلاصة:

الكلبسيلا الرئوية هي ممرض شائع يسبب عدوى المستشفيات. تعد مضخة التدفق، ومقاومة المضادات الحيوية، والقدرة على تكوين الأغشية الحيوية من عوامل الضراوة المهمة لبكتيريا الكلبسيلا الرئوية التي تلعب دورًا أساسيًا في استمرار العدوى. كان الهدف من هذه الدراسة هو تحديد توزيع جينات مضخة التدفق acrAB–

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tolC وما إذا كان هناك صلة بين هذه الجينات ومقاومة المضادات الحيوية وتكوين الأغشية الحيوية في الكلبسيلا الرئوية المعزولة من مصادر سريرية مختلفة. **طرق العمل:** تم جمع ١٧٤عينة سريرية مختلفة من مستشفيات بغداد. تم تحديد ٩٧ عزلة فقط على أنها *Klebsiella pneumoniae* بناءً على الخصائص المظهرية، والاوساط الزرعية، والاختبارات البيوكيميائية وتم تأكيد نتائج التشخيص باستخدام نظام Vitek-2 والتشخيص الوراثي بواسطة جين *rRNA* 16S. تم اختبار حساسية العزلات لأربعة عشر مضاد حيوي. كذلك تم فحص قابلية العزلات الأكثر مقاومة لإنتاج الأغشية الحيوية. تم فحص جينات مضخة التدفق *acr* A و *acr* B و *tol* C بواسطة تفاعل البلمرة المتسلسل (PCR). **النتائج:** كل العزلات كانت مقاومة للأمبيسيلين والأموكسيسيلين – كلافولانات بنسبة (100٪)، من ناحية أخرى كانت نسبة المقاومة للسيفوتاكسيم (95.87٪)، بينما كان للتيجيسيكلين أقل معدل مقاومة (11.43٪). إضافة الى ذلك، تباينت قدرة البكتريا على إنتاج الأغشية الحيوية حيث كانت 63.07٪ قوية، و29.23٪ كانت معتدلة، و7.69٪ ضعيفة، و23.52٪ كانت غير منتجة للغشاء الحيوي. في اختبار الكشف عن النمط الجيني لجينات مضخة التدفق، أوضحت النتائج أن النسب المئوية لـ *acr* A و *acr* B و *tol* C هي 100٪ و 100٪ و 85.1٪ على التوالي. **الاستنتاج:** هناك علاقة وثيقة بين مضخات التدفق وتكوين الأغشية الحيوية ومقاومة المضادات الحيوية.

Introduction:

Bacterial resistance is one of the most serious worldwide dangers to human health that must be addressed immediately [1]. A rise in the frequency of infections that pose a health danger due to persistent bacteria has also been observed in a number of patients. For instance, persistent *K. pneumoniae* is a pathogen that is widespread and causes recurring infections in patients with weakened immune systems [2]. *K. pneumoniae,* a gram-negative bacterium from the family Enterobacteriaceae, is an opportunistic pathogen that has the capacity to colonize the mucosal epithelium of the stomach and nasopharynx and spreads throughout the deep tissues and bloodstreams of susceptible patients, leading to the development of serious infections like pneumonia, meningitis, endophthalmitis, pyogenic liver abscesses and bacteremia [3, 4, 5].

It has been identified as one of the main nosocomial infections causes that is associated with the ESKAPE pathogens (*E. faecium*, *S. aureus*, *K. pneumoniae, A. baumannii*, *P. aeruginosa* and *Enterobacter species*), a class of clinically significant pathogens with the capacity for antibiotic resistance [6]. Existence of multidrug efflux pumps is one of the mechanisms causing an increase in bacterial resistance to numerous antimicrobial agents such as antibiotics, dyes, antiseptics and detergents [7]. Resistance nodulation cell division (RND) is one of the efflux pump systems found in *K. pneumoniae* that plays a critical role in aiding bacteria in resisting antibiotics which includes species of the Enterobacteriaceae family, particularly *K. pneumoniae* [8]. AcrAB-tolC is the most imported RND efflux pump in *K. pneumoniae* bacteria that is linked to antibiotic resistance [9]. It is made up of three different proteins: the outer membrane protein tolC, the periplasmic protein (accessory protein) acrA and the protein of the cytoplasmic membrane protein acrB [10]. The acrRAB operon of *K. pneumoniae* encodes for AcrAB-TolC and is regulated by the acrR gene [11]. The overproduction of acrAB-tolC in *K. pneumoniae* contributes to resistance to quinolones, tetracyclines, chloramphenicol, erythromycin and some other recently manufactured antibiotics [12]. Biofilms are bacterial communities that grow adhering to surfaces and are enveloped in a self-produced matrix of extracellular polymeric substances [13]. The capacity of *K. pneumoniae* to build biofilms protects bacteria from the human immune response and drugs in MDR isolates [14]. Several studies have found various associations between biofilm production and antibiotic resistance. Some of which have showed that higher resistance was associated with increased biofilm production [15, 16], while other investigations have demonstrated that a high resistance pattern was associated with lower biofilm production [17, 18]. This investigation aimed to determine a possible correlation between efflux pump genes and biofilm formation in antibiotic resistance.

Materials and Methods:

Isolation and Identification of Bacterial Isolates:

Baghdad College of Science's ethical committee (CSEC/0522/0062) accepted the project. In this study, clinical samples were collected from several Baghdad hospitals between April to July 2022. The 174 samples were collected from different clinical sample sources: urine, wound swabs, sputum samples, blood and burn swabs.

The collected samples were inoculated on Maconkey agar, blood agar, and CHROMagar, and then incubated at 37ºC for 24 hrs. These bacterial isolates were identified depending on gram stain, the cultural characteristics of the colonies, pigment production on CHROM agar, and biochemical tests including oxidase, catalase, urease, Simmon's citrate, Indole, Methyl Red (MR), and Voges-Proskaur tests (the growth at 37°C for 24 hrs). Finally, confirming identification tests included Vitek-2 compact system analysis and molecular detection by the 16S rRNA gene [19].

Antibiotics Susceptibility of *Klebsiella pneumoniae*

Disk diffusion method (Kirby-Bauer method) was used to examine the susceptibility of all isolates of *K. pneumoniae* using fourteen antibiotic disks, including: ampicillin AMP (10 µg), amoxicillin-clavulanate AMC (10/20 µg), cefotaxime CTX (30 µg), ceftazidime CAZ (30 µg), trimethoprim/sulfamethoxazole STX/ST (10/20 µg), ciprofloxacin CIP (5 µg), aztreonam ATM (30 µg), amikacin AK (10 µg), tigecycline TGC (15 µg), tetracycline TET (30 µg), imipenem IMP (10 µg), chloramphenicol CHL (30 µg), polymyxin B PB (300 µg), and colistin CL (10 µg). All disks were obtained from Bioanalyze in Turkey. The results were interpreted based on the Clinical Laboratory Standards Institute CLSI 2022 [20], except tigecycline which was interpreted in accordance with the FDA guidelines as there is no CLSI with Enterobacteriaceae [21].

Quantitative Biofilm Formation Assay: Microtiter plate (MtP), also known as 96-well plate, is a quantitative method for determining biofilm production using a microplate reader [22]. A single bacterial colony was propagated in 5 ml of brain heart infusion (BHI) broth and incubated for 24 h at 37°C. After incubation time, the bacterial suspension was then diluted in microtiter wells by combining 20 µl of bacterial broth with 180 µl of BHI broth enriched with 1% glucose in each well of polystyrene microtiter plates (three wells for each isolate). The wells were then sealed and incubated at 37°C for 24 hrs. The last three wells were just filled with 200 µl of BHI broth as a negative control. Microtiter plate was carefully washed three times with PBS to eliminate non-adherence bacteria without affecting the integrity of the biofilm. To fix the biofilm, two hundred microliters of 99% methanol were added to each well and left for 15 minutes to dry. It was then cleaned three times with PBS to eliminate non-adherence bacteria. The biofilm that was produced could be evaluated by staining the wells with 200 µl of 1% crystal violet. The wells were also washed with PBS to eliminate excess dye before the plate was dried. The dye was dissolved in 200 µl of 33% glacial acetic acid for 10 minutes. The absorbance was then measured at 630 nm using a micro-ELISA autoreader.

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).

Polymerase Chain Reaction (PCR):

PCR technique was performed to confirm *K. pneumoniae* identification using the *16S* rRNA gene and to examine for the presence of the *acrA*, *acrB* and *tolC* genes of the RND-AcrAB-TolC efflux pumps. Specific primers were designed for the *16S* rRNA, *acrA*, *acrB*, and *tolC* genes using Geneious Prime bioinformatics software. The primer sequence and amplicon size are described in Table 1. The conventional PCR procedure started with a denaturation step at 95°C for five min, just one cycle, and was then followed by 30 cycles of denaturation at 95°C. The reaction mixture then underwent 35 cycles of annealing at 54°C for 45 sec. The reaction mixtures then underwent 35 cycles of the extension stage for one minute. After seven minutes of the final extension step at 72°C, the amplification was finally stopped. Agarose gel (2%) containing RedSafe dye was used to find out the PCR products and a UV light was used to observe the gel.

Statistical Analysis:

Statistical Analysis System [23] program was used to detect the effect of numerous variables on research parameters. The least significant difference (LSD) test (ANOVA) was employed to compare means. Chi-square test was employed in this study to compare percentages (0.05 and 0.01 probability).

Table 1: The oligonucleotide primer sequences used in PCR amplification.

Primer	Sequence (5`-3`)	Amplicon Size (bp)	Reference
16Sr RNA	F: 5`-CGGTCTGTCAAGTCGGATGT-3` R: 5`-AGCGTCAGTCTTTGTCCAGG-3`	172bp	Designated in this study
acr A	F: 5`-ACCAGCCATTTATCGCCGAT-3` R: 5`-TACCGCAACAGGGTGTTACC-3`	124bp	
acr B	F: 5`-CCCAGCTCAATTTTGGCGAC-3` R: 5`-GAAAGGCCAGCAGCTTAACG-3`	138bp	
tol C	F: 5`-TTAGAGTTGACGCCGTTGCT-3` R: 5`-ACCCCGATCTGCGTAAATCG-3`	148bp	

Results and Discussion:

Isolation and Identification of *K. pneumoniae*

One hundred seventy-four bacterial isolates were obtained from different clinical sources: urine, wound swabs, sputum samples, blood and burn swabs. Only 97 isolates were identified as *K. pneumoniae* based on morphological characteristics and by using biochemical tests. On MacConkey agar, 97 *K. pneumoniae* strains appeared as mucoid, huge, pink-colored colonies due to lactose fermentation and the presence of capsules. However, the colonies appeared big, white-gray and mucoid without hemolysis on blood agar. On the other hand, some scientists have asserted that experiments on blood agar demonstrated the ability of specific *K. pneumoniae* isolates to produce hemolysin [24, 25]. Additionally, they were streaked on CHROMagar (Figure 1) where they appear as colonies with a metallic blue color [26]. In addition, it was negative for oxidase, indole, and methyl red, while positive for urease, citrate utilization, catalase, and Voges-Proskauer tests. Vitek-2 compact system test proved that the 97 isolates were *K. pneumoniae*, with a probability ranging between 91 and 99 percent, and the results of PCR confirmed these results. The PCR findings showed that the *16S* rRNA gene has a volume of 172 base pairs by comparing the molecular size of the bands to a 100 - 200 bp DNA ladder (Figure 2). The results shown here coincide with those found by Ghaima and Tamara [27] who collected 260 clinical samples from urinary tract infections. Using both conventional methods and a molecular methodology based on the *16S*rRNA gene, researchers showed that only 76 of the 260 bacterial isolates belonged to *K. pneumoniae* by using both conventional and molecular diagnostic methods based on the *16S* rRNA gene (159 bp). *K.*

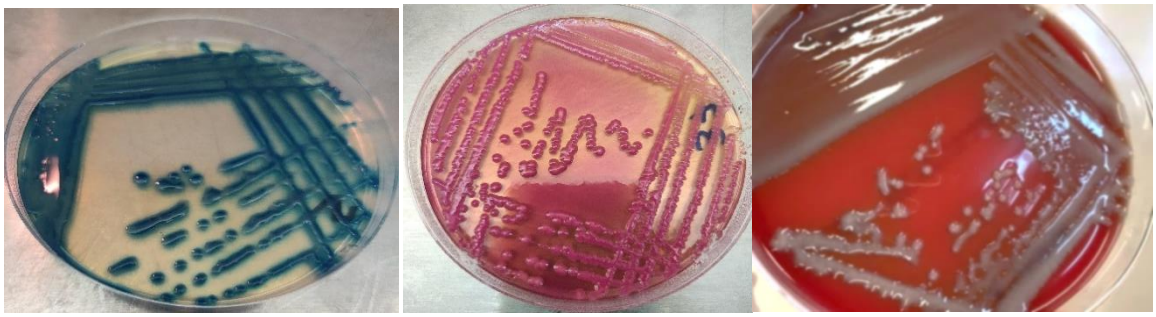
pneumoniae was distributed as shown in Table 2, indicating that it is more frequently found in UTIs which represented approximately 42.26% (41/97), followed by sputum which represented approximately 26.80% (20/97). While 9.27% (9/97), 13.40% (13/97), and 14.43% (14/97) of *K. pneumoniae* were isolated from blood, burns, and wounds, respectively. This result agrees with Seifi *et al.*[28] who collected clinical specimens from two Tehran hospitals and indicated that specimens of *K. pneumoniae* were isolated from urine, sputum, and blood with percentages of 61.7%, 11.7% and 8.5% respectively. A statistically significant difference was seen between the different types of samples. *K. pneumoniae* isolates were significantly higher (P = 0.0001) than urine *K. pneumoniae* isolates (p = 0.0017) among the total of sputum, burns, blood and wounds samples.

Table 2: The prevalence of *K. pneumoniae* based on source of infection.

Type of Clinical Samples	Total Samples Number	Bacterial Isolates Number	Percentage (% *)	Percentage (%**)	Percentage (%***)	*P*-value
Sputum	26	20	76.92%	20.61%	11.94%	0.0001 **
Burns	19	14	73.68%	14.43%	8.04%	0.0001 **
Urine	77	41	53.24%	42.26%	23.56%	0.0017 **
Wounds	27	13	48.14%	13.40%	7.47%	0.0001 **
Blood	25	9	36.00%	9.27%	5.17%	0.0001 **
***P*-value**	---	---	0.0001 **	0.0001 **	0.0001 **	---

**** ($p \leq 0.01$).**

Percentage (%*) of *K. pneumoniae* relative to the source; Percentage (%**) of *K. pneumoniae* relative to total no. of *K. pneumoniae* isolate; Percentage (%***) of *K. pneumoniae* relative to total no. of clinical samples.



(a) **(b)** **(c)**

Figure 1: The colonies of *K. pneumoniae* on (a) Chromagar, (b) MacConkey agar and (c) Blood agar, after 24 hours of incubation at 37°C.

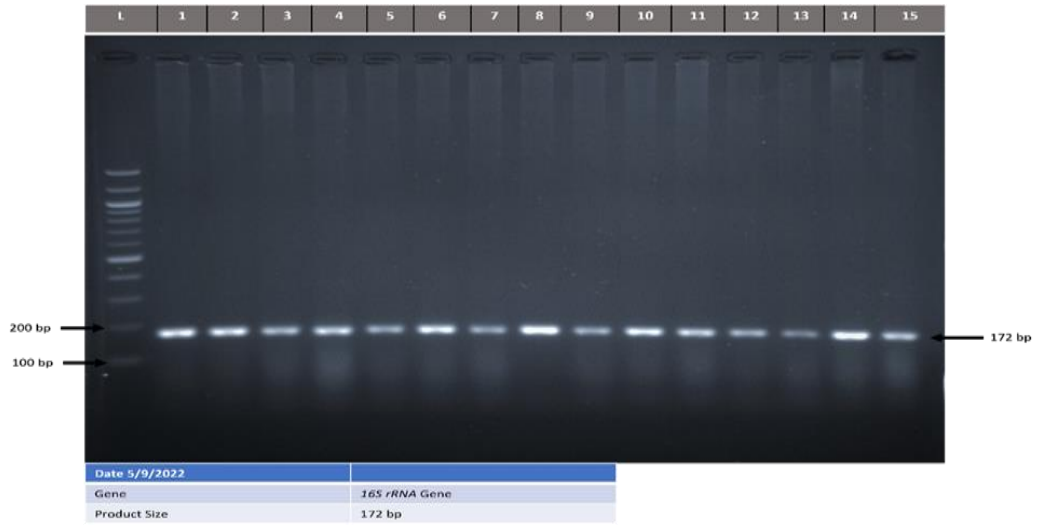


Figure 2: The results of the amplification of the 16S rRNA gene of bacterial isolates were fractionated on 2% agarose gel electrophoresis and stained with Red Safe. L: 100–200 bp ladder marker. Lanes 1–15 resemble 172 bp PCR products (agarose 2%, 80V for 80 min).

Antibiotic Susceptibility of *K. pneumoniae* Isolates:

Ninety-seven bacterial isolated were found to be resistant to ampicillin and amoxicillin-clavulanate (100%), cefotaxime (95.87%), ceftazidime (94.84%), trimethoprim/sulfamethoxazole (89.69%), aztreonam (84.53%), polymyxin B (62.8%), tetracycline (55.67%), ciprofloxacin (59.79%), amikacin (50.51%), chloramphenicol (39.17%), imipenem (39.92%) and colistin (36.08%). Tigecycline and colistin proved to be the most effective antibiotics against *K. pneumoniae* (Figure 3). Several mechanisms such as drug inactivation, target transformation, decreased permeability, and increased efflux pump activity make bacteria resistant to the antibiotics. The efflux pump serves as a crucial multiple-drug-resistant mechanism. The efflux pump's ability to eliminate a variety of antibiotics due to its multiple substrate properties also allow it to provide extra resistance mechanisms that lower the intracellular concentration of antibiotics and boost mutation accumulation [29].

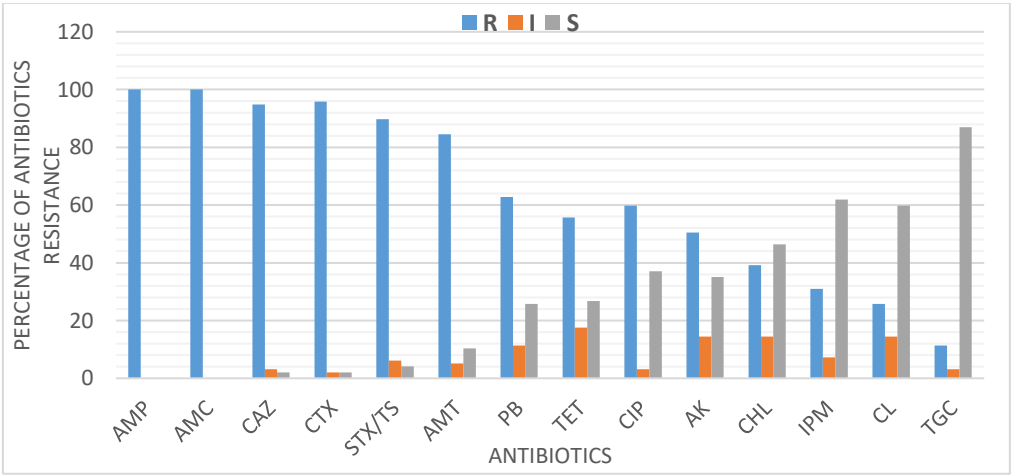


Figure 3: Antibiotic resistance pattern of *K. pneumoniae.* Ampicillin (AMP), amoxicillin-clavulanate (AMC), cefotaxime (CTX), ceftazidime (CAZ), trimethoprim/sulfamethoxazole (STX/ST), aztreonam (ATM), ciprofloxacin (CIP), amikacin (AK), tigecycline (TGC), tetracycline (TET), imipenem (IMP), chloramphenicol (CHL), pPolymyxin B (PB), and colistin (CL), R = Resistant, I = Intermediate, S = Sensitive.

Detection of Biofilm Formation by MDR-*K. pneumoniae*:

The highly resistant *K. pneumoniae* isolates (81/97 isolates) were tested for biofilm production using the microtiter plate (MtP) quantitative method. Results showed that 65/81 (80.24%) isolates were biofilm producers, with a percentage of 63.07% (41/65) being strong, 29.23% (19/65) being moderate, 7.69% (5/65) being weak, and the remaining isolates (19.75% (16/81)) were non-biofilm producers (Figure 4). This result agrees with those obtained by Khoshnood *et al.* [30] who found that 77.2% (88/114) of *K. pneumoniae* isolates were biofilm producers, with different categories including 65.9% (58/88), 27.3% (24/88) and 6.8% (6/88) which were strong, moderate, and weak biofilm production respectively. Additionally, the findings were in accordance with the results reported by Shadkam *et al.* [31] who found that 25% of isolates failed to produce biofilm and 75% of them were biofilm producers. Biofilm formation is an important factor for these bacteria as it is primarily to blame for persistent infections due to their resistance to phagocytosis and death as a result of humoral and cellular immunity [32, 33]. The previous investigation showed a significant correlation between MDR capacity and biofilm formation in *K. pneumoniae* [34]. According to Li *et al*. [35] the expression of various adhesions, cognate receptors and exopolymer components by specific cell types within a biofilm community can contribute to overall biofilm formation. Many bacteria, in particular, can use a quorum sensing mechanism to control biofilm production and other social activities. The prevalence of biofilm-producing isolates among highly resistant *K. pneumoniae* is also investigated in the present study (Table 3). The results demonstrated that the biofilm producers, *K. pneumoniae*, were mainly isolated from sputum and urine with percentages of 95% (19/20) and 68.29% (28/41) respectively. While isolates from burns, wounds, and blood had percentages of 57.14% (8/14), 53.84% (7/13) and 33.33% (3/9) respectively.

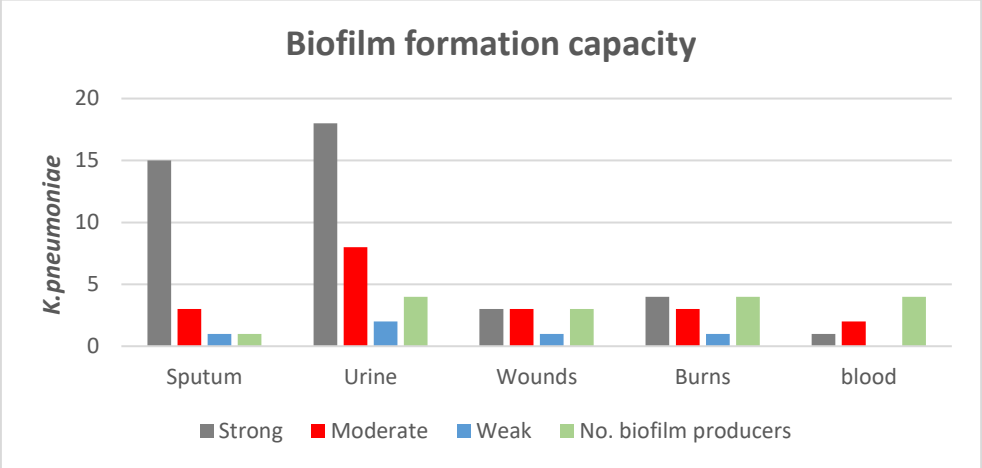


Figure 4: Biofilm-forming capacity utilizing the MTP method with various clinical sample sources.

Table 3: Biofilm capacity based on the estimated cutoff value* of *K. pneumoniae* isolates.

N.o	Biofilm Capacity	Measurement Isolates on the OD630	N. of Isolate	Percentage %
1	Non-producer	< 0.0442	16	19.75
2	Weak	0.0442-0.0884	5	6.17
3	Moderate	0.0884-0.1768	19	23.45
4	Strong	≤ 0.1768	41	50.61

*Cutoff is defined as the mean of the control (optical density 630) in addition three standard deviations.

Relationship between Multidrug Resistance and Biofilm Production Capacity

Klebsiella pneumoniae is one of the major prevalent bacteria in hospital-acquired infections that is resistant to several antibiotics [36]. Upon investigating the correlation between biofilm formation and antibiotic resistance, the results of the current study indicated that 49/65 (75.38%) of the biofilm producer isolates were MDR and 11/65 (16.92%) were XDR (Table 4). While only five of the isolates (7.69%) were susceptible to antibiotics (non-MDR). In addition to all strong biofilm producers, isolates were resistant to amoxicillin-clavulanate, ampicillin, vancomycin, cefotaxime, and cefixime, while most isolates were sensitive to tigecycline. A strong relationship was detected between biofilm production and antibiotic resistance ($p \leq 0.01$) was detected in the present study. The results agree with many previous investigations including the recent research by [37] which showed that bacteria producing biofilms had significant levels (p-value ≤ 0.05) of antibiotic resistance. Paudel *et al.* [38] reported a significant association between the formation of biofilms and antibiotic resistance ($p \leq 0.05$).

Another study by Nirwati *et al*. [39] reported no significant association between *MDR-K. pneumoniae* and biofilm production capacity. These findings are in contrast with the present study. Gram-negative bacteria, especially *K. pneumoniae*, can use biofilm as a critical virulence component to produce chronic infections. The ability of bacterial cells to survive at high antibiotic concentrations is a crucial characteristic of biofilms. Compared to the planktonic form, the bacteria in a biofilm are 1000 times more resistant to antibiotic treatment [40]. The prevalence of microbes that form biofilms and are multidrug-resistant, gives a preview of the global threat to come. The tight implementation of infection control and prevention actions in clinical laboratories, together with the routine monitoring of biofilm and antibiotic resistance among microorganisms, is advised [41].

Table 4: The prevalence of MDR and XDR-*K. pneumoniae* based on biofilm formation capacity.

R^{es} Isolates	**Strong Biofilm**	**Moderate Biofilm**	**Weak Biofilm**	***P*-value**
MDR	34/65 (52.30%)	13/65 (20%)	2/65 (3.07%)	0.0001 **
XDR	7/65 (10.76%)	4/65 (6.15%)	0/65 (0%)	0.0096 **
S*	0/65 (0 %)	2/65 (3.07%)	3/65 (4.61%)	0.091 NS
Total = 65	41	19	5	---
P-value	0.0001 **	0.0087 **	0.417 NS	---

** (P≤0.01), NS: Non-Significant.

R*= Resistant, S* = Sensitive

DNA extraction and purification

DNA concentration and purification from clinical bacterial isolates were estimated using Qubit 4.0 and through PCR analysis which was then confirmed by gel electrophoresis. The results showed visible bands (Figure 5), where the concentration and purity of each isolate ranged from 10 to 100 ng/µl. These findings are similar to the findings of Ghaima [27] who used the Qubit 4.0 technique to purify and quantify the samples.

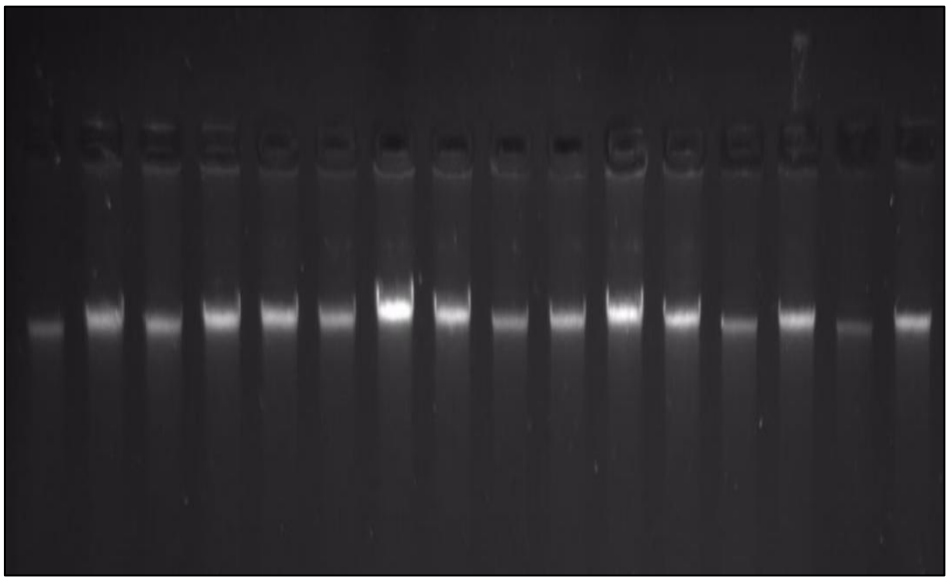


Figure 5: Gel electrophoresis of genomic DNA from *K. pneumoniae* isolates.

PCR Results: In order to detect the presence of efflux pump genes (*acr*A, *acr*B, and *tol*C) and determine the frequency of each gene among more resistant *K. pneumoniae* isolates, polymerase chain reaction (PCR) techniques were used for each DNA-extracted sample. The PCR results were confirmed by analyzing the bands on gel electrophoresis and by comparing the molecular weight of the bands with a 100–200 bp DNA ladder. The findings of the polymerase chain reaction for *acr*A (124 bp), *acr*B (138 bp) and *tol*C (148 bp) genes are shown in Figures 6, 7, and 8, and Table 5. Efflux pump genes were found to be prevalent among 61 *K. pneumoniae* isolates. The results showed that the percentages of *acr*A, *acr*B, and *tol*C were 100%, 95.08%, and 85.24% respectively. These results coincided with many previous studies: Abid Fazaa Almiyah *et al*. [42] who isolated 55 *K. pneumoniae* from patients suffering from acute and chronic wounds and burns at Al-Diwaniyah teaching hospital and private clinics in Al-Diwaniyah province, they found that all bacterial isolates were positive for *acr*A and *acr*B genes according to PCR technique. Another study conducted by Ranjbar *et al*. [43] reported that the proportions of *acr*AB and *tol*C genes were 96.52% and 85.21% respectively. In terms of gene prevalence, these outcomes are the most similar to those of the present study. The distribution of efflux pump genes among highly resistant *K. pneumoniae* strains isolated from various clinical sources was also investigated in the current study. Results are indicated in Table 5. A statistically significant difference was observed in the present study between the source of infection and the prevalence of efflux pump genes ($p \leq 0.01$).

The correlation between efflux pumps, biofilm formation and antibiotic resistance was also determined in the current study. The results showed that all *Klebsiella pneumoniae* isolates that had efflux pump genes with the ability to produce biofilm, were 100% resistant to ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, aztreonam, trimethoprim, and sulfamethoxazole. And the percentage of the isolates' resistance to ciprofloxacin,

nitrofurantoin, tetracycline, amikacin and tobramycin were 85.24%, 81.96%, 78.68%, 73.77% and 68.85% respectively. Our results coincided with those of Akinpelu *et al*. [44] who found that all 18 *Klebsiella* isolates had an active efflux pump and the ability to produce biofilm.
Several previous investigations have shown that efflux pumps play an important role in the production of bacterial biofilm. Mirzaie and Ranjbar [45] found a significant relationship between MDR phenotype, biofilm production and efflux pump among *K. pneumoniae* strains ($p< 0.05$). Another study by Tang *et al*. [46] indicated that efflux pump genes *acrA*, *emrB oqxA*, and *qacE1* were much more expressed in *K. pneumoniae* biofilms than in planktonic cells, indicating that biofilms can enhance efflux pump gene expression. Several previous studies have confirmed that overexpression of *AcrAB* is associated with beta-lactam resistance [47, 48]. However, Mirzaie and Ranjbar [45] found that the efflux pump system (acrAB-tolC) in *K. pneumoniae* strains was responsible for antibiotic resistance such as fluoroquinolones like ciprofloxacin, tetracycline and beta-lactam antibiotics, in MDR *K. pneumoniae* isolates. This result is closely related to the results of the present study. Pakzad *et al.* [49] mentioned that the intrinsic resistance of *K. pneumoniae* isolates to fluoroquinolones, particularly ciprofloxacin, is mostly a result of pumps, one of which is the AcrAB efflux pump. Furthermore, the use of this pump results in resistance to the drugs tetracycline, chloramphenicol, trimethoprim, macrolides, and lactams. Padilla *et al*. [9] reported that *acr*B deficiency increases sensitivity to erythromycin, tetracycline, chloramphenicol and aminoglycosides, as well as bronchoalveolar lavage fluid and antimicrobial peptides.

Table 5: *AcrAB-tol C* efflux pump genes in *K. pneumoniae* isolates,

Gene Name	Highly Resistant *K. pneumoniae* Clinical Isolates		
	N.O of +ve Isolates	N.O of –ve Isolates	*P*-value
acrA	61 (100%)	0 (0%)	0.0001 **
acrB	58 (95.08%)	3 (4.91%)	0.0001 **
tolC	52 (85.24%)	9 (14.75)	0.0001 **

** ($p \leq 0.01$).

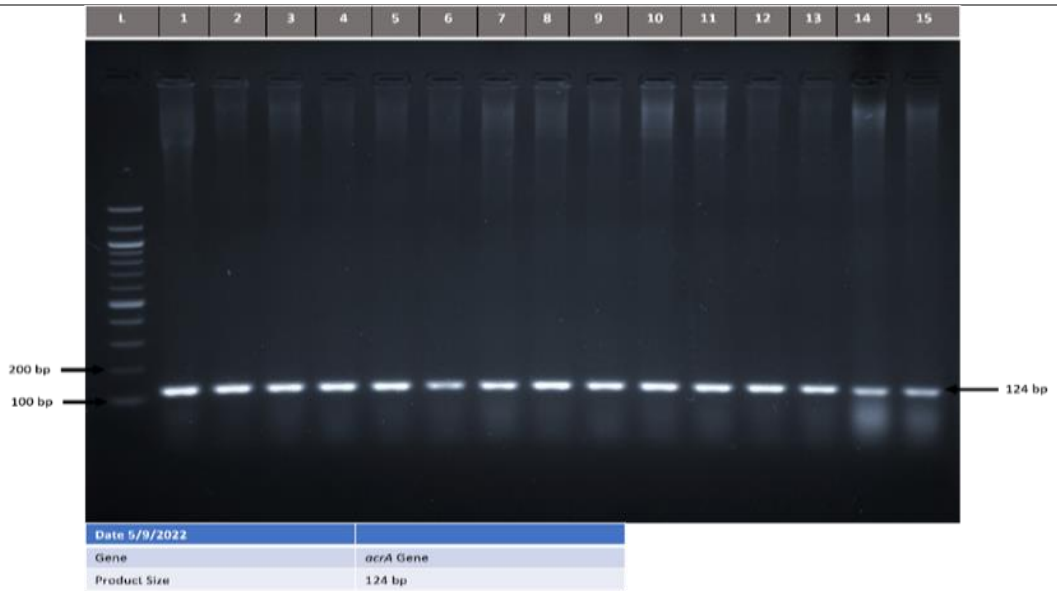


Figure 6: Agarose gel electrophoresis for *acr*A gene of *K. pneumonia* isolates. lane L: 100-200 bp DNA ladder, lanes 1-15 are positive results with 174 bp amplicon (agarose 2% and 80 V for 80 min).

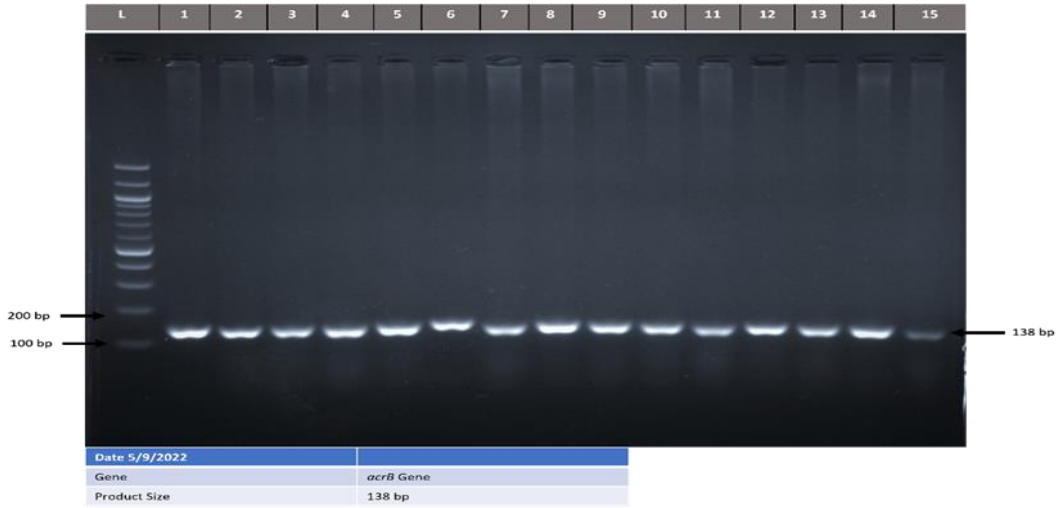


Figure 7: Agarose gel electrophoresis for *acr*B gene of *K. pneumonia* isolates. lane L: 100-200 bp DNA ladder, lanes 1-15 are positive results with 138 bp amplicon (agarose 2% and 80 V for 80 min).

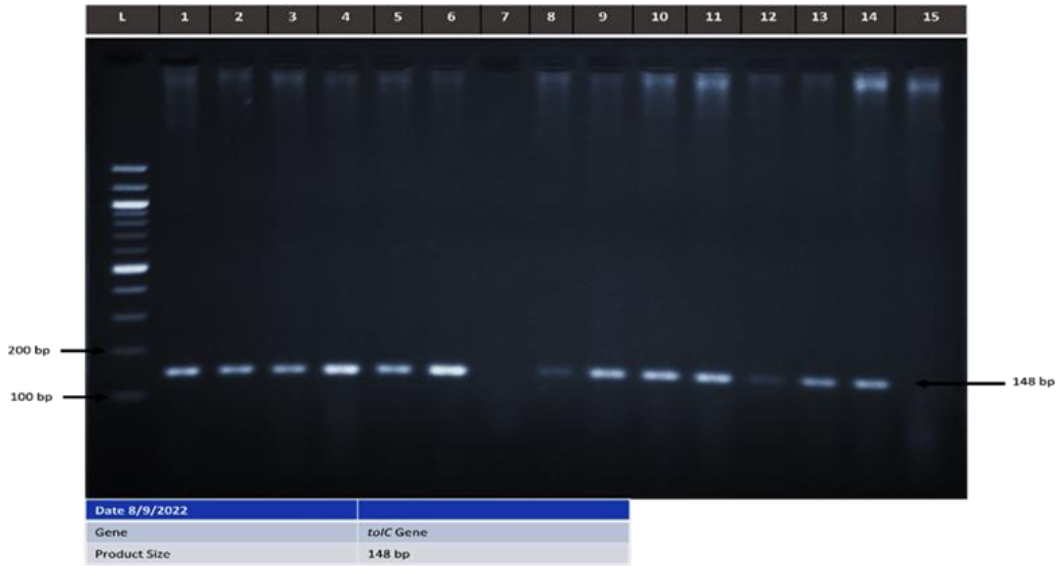


Figure 8: Agarose gel electrophoresis for *tol*C gene of *K. pneumonia* isolates. Lane L: 100-200 bp DNA ladder, lanes 1-15 resemble 148 bp PCR product (agarose 2% and 80 V for 80 min).

Conclusion

All isolates of *K. pneumoniae* were 100% resistant to amoxicillin-clavulanate and ampicillin, while the lowest resistance was found against tigecycline with a percentage of 11.43%. Most MDR *K. pneumoniae* isolates produce biofilm and have an active efflux pump system. The distribution of *acrA* genes was more frequent than that of other efflux pump genes (*acr*B and *tol*C) detected in the present study. Close connection was observed between efflux pumps, biofilm formation and antibiotic resistance as the isolates that had the ability to produce biofilm and possessed efflux pump genes were resistant to most antibiotics.

Conflict of Interest Statement

There are no reported engagements of interest.

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