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Molecular Detection of the K1 and K2 Capsular Serotypes and its Correlation with Biofilm Formation in Clinical Isolates of *Klebsiella Pneumoniae*

Mustafa Jasim Mohammed[1]*, Suhad Saad Mahmood[2]
[1]*Department of Molecular Biology, Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad - Iraq*
[2]*Department of Biotechnology, College of Science, University of Baghdad, Baghdad – Iraq*

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Abstract
Klebsiella pneumoniae is a Gram-negative bacterium and a member of the *Enterobacteriaceae* that can cause many infections. This research aimed to examine the effect of the presence of the *K1* and *K2* capsule serotypes on biofilm formation and its relation with resistance to antibiotics. In this study, 130 clinical specimens were collected from different clinical sources from patients in hospitals, and only 61 isolates were identified as *K. pneumoniae* by selective media, biochemical test, and Vitek II. Disc diffusion technique was used to assess the resistance of 61 isolates to ten distinct antibiotics. The greatest resistance was observed against Amoxicillin-clavulanate (98.36%), while the least resistance was noted for Meropenem (13.11%). Additionally, 95.08% of the isolated bacteria exhibited multidrug resistance (MDR). Microtiter plate method (MTP) was used to assess biofilm formation; 100% of the isolates were able to form biofilm, (13.11% 8/61) were strong biofilm producers, in comparison (65.57% 40/61) were moderate biofilm producers, and (21.31% 13/61) were weak biofilm producers. Using standard PCR, the *wzck1* and *cpsk2* genes were identified; the findings revealed that only three of the 61 isolates have been positive for K1 (*wzck1*), fifty-two for K2 (*cpsk2*), and Six for non-K1/K2. This study concludes that *K. pneumoniae* has a high ability to form a biofilm with varying levels, which in turn increases antibiotic resistance, as 95.08% of the isolates were MDR. Also, the capsule affects the biofilm formation, especially the K1 and K2 serotypes, as it shows higher levels of biofilm.

Keywords: *Klebsiella pneumoniae*, K1 and K2 capsular, PCR, biofilm, microtiter plate.

الكشف الجزيئي عن النمط المصلي الكبسولي K1 و K2 وعلاقته بتكوين الأغشية الحيوية في العزلات السريرية لـ *Klebsiella Pneumoniae*

مصطفى جاسم محمد[1]*, سهاد سعد محمود[2]
[1]قسم الاحياء الجزيئي، المركز العراقي لبحوث السرطان و الوراثة الطبية، الجامعة المستنصرية، بغداد – العراق
[2] قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد – العراق

*Email: mustafaghost33@gmail.com

الخلاصة

Klebsiella pneumoniae هي عضو في فصيلة المعويات وهي بكتيريا سلبية الجرام يمكن أن تسبب العديد من الالتهابات. تهدف هذه الدراسة إلى معرفة تأثير وجود جينات الكبسولة *K1* و *K2* على تكوين الأغشية الحيوية وعلاقتها بمقاومة المضادات الحيوية. وفي هذه الدراسة، من 130 عينة سريرية تم جمعها من مصادر سريرية مختلفة من المرضى في المستشفيات، تم التعرف على 61 عينة فقط على أنها *K. pneumoniae* عن طريق الاوساط الانتقائية والاختبار الكيميائي الحيوي واختبار Vitek II. استعملت طريقة الانتشار القرصي لتقييم حساسية 61 عزلة لـ عشرة مضادات حيوية. المقاومة الأكبر كانت ضد الأموكسيسيلين–كلافولانات (98.36%)، في حين كانت المقاومة الأقل ضد الميروبينيم (13.11%). بالإضافة إلى ذلك، أظهرت 95.08% من العزلات مقاومة متعددة للأدوية (MDR). تم استخدام طريقة لوحة الميكروتيتر (MTP) لتقييم تكوين الأغشية الحيوية، وكانت 100٪ من العزلات قادرة على تكوين الأغشية الحيوية، (8/61 13.11%) كانوا منتجين أقوياء للغشاء الحيوي، بالمقارنة مع (40/61 65.57%)كانوا منتجين متوسطين للغشاء الحيوي، و (13/61 21.31%) كانوا منتجين ضعفاء للغشاء الحيوي .وباستخدام تفاعل البوليميراز المتسلسل التقليدي، تم التعرف على الجينات *wzk1*و*cpsk2*؛ وكشفت النتائج أنه من بين 61 عينة، كانت ثلاث عينات فقط إيجابية لـ K1، واثنين وخمسين عينة لـ K2، وست عينات لغير K1/K2. استنتجت هذه الدراسة إلى أن *K. pneumoniae* تتمتع بقدرة عالية على تكوين الأغشية الحيوية بمستويات متفاوتة مما يؤدي بدوره إلى زيادة مقاومة المضادات الحيوية حيث كانت 95.08٪ من العزلات مقاومة للمضادات الحيوية المتعددة، كما تؤثر الكبسولة على تكوين الأغشية الحيوية وخاصة النمطين المصليين *K1* و *K2* حيث تظهر مستويات أعلى من الأغشية الحيوية.

1. Introduction

Klebsiella pneumoniae, an *Enterobacteriaceae* bacterial pathogen responsible for health-associated disorders, represents one of the strongest resistant bacteria to antibiotics that produce outbreaks in both local communities and hospitals [1]. *K. pneumoniae* is an opportunistic pathogen responsible for causing a spectrum of hospital community acquired and nosocomial infection and especially infect patients with indwelling medical devices such as urinary catheters [2]. On agar plates, *K. pneumoniae* appears bright and mucoid, has a noticeable polysaccharide capsule of significant size, and is negative for Gram staining [3]. In the general population, *K. pneumoniae* bacteremia causes significant morbidity and mortality [4]. In hospitals, *K. pneumoniae* is regarded as a serious pathogen because they are resistant to multiple antibiotics [5]. It causes hospital-acquired infections such as pneumonia, wound infections, and urinary tract infections (UTIs) as well as community acquired infections such as liver abscess, bone infections, pneumonia, meningitis, and soft tissue infections [6].

Biofilm is characterized by the aggregation of numerous organisms encased in a polysaccharide matrix, along with the presence of exogenous DNA (eDNA) and proteins. This structure plays a significant role in enhancing resistance to the immune system and contributing to antibiotic resistance [7]. The process of forming biofilm begins with reversible binding of bacterial cells, then irreversible binding, followed by the growth of microcolonies, and finally, biofilm maturation [8]. Cells associated with biofilm can be distinguished from those in suspension by producing an extracellular polymeric substance (EPS) matrix, growing at a slower pace, and regulating certain genes up or down [9]. Biofilm production and composition are influenced by a variety of environmental conditions and substances, including glucose, which promotes biofilm formation [10].

K. pneumoniae has the ability to develop a dense coat of external biofilm that helps the bacteria adhere to either living or not surfaces, preventing antibiotic penetration and lessening its impact [11]. The development of biofilms and alterations in bacterial phenotypes are vital

contributors to antibiotic resistance. In contrast to planktonic bacteria, the biofilm phenotype was strongly related to more excellent antibiotic resistance, highlighting biofilm's significance for pathogen survival in dangerous conditions [12].

In *K. pneumoniae*, biofilm development is influenced by a number of factors. The polysaccharide capsule, fimbriae and pili, the metabolism of iron, and the existence of several bacterial species are a few examples of these [13]. The expression of the capsule is crucial to the pathogenesis of *K. pneumoniae* [14]. Serious infections often harbor only a small number of the over 80 capsular serotypes identified in *K. pneumoniae*. The principal pathogenic component of this bacterium is its polysaccharide capsule. The capacity of the capsule to withstand complement deposition, peptides that inhibit bacteria, and phagocytosis has been demonstrated in laboratory settings [8]. Taiwan, Europe, and North America have high death rates associated with K1 and K2 capsule types, which have been associated to bacteremia [15]. According to some researches in Iraq and Iran K2 was more prevalent than K1 [16-18] while some studies in Singapore, Taiwan and also in Iraq the K1 was more prevalent than K2 [19, 20]. K1 is the most predominant serotype in a study from east China (68.9%) followed by K2 serotype (20%). They were isolated from liver abscess patients. In Asia, K1 is the main serotype followed by K2 [21]. Based on the same virulence factors backgrounds except serotypes, K2 *K. pneumoniae* is more virulent than K1 [22] *K. pneumoniae* genotype K1 is capable of causing catastrophic septic ocular or central nervous system complications from pyogenic liver abscess independent of underlying diseases in the host [23] while K2 capable of causing hepatic abscesses and severe cases of pneumonia and other hvKP-associated infections such as necrotizing fasciitis [24]. It is demonstrated that the initial surface adherence and maturation stages of *K. pneumoniae* biofilm development are affected by the presence of the capsule [25]. Impairment in the development of biofilm was observed in mutant stains with defects or abnormalities in capsule synthesis [26]. Research on the interference of the capsule in biofilm development reveals that the expression of the capsule affects the adhesive characteristics of the fimbriae because the capsule hides the fimbriae, which lowers adhesive characteristics and impedes biofilm creation [27]. Capsular polysaccharides from *K. pneumoniae* not only directly aid in producing biofilms but also exhibit anti-biofilm characteristics towards other microbes, giving them an edge in habitats containing a mixture of bacteria [28]. This research aimed to examine the effect of the presence of the *K1* and *K2* capsule serotypes on biofilm formation and its relation with resistance to antibiotics.

2. Materials and methods

2.1 Bacterial isolation and identification

One hundred thirty samples have been collected from the Medical City in Baghdad, Iraq, by growing them in Brain Heart Infusion Broth (BHI Broth) from sputum, blood, burns, urine, and wounds, as illustrated in Figure 1. All isolates were identified using selective media (MacConkey agar, Eosin methylene blue agar (EMB), and CHROMagar) and biochemical tests (urease, Simmon's citrate, oxidase, catalase, and indole), with confirmation provided by Vitek II.

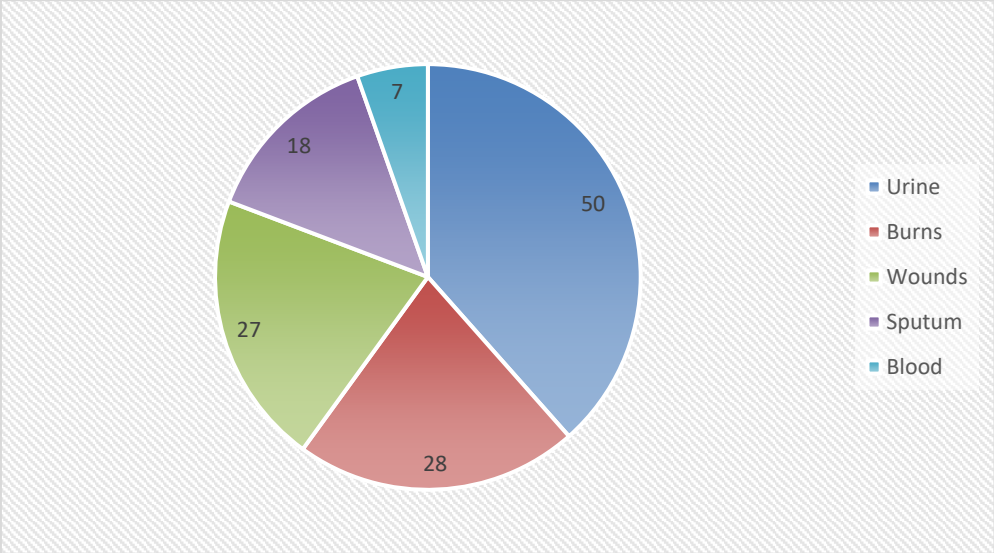


Figure 1: The total number of clinical samples taken from various clinical sources.

2.2 Antibiotic susceptibility test

The susceptibility of all *K. pneumoniae* isolates was tested using the disk diffusion method with ten antimicrobial disks, including Imipenem, Meropenem, Amoxicillin-clavulanate, Ceftazidime, Cefixime, Aztreonam, Gentamicin, Azithromycin, Levofloxacin, Trimethoprim-sulfamethoxazole. After reactivation, a colony of bacteria was transferred into a 5ml normal saline tube, and the bacterial suspension's turbidity was equaled to the McFarland tube (0.5), which was provided directly by the manufactured company (HIMEDA/ India) with a different optical density of 0.5 to 2. The diameter of the inhibition zone in millimeters was used to read the result and compared with the size of the National Community for Clinical Laboratory Standard (CLSI, 2022)

2.3 Biofilm detection by Microtiter plate method (MTP)

MTP is a quantitative method for the detection of biofilm. A culture that was grown overnight in tryptic soya broth with 1% glucose (Himedia/India) was used to create a 0.5 McFarland standard, a 96-well microtiter plate containing 200 µl of diluted culture. It was incubated at 37 °C for a full day (3 wells containing only broth were used as a negative control). Phosphate buffer saline (PBS) was used to wash the microtiter plate three times after removing the bacterial suspension, and then the plate was allowed to dry. Two hundred microliters of 96% methanol was added to all the microtiter plate wells at room temperature for 15 minutes and then removed. Adhering cells were air-dried in each well before being stained with 0.1 percent crystal violet. The plate was washed twice with deionized water to remove the excessive dye, and then two hundred microliters of 96% ethanol was added to every well to detach the bacterial cells [29]. At 630 nm, the optical density was determined using an ELISA plate reader. This method was carried out three times in triplicate [30]. The standards used to analyze the results are shown in Table 1.

Table 1: Biofilm formation interpretation using the MTP method.

Average of OD value	The level of biofilm intensity
OD≤ ODc*	Non -producer
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD> 4ODc	Strong

Optical density cut-off value (O.Dc) = Average of negative control + 3 Standard deviation of negative control, OD = Optical density 630 nm [31].

2.4 DNA Extraction

ABIOpure Extraction Genomic DNA Mini Kit (ABIOpure, USA) was used to extract DNA from *K. pneumoniae,* and the extracted DNA was stored at -20ºC prior to use.

2.5 Molecular identification of wzck1 and *cpsk2* genes

Traditional PCR has been employed to investigate the existence of both *wzck1* and *cpsk2* genes, with the amplicon sizes detailed in Table 2.

Table 2: Primers utilized for this study for gene identification and their corresponding sizes of products.

Gene	Sequence of the primer in the 5′ to 3′ direction		Size of the product (base pair)	Reference
wzck1	F	CTGGAGGAGTTGGGAATAAAT	329 bp	This research
	R	CCTTTCCTTAAATCAGCATCG		
cpsk2	F	AAAGGCAATTCCAAAGGAGA	466 bp	This research
	R	GACGGAGTATTCGGAGAGAT		

Two unique pairs of primers have been developed for the molecular identification of K1 (*wzck1*) and K2 (*cpsk2*) capsule types employing the isolated DNA of *K. pneumoniae*. Twenty microliters of reaction mixture were used, ten microliters of the Master Mix, one microliter of each forward and reverse primer, two microliters of DNA, and six microliters of nuclease-free water were added. The PCR reaction was carried out in the thermal block.
The PCR program steps are illustrated in Table 3.

Table 3: Conditions of the PCR

Steps	Temperature (°C)	Time	Cycle number
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	30
Annealing of *wzck1*	48	30 sec	
Annealing of *cpsk2*	49	30 sec	
Extension	72	30 sec	
Final Extension	72	7 min	1

The PCR products were observed on a gel made of 1.5% agarose infused with red safe dye. Afterward, the gel was examined under an ultraviolet (UV) light.

2.6 Statistical analysis

The Statistical Analysis System was employed to identify the influence of various variables on research variables. The chi-square test was employed to assess the significance of percentages at the 0.05 and 0.01 probability levels in this investigation. A P value of $\leq$ 0.05 was deemed statistically significant, while a P value of $\leq$ 0.01 was regarded as extremely significant.

3. Results and discussion

3.1 Isolation and identification of Klebsiella pneumoniae

In this research, out of the 130 specimens collected from different clinical sources, including urine (28), burns (8), wounds (11), sputum (10), and blood (4), only 61 were identified as *K. pneumoniae.* Isolated bacteria were obtained from hospitals through cultivating in BHI broth, and three different kinds of selective synthetic media (CHROMagar, MacConkey agar, and Eosin methylene blue agar (EMB)) were used to confirm the identification of 61 bacterial isolates. Figure 2 shows *K. pneumoniae* growth and its characteristics.

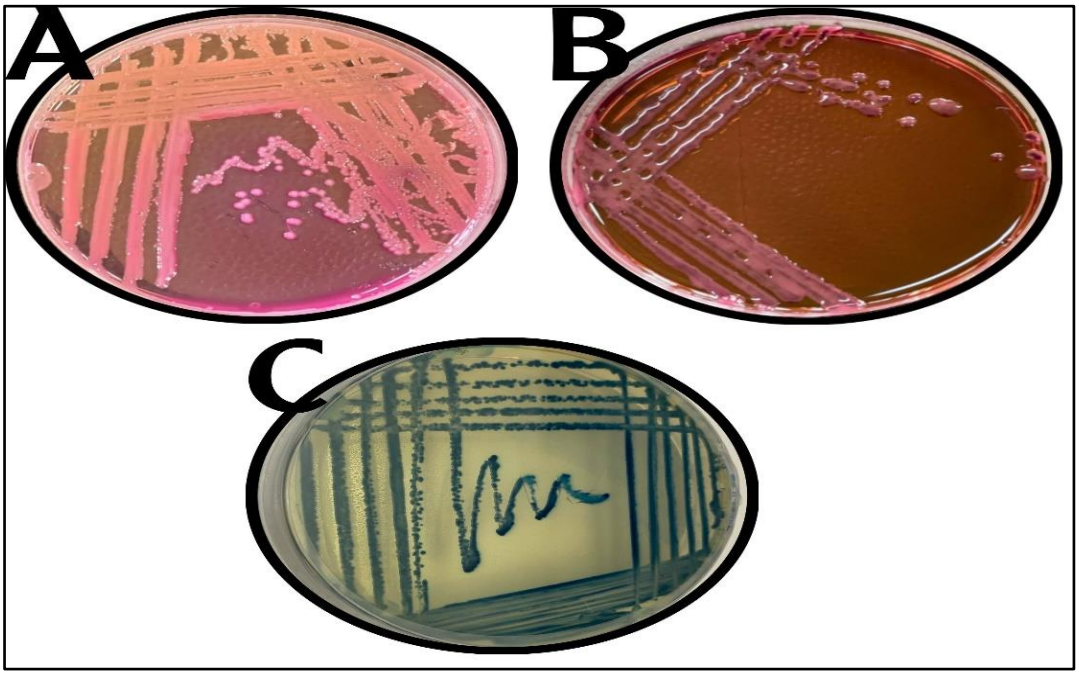


Figure 2: *K. pneumoniae* on different agar media. (A) *K. pneumoniae* on MacConkey agar appeared as a mucoid lactose fermenter colonies. (B) *K. pneumoniae* on EMB, which gives a dark purple color. (C) *K. pneumoniae on* CHROMagar, which gives a metallic blue color.

Subsequently, each isolate underwent a series of biochemical tests. Oxidase and indole were found to be negative in the biochemical tests, while catalase, Simmon citrate, and urease were found to be positive. The results of the biochemical tests used in this study are shown in Figure 3. Vitek II further validated the findings.

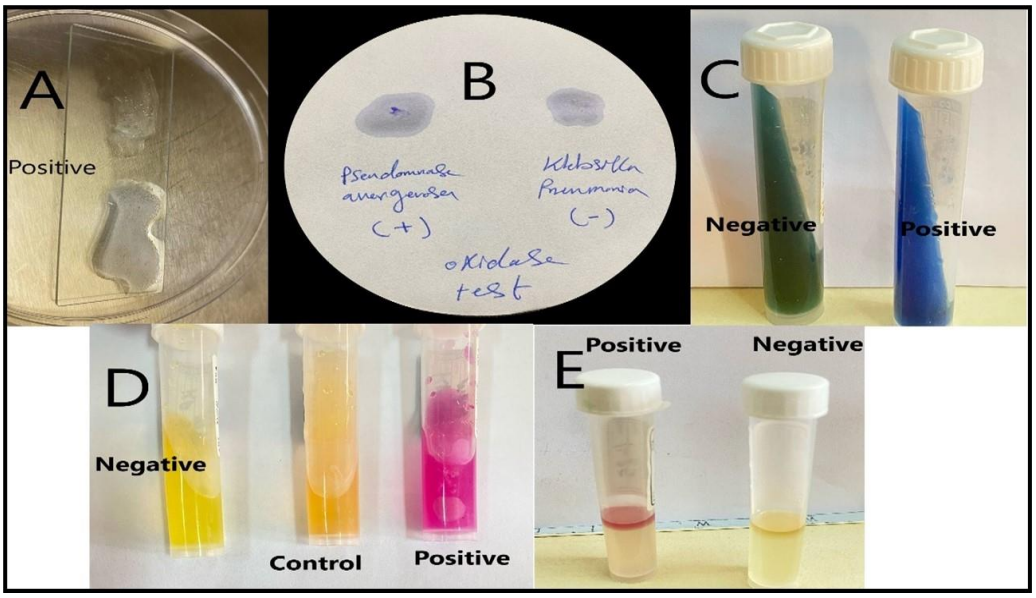
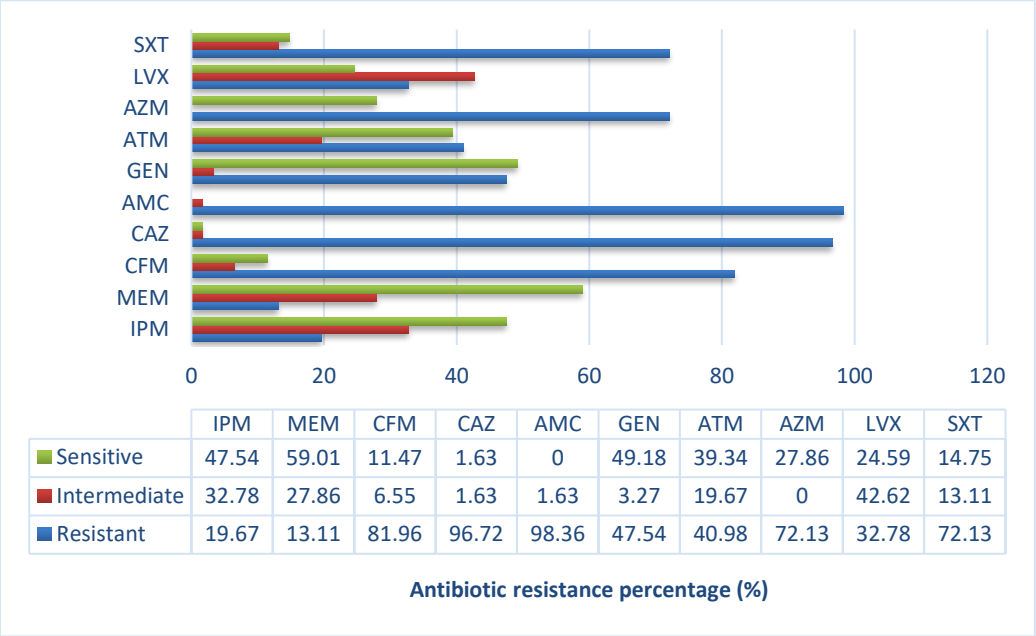


Figure 3: Identification of *K. pneumoniae* using biochemical tests. (A) Catalase test, (B) Oxidase test, (C) Simmon citrate test, (D) Urease test, and (E) Indole test.

3.2 Antibiotic susceptibility results

The resistance ratios of *K. pneumoniae* have been organized in descending order as follows: Amoxicillin-clavulanate at 98.36%, followed by Ceftazidime at 96.72%. The resistance levels for other antibiotics continued to decrease, with susceptibility dropping to 81.96% for Cefixime, 72.13% for both Azithromycin and Trimethoprim-sulfamethoxazole. Gentamicin showed a resistance level of 47.54%, Aztreonam at 40.98%, Levofloxacin at 32.78%, and Imipenem at 19.67%. Finally, the percentage for Meropenem was 13.11%. The findings showed that 95.08% of the isolated bacteria were MDR. Resistance to antibiotics may arise from a number of factors, with the primary cause being the presence of numerous virulence factors. These include capsules, lipopolysaccharides (LPSs), the creation of biofilm, adhesion molecules, exopolysaccharides associated with mucoviscosity, and mechanisms for iron absorption, all of which contribute to significant resistance to antibiotics [14]. The study by Ali and Al-Jaff [32] reported that *K. pneumoniae* resistance was 15.8% to Imipenem, 21.1% to Meropenem, 94.7% to Amoxicillin-clavulanate and 84.2% to Trimethoprim-sulfamethoxazole which is consistent with this study. In comparison, the resistance was 57.9% to Azithromycin and 73.7% to Gentamicin and Ceftazidime, which is inconsistent with this study. Another study by Adeosun *et al.*, [1] reported that the resistance was 46.7% to Aztreonam and 53.2% to Levofloxacin, which agrees with the current study. A study by Fatima *et al.,* [33] recorded that the resistance towards Cefixime was 100%, which is inconsistent with this report. The statistical analysis revealed that the P values for each antibiotic were greatly significant, with the exception of Levofloxacin, which showed an insignificant value of 0.341. Figure 4 presents the results of resistance to antibiotics.



	IPM	MEM	CFM	CAZ	AMC	GEN	ATM	AZM	LVX	SXT
Sensitive	47.54	59.01	11.47	1.63	0	49.18	39.34	27.86	24.59	14.75
Intermediate	32.78	27.86	6.55	1.63	1.63	3.27	19.67	0	42.62	13.11
Resistant	19.67	13.11	81.96	96.72	98.36	47.54	40.98	72.13	32.78	72.13

Figure 4: *K. pneumoniae* antibiotic resistance result.

3.3 Detection of biofilm by MTP method

K. pneumoniae has a propensity to build biofilms on living and non-living surfaces, including surgical instruments, contributing to antibiotic resistance [34]. Because of its higher sensitivity and specificity, the MTP test shows that it was more successful than the Congo Red agar (CRA) test in identifying biofilms *in vitro* [35]. The MTP assay is the most commonly used and widely accepted test for determining whether biofilms are being produced.

According to reports, this technique is the most sensitive, precise, and repeatable screening approach for determining the development of biofilms [36].

In the MTP method (100% 61/61) of isolates formed biofilm, and according to that method, only (13.11% 8/61) were strong biofilm producers; in comparison (65.57% 40/61) were moderate biofilm producers, and (21.31% 13/61) were weak biofilm producers, which indicates that the majority are moderate biofilm producers. Table 4 shows the biofilm capacity of the isolates.

Table 4 : Distribution of *K. pneumoniae* isolates according to biofilm intensity.

Biofilm Capacity	MTP method n=61 (%)
Producer	61 (100%)
Strong	8 (13.11%)
Moderate	40 (65.57%)
Weak	13 (21.31%)
Non-producer	0 (0%)
P-value	0.0001 **
**** (P≤0.01).**	

A study by Ochońska *et al.*, [37] in which clinical *K. pneumoniae* strains found in tracheostomy tubes found that (44.4%) of the isolated produced biofilm and that none of them were strong producers while (22%) of them were moderate and (22%) were weak producers, which is inconsistent with the current results that (100%) of the isolates produced biofilm. Seifi *et al.,* [38] reported that among the 94 isolates of *K. pneumoniae* that were obtained from medical centers in Iran, only (33%) fully formed biofilm, (52.1%) had moderate, (8.5%) weak, while (6.4%) of them were able to form biofilm. In another research

by Nirwati *et al.*, [11],167 isolates of *K. pneumoniae* were isolated, and the majority of them had high levels of antibiotic resistance (85.63%) and were capable of forming biofilm, which agrees with the current findings indicating more than (80%) of the isolates produced biofilm. Nevertheless, according to a study by Makhrmash *et al.*, [39], all the isolates (100%) produced biofilm, which entirely agrees with the current findings.

In this study, all the isolates formed biofilm, and the prevalence of MDR isolates among them was (95.08% 58/61), while only (4.91% 3/61) of them were non-MDR, (10.34% 6/58) of MDR *K. pneumoniae* formed strong biofilm, (67.24% 39/58) of MDR *K. pneumoniae* formed moderate biofilm and (22.41% 13/58) MDR *K. pneumoniae* formed weak biofilm. The study conducted by Shadkam *et al.*, [40] reported that biofilm generation was significantly more common in MDR populations than in non-MDR populations and that resistance to antibiotics is substantially higher in biofilm former strains than in non-biofilm former strains.

Capsular polysaccharides of *K. pneumoniae* are significant virulence components that aid in developing biofilms [37].

3.4 Molecular detection of wzck1 and *cpsk2* genes

K1 and *K2* molecular detection was performed for sixty-one isolates of *K. pneumoniae* by using a conventional PCR technique and specific primers listed in Table 2. The results showed that the dominant serotype was the K2 serotype, which was present in 85.24% (52/61), and only 4.91% (3/61) of the isolates were positive for the K1 serotype while 9.81% (6/61) belonged to non-K1/K2 serotypes.

The study of Kot *et al.*, [41] was consistent with the current results that the K2 serotype was the dominant serotype, with 22.9% of isolates showing K2 capsule serotype. In the study of Razzaq *et al.*, [42], 30.43% of isolated bacteria tested for the K2 serotype, and 17.39% tested positive for the K1 serotype, which agrees with this research that K2 is the dominant capsule type. Nevertheless, a report by Qasim and Khalid, [20] revealed that the K1 serotype was dominant, which is inconsistent with the current results. In general, K1 and K2 strains are more virulent than isolates from different serotypes. They may prevent neutrophils in humans from producing reactive oxygen species, allowing them to live in tissues better than the other serotypes [41]. Figures 5 and 6 show the gel electrophoresis results of the *wzck1*/*cpsk2* genes.

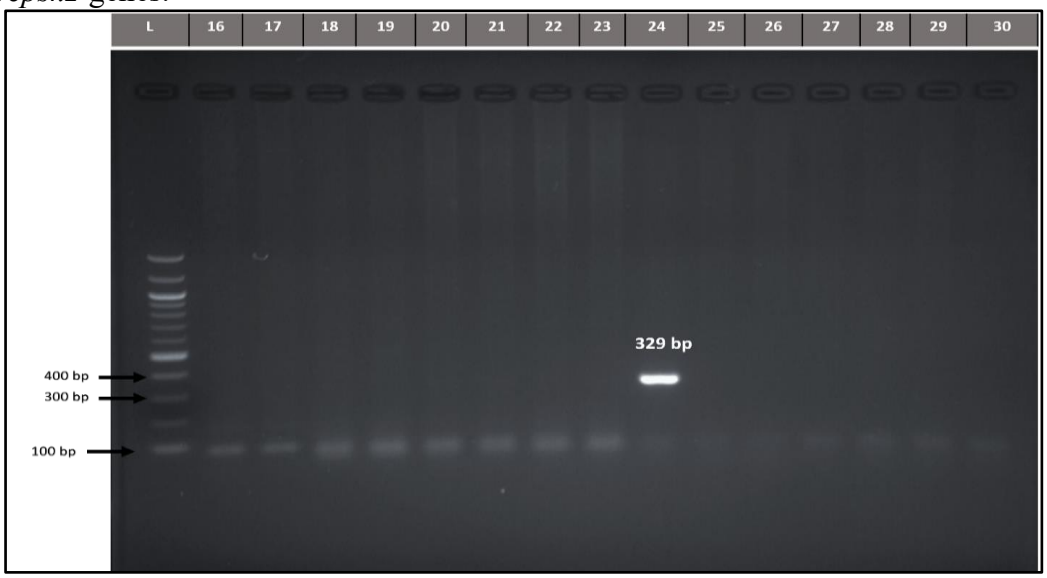


Figure 5: Visualization of *K. pneumoniae wzck1* gene by Agarose gel at 1.5% concentration, stained with red safe dye for sixty minutes. The bands shown correspond to the PCR product (329 bp).

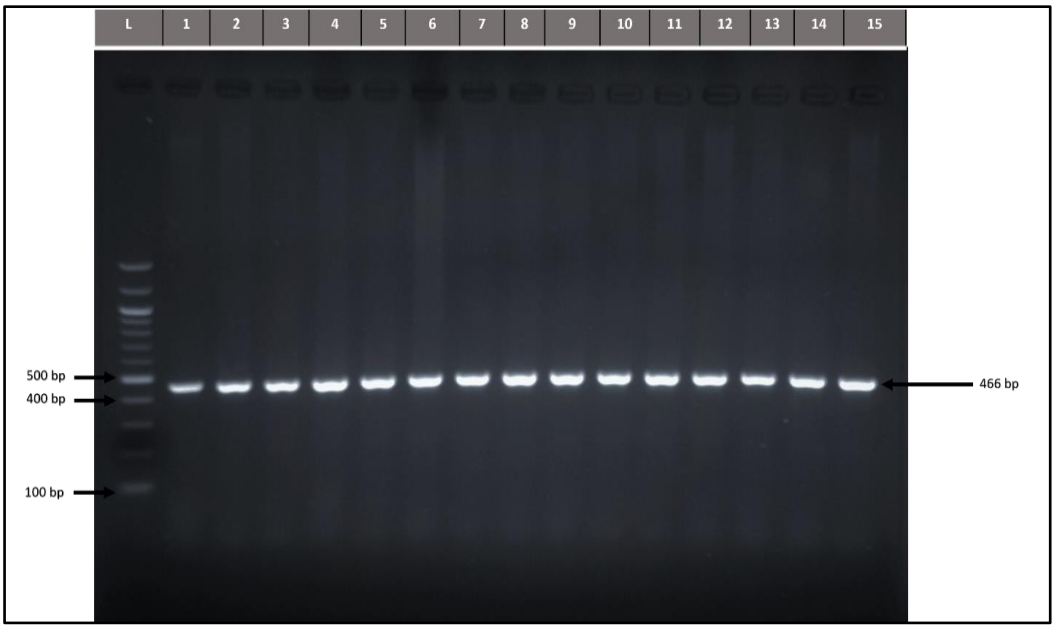


Figure 6: Visualization of *K. pneumoniae cpsk2* gene by Agarose gel at 1.5% concentration, stained with red safe dye for sixty minutes. The bands shown correspond to the PCR product (466 bp).

3.5 Relationship between biofilm production and K1, K2 serotypes

The process of biofilm formation is strongly related to the membrane proteins, as the ability to form biofilm by *K. pneumoniae* serotypes K1 and K2 may be directly influenced by the capsules' existence [43]. In the current findings, all isolates formed biofilm (61/61); 3 of them belonged to the K1 serotype, 52 of them belonged to K2 and 6 were other than K1/K2 serotypes. According to the study of Hyun and Kim, [44], the K1 and K2 serotypes displayed greater levels of biofilm generation than the non-K1/K2 serotypes, which agrees with this study. In another study by Cubero *et al.*, [43], Biofilm development was more closely related to the K1 serotype than the K2 serotype, another report by Zheng *et al.*, [45] suggested that biofilm was more pronounced in K1 serotype than the K2.

4. Conclusion

This research shows that *K. pneumoniae* exhibits a significant capacity for biofilm formation, with varying levels observed, which in turn increases antibiotic resistance as 95.08% of the isolates were MDR. Also, the capsule affects the biofilm formation, especially the K1 and K2 serotypes as it shows higher levels of biofilm.

Ethical clearance

The ethical committee of the College of Science, University of Baghdad, has approved this study, as indicated by the reference number (CSEC/0123/0001).

Conflict of interest

No conflict of interest.

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