P- ISSN 1991-8941 E-ISSN 2706-6703 *Journal of University of Anbar for Pure Science (JUAPS)* Open Access
2025,(19), (01):23 – 31

Abundance of Capsule-Associated Genes in *Klebsiella pneumoniae* and its Relationship with Biofilm Formation

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ARTICLE INFO

Received: 05 / 07 /2024
Accepted: 12/ 08/2024
Available online: 08/ 06 /2025

DOI: 10.37652/juaps.2024.151564.1285

Keywords:
Rosmarinus Klebsiella pneumoniae, capsule, associated genes, biofilm, resistance profile

ABSTRACT

Klebsiella pneumoniae is a member of Enterobacteriacea that causes pneumonia and urinary tract infections, especially in patients with immune deficiency. It is characterized by the presence of a polysaccharide capsule, which is an important factor for enhancing its pathogenicity. This study aims to detect the abundance of capsule-associated genes in *K. pneumoniae* strains and its relationship with biofilm formation. Antibiotic resistance profiling was performed by using the Kirby–Bauer method, and biofilm formation was assessed by employing the crystal violet method in a 96-well microtiter plate. Gene detection in 50 *K. pneumoniae* isolates was performed through PCR. Four capsule-associated genes, namely, *rcsA*, *galF*, *manC*, and *wzi*, were included in this study. Results showed that the isolates had high resistance against most of the antibiotics used in this study. Biofilm formation assay results indicated that 39 out of the 50 isolates have the ability to form biofilms, with 68% being weak biofilm producers and 10% being moderate producers. No strong biofilm producer was found among the 50 strains. PCR results revealed that *rcsA* (100%) was the most abundant gene because it was detected in all the 50 isolates, followed by *galF* (90%), *manC* (72%), and *wzi* (64%). Interestingly, the four genes were highly redundant given that they were all detected in 24 isolates of the same strain (48%). Statistical analysis indicated that biofilm formation had nonstatistically significant relationships with the detected genes and antibiotic resistance profiles. In conclusion, capsule-associated genes are highly abundant in local clinical *K. pneumoniae* strains, and no correlation exists between these genes and the ability for biofilm formation.

Introduction:

Klebsiella pneumoniae was recognized in 1883, when the German pathologist Friedlander described a bacterium from a patient's lungs that induced lung inflammation [1]. It is an important Gram-negative bacillus. Its capability to adhere and attach to different host structures is crucial for causing infections, which are persistent threats to human health and correlated with hospital outbreaks [2]. *K. pneumoniae* is an opportunistic pathogen that causes blood, respiratory tract, nosocomial, and urinary tract infections. It produces carbapenemases that hydrolyze and inactivate several antibiotic agents [1].

A prominent capsular polysaccharide (CPS) envelops the complete bacterial surface, leading to a distinctive mucoid appearance when cultivated on agar plates [3]. The CPS is one of the most essential virulence factors of *K. pneumoniae* strains, including community-acquired and health care–associated strains [4]. The capability of particular *K. pneumoniae* capsules to obstruct and resist phagocytosis by host cells has been studied in detail [5]. Different factors, including CPSs, lipopolysaccharides, secreted toxic materials, and multidrug resistance, have been identified and characterized [6]. Characteristics related to CPSs have been linked with the virulence of *K. pneumoniae*. They include the presence of a mucopolysaccharide web surrounding the capsule, which plays a role in facilitating biofilm formation [7]. The CPS matrix is the main motif of the intercellular matrix of biomembranes;

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it envelops the cell surface, functions in protection from host immunity, and helps bacteria adhere to host cells [8]. In *K. pneumoniae*, the bacterial capsule plays a crucial role in biofilm development and is a crucial factor for enhancing bacterial pathogenicity [9]. Biofilm production could be seen as an additional factor contributing to pathogenicity, enabling bacteria to persist in the urogenital tract for extended periods and impeding their elimination by expressing virulence and resistance genes [10, 11]. Biofilm structures, which often comprise a compact arrangement of different molecules, including DNA, polysaccharides, and proteins, decrease the effectiveness of antibiotics and bacterial exposure to antibiotic agents [12]. The transformation of genetic material among bacterial cells may increase within biofilms, thus facilitating the acquisition of new DNA fragments, among which are genes responsible for antibiotic resistance [13].

Capsule synthesis in *K. pneumonia* is transcriptionally regulated by a number of proteins (*cps* cluster) localized on the bacterial chromosome [14]. The region of the *cps* cluster is composed of over 20 genes (from *galF* to *ugd*) that are mainly controlled by three promoters located upstream of *galF*, *wzi*, and *manC* (Figure 1). These three promoters are collectively responsible for capsule synthesis and known as K-antigens [15]. The *rcsA* gene regulates capsule synthesis in numerous Gram-negative bacteria [16].

The *wzi* gene, which starts and initiates with the codon GTG, encodes an outer membrane protein that was originally specified and explicated in *K. pneumoniae* k2 [17, 18]. It is responsible for the transportation, processing, and surface assembly of CPSs [4]. The determination of K antigens has traditionally relied on serological techniques. Recently, the typing of K antigens is often performed by the sequencing of the *wzi* locus because it is present in all capsular types of *K. pneumoniae* [19]. This gene encodes a surface protein that plays a crucial role in attaching the capsule to the outer membrane, and its knockout results in a strain lacking the ability to form capsules [20, 21]. The *galF* gene, which codes for a UDP–glucose pyrophosphatase, is the first gene in all *cps* cluster that participates in expressing capsule synthesis precursors important for CPS translocation and treatment [22, 23]. A previous study [24] reported that the expression levels of the *galF* and *manC* genes decreased in *ΔrmpA* mutants, showing that the *rmpA* gene mediates the regulation of these genes. A unique characteristic of *K. pneumoniae* is the existence of *rcsAB* sequences up to the *galF* gene. This sequence elevates the level of *galF* transcription, leading to increases in the amount of UDP–glucose and UDP–glucuronic acid that subsequently increase the level of capsule production [17]. The *manC* gene is the third of the three characterized promoters in the capsule locus; it encodes for mannose-1-phosphate guanylyl transferase [15].

The immediate connection between the *wzi* and *manC* genes affects capsule expression [20, 25]. Therefore, this study focused on determining the abundance of capsule-associated genes in *Klebsiella* strains isolated from hospitals in Ramadi and studied its correlation with biofilm formation.

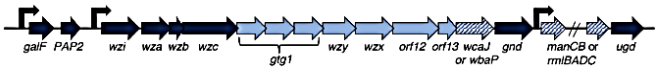

Figure 1. Arrangement of capsule-associated genes in *K. pneumoniae*: *galF*, *wzi*, and *manC* [16].

Materials and methods

Bacterial strains and growth conditions

A total of 50 *K. pneumoniae* strains were obtained from the microbiology laboratory of the biology department. Bacterial strains were isolated from different patient sources, including urinary tract infections, sputum, blood, and wounds [26]. All strains were activated and maintained by using nutrient agar and MacConkey agar at 37 °C for 24 h.

Antibiotic susceptibility test

The results of the susceptibility test from a previous study [26] were cited and confirmed in this work. The Kirby–Bauer method was performed in accordance with CLSI [27] to confirm the antibiotic susceptibility results (Table 1).

Table 1. Antimicrobial agents used to determine antibiotic resistance profiles.

Antibiotic	Concentrations (µg)
Gentamicin	30
Amikacin	30
Amoxicillin–clavulanate	20/10
Piperacillin–tazobactam	100/10
Trimethoprim–sulfamethoxazole	1.25/23.75
Ceftriaxone	30
Cefotaxime	30
Cefepime	30
Ceftazidime	30
Imipenem	10
Aztreonam	30
Levofloxacin	5
Ciprofloxacin	5

Detection of capsule-associated genes by PCR

The genomic DNA (gDNA) of all isolates was extracted by employing Easy Pure Bacteria Genomic DNA Kit in accordance with the manufacturer's instructions. Nanodrop was used to quantify the concentrations and purity of the extracted gDNA. PCR amplifications of DNA fragments were performed by utilizing Hot Start Green Master Mix (Promega, the USA). The extracted gDNA was used as a template for the amplification of all capsule-associated genes selected in this study, including *galF*, *wzi*, *manC*, and *rcsA*. PCR primers were designed with Primer3Plus software and ordered from Macrogen (Korea). The sequences are shown in Table 2. PCR amplification was performed in a 20 µL reaction prepared by adding 10 µL of 2× Master Mix, 1 µL (0.2 µM) of the forward primer, 1 µL (0.2 µM) of the reverse primer, and 20 ng/µL gDNA. Nuclease-free water was used to adjust the volume of the reaction to 20 µL. The following PCR cycling conditions were applied in a Bio-Rad T100 Thermal Cycler (the UK) instrument: initial denaturation at 95 °C for 5 min (one cycle); denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s (30 cycles); and final extension at 72 °C for 10 min (one cycle). PCR products were analyzed through electrophoresis using 1.5% agarose gels, and a gel documentation system was employed to visualize the resulting DNA fragments.

Table 2. Oligonucleotide sequences of capsule-associated genes.

Primer	Oligo Sequence 5′→3′	Size (bp)	Tm (C)	References
galF	F-CTGCCGGATATCATCCTTGAC	139	58	This study
	R-CGGAGTATTCGGAGAGATCG			
Wzi	F-CTTCACCTTTACGCCGTTT	150	58	This study
	R-TGGTTACCCGGTTCGTTATC			
manC	F-CAGCGGCATGTTTATGTTCC	120	58	This study
	R-ATGAAGTCGCTGCCGTTGTC			
rcsA	F-GGCATGGTACTTCGCAAATC	144	58	This study
	R-AGGTGATGTTTTCGGTCAGC			
rpoD	F-AGATGGTTGAAGCGAACCTG	115	58	This study
	R-CGAACTTATCGACCGCTTTC			

Biofilm formation assay

The capability of *K. pneumoniae* to form biofilms was assessed by using the crystal violet method in a 96-well microtiter plate following the procedure mentioned in a previous work [11] with minor modifications. Briefly, *Klebsiella* isolates were grown in nutrient broth (NB) at 37 °C under shaking at 180 rpm for overnight incubation. For each isolate, bacteria were diluted with NB to obtain a sample with a cell concentration of 1.5×10^8 CFU/mL. Concentrations were measured at an optical density (OD) of 600 nm by using a spectrophotometer. Each well of the microtiter plate was dispensed with 200 µL of the diluted broth and incubated at 37 °C for 48 h. After the incubation period, the plate was washed with sterilized dH_2O to remove unbound cells. The remining bacterial cells in each well were treated with 200 µL of 1% (w/v) crystal violet dye, and the plate was incubated for 30 min. Subsequently, the plate was washed to remove excess and unbound dye. Each well was treated with 200 µL of 95% ethanol to solubilize the dye. The results were recorded by measuring the OD of the supernatant at 570 nm, as explained below. The experiment was performed in triplicate, and the mean absorbance value was determined.

OD ≤ ODc	**= nonbiofilm producer**
ODc < OD ≤ 2 ODc	**= weak biofilm producer**
2 ODc < OD ≤ 4 ODc	**= moderate biofilm producer**
4 ODc < OD	**= strong biofilm producer**

Results

General characteristics and antimicrobial resistance profiles

Under microscopy examinations, including Gram staining, the obtained isolates showed the general characteristics of *K. pneumoniae* after activation at −80 °C and culture on MacConkey agar (Figure 2). Of the 50 strains, many were highly resistant to amoxycillin–clavulanic acid (98%), ceftazidime (98%), trimethoprime–sulfamethaxazone (90%), ceftriaxone (90%), and cefotaxime (84%). Some strains exhibited low resistance to gentamicin (58%), aztreonam (46 %), and amikacin (22%), as shown in Table 3. Interestingly, all strains were sensitive to imipenem.

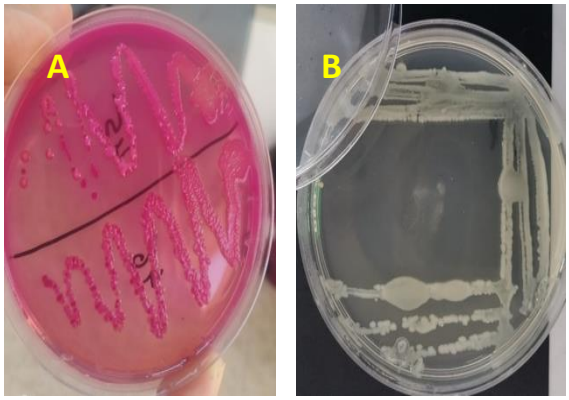

Figure 2. *K. pneumoniae* grown on (A) MacConkey agar. (B) Nutrient agar.

Abundance of capsule-associated genes

Four capsule-associated genes, namely, *manC*, *rcsA*, *wzi*, and *galF*, were detected in *K. pneumoniae* strains by using PCR. *rcsA* (100%) was the most abundant gene because it was detected in all 50 isolates, followed by *galF* (90%), *manC* (72%), and *wzi* (64%), as indicated in Figure 3. Interestingly, the four genes were highly redundant given that they were all detected in the 24 isolates of the same strain (48%). DNA fragments and gene sizes are indicated in Figures 4a, b, c, and d.

Table 3. Antimicrobial resistance profiles of the *Klebsiella* isolates.

Antibiotic		**No. of resistance isolates (n = 50)**
Aminoglycosides	Gentamicin	29 (58%)
Penicillins (β-lactamase)	Amikacin	11 (22%)
	Amox–clavu	49 (98%)
	Piper-tazoba	25 (50%)
Folate pathway inhibitors	Trim–sulfa	45 (90%)
Cephalosporins	Ceftriaxone	45 (90%)
	Cefotaxime	42 (84%)
	Cefepime	35 (70%)
	Ceftazidime	49 (98%)
Carbapenem	Imipenem	0
Monobactam	Aztreonam	23 (46%)
Quinolones	Levofloxacin	29 (58%)
	Ciprofloxacin	**35 (70%)**

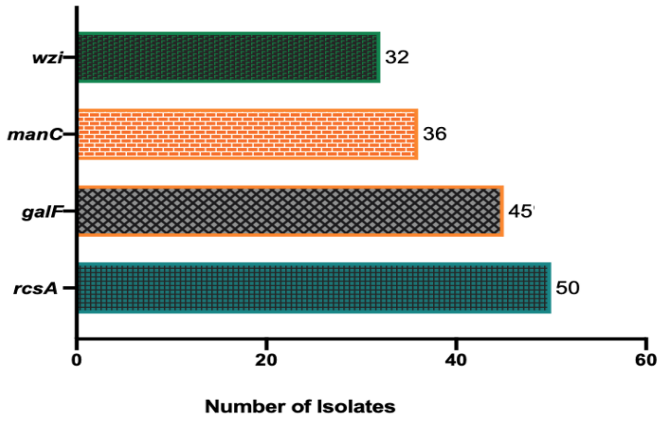

Figure 3: Number of capsule-associated genes in *K. pneumoniae* isolates.

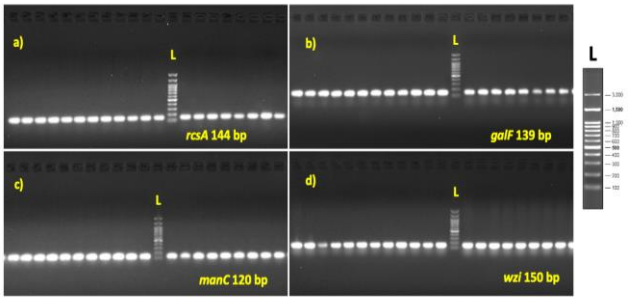

Figure 4: Gel electrophoresis of the PCR products using 1.5% agarose: a) *rcsA*, b) *galF*, c) *manC*, and d) *wzi*.

Biofilm formation and its relationship with capsule-associated genes and antibiotic resistance profiles

The results of the biofilm formation assay indicated that 39 out of the 50 isolates have the ability to form biofilms. Figure 5 shows that out of the 50 strains, 34 (68%) were weak biofilm producers, five (10%) were

moderate producers, and none were strong biofilm producers. The results of statistical analysis revealed a nonstatistically significant relationship between biofilm formation and antibiotic resistance profiles, even in the strains with the high ability to resist most of the antibiotics used in the present study (Figure 6). Similarly, a nonstatistically significant relationship was found between biofilm formation and the presence of capsule-associated genes, except with cefepime (Figure 7).

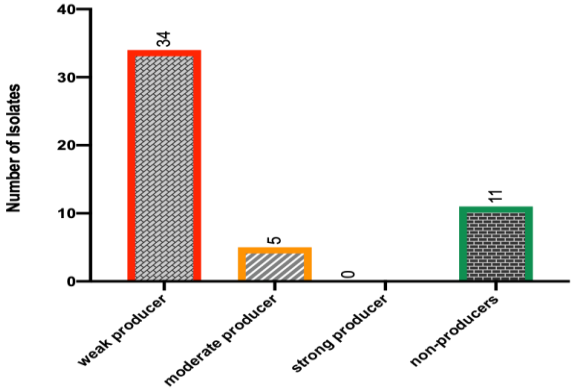

Figure 5. Biofilm formation abilities of *K. pneumoniae* isolates.

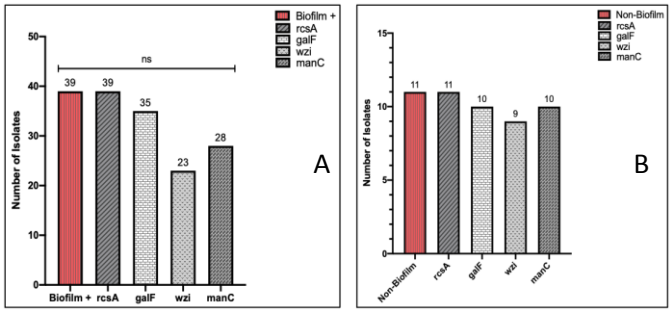

Figure 6. Relationship between biofilm production and capsule-associated genes: A) Biofilm producers. B) Nonbiofilm producers.

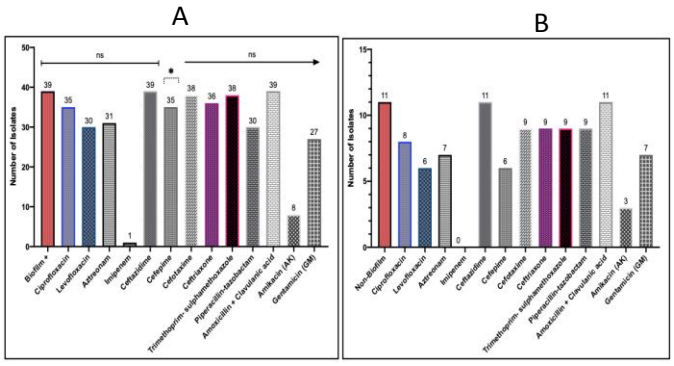

Figure 7. Relationship between biofilm production and antibiotic resistance profiles: A) Number of biofilm producers. B) Number of nonbiofilm producers.

Discussion

Klebsiella pneumoniae is an enteric bacillus that causes pneumonia and urinary tract infections, especially in people with immune deficiency [10]. It is an opportunistic microorganism that can form biofilms, resulting in host colonization. Biofilms help bacteria survive in severe environments, enabling the colonization of catheters, wounds, and the urinary tract. Capsule formation is one of the most important virulence factors that increases the ability of *Klebsiella* to survive and cause infections. It is a complex process involving numerous genes and genetic pathways. Four capsule-associated genes were detected using PCR: *rcsA*, *galF*, *manC*, and *wzi*. As mentioned in the Results section, *rcsA* was the most abundant gene and found in all of the 50 isolates, followed by *galF*, *manC*, and *wzi*. *rcsA* is responsible for mucuoid morphology and regulates other capsule-associated genes. The findings of this work agree with those of Mccallumt and Whitfield [28], who indicated that *rcsA* is highly abundant in *K. pneumonia* isolates. The close similarity between the percentages of *rcsA* and *galF* genes could be attributed to the control of *galF* expression, which is related to CPSs, by *rcsA*. In *Klebsiella* isolates, *galF* gene deletion resulted in decreased CPS synthesis [6]. The similarity in the abundances of *manC* and *wzi* in *Klebsiella* isolates confirmed the interaction and association of these two genes in capsule formation. *manC* is responsible for producing a type of sugar precursor required for K2 capsule formation, whereas *wzi* encodes a protein responsible for anchoring the formed CPS to the outer membrane [16, 29]. Zhu et al. [20] concluded and confirmed the direct interaction between the *manC* and *wzi* promoters.

Of the strains, 78% were biofilm producers (68% and 10% were weak and moderate producers, respectively) and 22% lacked the ability to form biofilms. A previous study [30] on biofilm formation by local *K. pneumoniae* isolates found that 23 of the isolates showed weak biofilm formation ability, 14 had moderate biofilm formation ability, three were strong biofilm producers, and 10 were nonbiofilm producers.

This result, except for the presence of strong producers, is almost aligned with the findings of the present study. However, a different biofilm formation pattern was found in another study [31], which reported that 73.33% of the isolates were strong producers. This discrepancy could be attributed to the differences in the size of the sample, source of isolates, and health history of the patients included in the study.

Statistical analysis indicated a nonstatistically significant relationship between the ability for biofilm formation and presence of capsule-associated genes, as shown in Figures 4–6. This result could be due to the high redundancy of the selected genes in *Klebsiella* isolates in the present study. All genes in the present study found to be responsible for capsule production are encoded in the *cps* cluster and regulated by the *rcsA* gene [17]. No correlations were found between these genes and biofilm formation. The capsule is not correlated with the adhesion of *Klebsiella* given that strains with the knock out of genes responsible for capsule formation showed greater adhesion than the wild-type strain [32]. Therefore, the surface polysaccharides present in the mucoid *K. pneumoniae* strain could mask the adhesive effect of fimbriae and reduce biofilm production. This result suggests that other virulence factors are important for the persistence and biofilm formation of *Klebsiella*

In *Porphyromonas gingivalis* and *K. pneumoniae* MGH 78578, biofilm formation is enhanced in the absence of capsules [33]. Moreover, biofilm formation is facilitated by the presence of cell-surface adhesins, like fimbriae [34]. Therefore, the surface polysaccharides present in the mucoid hypervirulent *K. pneumoniae* strain could mask the adhesive effect of fimbriae and reduce biofilm production. Genes encoding polysaccharide capsules and siderophores support the ability of *Klebsiella* species to establish infection. The capsule functions in protecting cells against phagocytosis, and adherence and the ability to form capsules are inversely related in *K. pneumoniae* isolates [34].

Similarly, the results indicated a nonstatistically significant relationship between biofilm formation ability and antibiotic resistance profiles. Another study reported that 85% of *Klebsiella* isolates are biofilm producers and 11.7% are nonbiofilm producers that can produce enzymes that resist antibiotics [35]. This characteristic could be a reason behind our results indicating that this nonstatistically significant relationship is due to high antibiotic resistance, even in nonbiofilm producers. Moreover, ampicillin and cefotaxime resistance rates of 83.3% and 73.3%, respectively, were found among biofilm-forming isolates, whereas ampicillin and cefotaxime resistance rates of 60% and 35%, respectively, were found among nonbiofilm producers [36]. Therefore, the relationship between biofilm producers and antibiotic resistance profiles in *K. pneumoniae* isolates remains controversial and needs further studies to clarify. This relationship varies depending on the source of isolates and the gene types responsible for resistance [37, 38, 39]. *K. pneumoniae* is one of the most common species carrying plasmids that contain genes encoding enzymes responsible for antibiotic resistance, such as extended-spectrum β-lactamases, which are a major cause of hospital-acquired infections [2, 13, 40].

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P- ISSN 1991-8941 E-ISSN 2706-6703 *Journal of University of Anbar for Pure Science (JUAPS)* Open Access
2025,(19), (01):23 – 31

تردد الجينات المرتبطة بالكبسول لبكتريا الالتهاب الرئوي السريرية وعلاقتها مع تكوين الغشاء الحيوي

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الخلاصة:

بكتريا الالتهاب الرئوي احد أنواع البكتريا المعويه والتي تسبب بالاضافه للالتهاب الرئوي التهاب المسالك البوليه خصوصا في المرضى الذين لديهم نقص في المناعة. تمتاز بامتلاكها كبسول متعدد السكريات والذي يعتبر احد العوامل التي تزيد من امراضية هذه البكتريا. الهدف من الدراسه هو الكشف عن تردد بعض الجينات المرتبطه بتكوين الكبسول وعلاقتها مع تكوين الغشاء الحيوي. الكشف عن المقاومة للمضادات الحيوية تمت باستخدام طريقة Kirby-Bauer وتكوين الغشاء الحيوي بطريقة استخدام صبغة crystal violet. استخدم PCR للكشف عن الجينات في ٥٠ عزلة محلية لبكتريا الالتهاب الرئوي وشملت الجينات *rcsA*, *galF*, *manC*, and *wzi*. أظهرت النتائج وجود مقاومة عالية لاغلب المضادات المستخدمه قيد الدراسه. نتائج تكوين الغشاء الحيوي بينت ان هنالك ٣٩ عزله كانت قادره على تكوين الغشاء الحيوي بنسبة ٦٨٪ تكوين ضعيف، ١٠٪ متوسطه في حين لم تكن هنالك أي عزله لها القابليه على انتاج الغشاء بصوره قويه. أظهرت نتائج الـPCR ان جين *rcsA* وجد بنسبة ١٠٠٪، يليه *galF* (٩٠٪)، *manC* (٧٢٪)، و *wzi* (٦٤٪). كما بينت النتائج ان هنالك ٢٤ عزلة تمتلك الأربع جينات سوية. بينت نتائج التحليل الاحصائي بعدم وجود أي علاقه او ترابط بين امتلاك الجينات وتكوين الغشاء الحيوي او المقاومه للمضادات والغشاء الحيوي. الجينات المستخدمه قيد الدراسه ترددها عالي بين عزلات بكتريا الالتهاب الرئوي ولاتوجد علاقه بينها وبين تكوين الغشاء الحيوي.

كلمات مفتاحية: بكتريا الالتهاب الرئوي، كبسول، جينات مرتبطه، الغشاء الحيوي، المقاومة للمضادات