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Research Article

Overexpression of *lasB* Gene in *Klebsiella pneumoniae* and its Effect on Biofilm Formation and Antibiotic Resistance

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Abstract

Background: *Klebsiella pneumoniae* is the second most frequent pathogenic bacterium in the Enterobacteriaceae family, after *E. coli*. It is also regarded as a major pathogen responsible for healthcare-associated infections around the world. ***Objective***: To look at how overexpressing the elastase gene (lasB) in *K. pneumoniae* affects biofilm development and antibiotic resistance. ***Methods***: 25 clinical isolates of *K. pneumoniae* were received from Baghdad's Chemistry Analysis Center (CAC) and re-identified using the Vitek-2 method. The microtiter plate was used to measure biofilm production with ELISA. The disc diffusion method was used in antibiotic sensitivity tests in accordance with the CLSI 2022 criteria. The PlasB plasmid was transformed into *K. pneumoniae* via electroporation. ***Results***: Out of 25 isolates, 11 (44%), 11 (44%), and 3 (12%) produced strong, moderate, and weak biofilms, respectively. One strong biofilm producer (KA1) was chosen for further investigation. The *lasB* plasmid was successfully transformed into KA1, yielding the KA1 (plasB) isolate. KA1 (plasB) formed considerably fewer biofilms than KA1, and it was more susceptible to tetracycline, doxycycline, and amoxicillin-clavulanic acid than KA1. Furthermore, KA1 (plasB) has shown a significant decrease in ampicillin resistance and an increase in ciprofloxacin sensitivity, but no variations in susceptibility to levofloxacin, cefotaxime, piperacillin-tazobactam, amikacin, or erythromycin when compared to KA1. ***Conclusions***: Overexpression of the elastase gene (plasB) has a major impact on biofilm development and antibiotic resistance in *K. pneumoniae*.

Keywords: Antibiotic resistance, Biofilm formation, *K. pneumoniae*, *lasB*.

الإفراط في تعبير جين الإيلاستيز في الكلبسيلا الرئوية وتأثيره على تكوين الغشاء الحيوي ومقاومة المضادات الحيوية

الخلاصة

الخلفية: تعتبر الكلبسيلا الرئوية ثاني أكثر البكتيريا الممرضة شيوعًا في عائلة Enterobacteriaceae بعد الإشريكية القولونية. كما تعتبر واحدًا من الكائنات الدقيقة الممرضة المهمة التي تُسبب العدوى المرتبطة بالرعاية الصحية في جميع أنحاء العالم. **الأهداف**: دراسة تأثير الافراط في تعبير جين الإيلاستيز (*lasB*)في الكلبسيلا الرئوية على مقاومة المضادات الحيوية وتكوين الغشاء الحيوي. **الطرق**: تم الحصول على 25 عزلة سريرية من الكلبسيلا الرئوية من مركز التحليل الكيميائي (CAC) في بغداد، العراق وإعادة تشخيصها باستخدام نظام Vitek-2. تم استخدام طبق المايكروتيتر لقياس تكوين الغشاء الحيوي باستخدام اختبار ELISA. تم استخدام طريقة انتشار القرص في اختبارات الحساسية للمضادات الحيوية وفقًا لإرشادات CLSI 2022. تم ادخال البلازميد plasBإلى الكلبسيلا الرئوية باستخدام طريقة الادخال الكهربائي. **النتائج**: من بين 25 عزلة، كانت 11 (44%)، 11 (44%)، و3 (12%) منتجة للغشاء الحيوي بشكل قوي، متوسط، وضعيف على التوالي. تم اختيار إحدى منتجات الغشاء الحيوي القوية (KA1)للدراسة اللاحقة. تم ادخال بلازميد plasBبنجاح إلى KA1 لإنتاج عزلة KA1(plasB). كان تكوين الغشاء الحيوي لدى KA1(plasB)أقل بشكل كبير من KA1، كما أظهرت KA1(plasB) حساسية تجاه التتراسيكلين والدوكسيسيكلين وحمض الأموكسيسيلين-الكلافيولانيك بالمقارنة مع KA1. علاوة على ذلك، أظهرت KA1(plasB)انخفاضًا كبيرًا في مقاومتها للأمبيسيلين وزيادة كبيرة في حساسيتها تجاه سيبروفلوكساسين، بينما لم تظهر اختلافات في قابلية التحسس تجاه ليفوفلوكساسين، سيفوتاكسيم، بيبراسيلين-تازوباكتام، أميكاسين، وإريثرومايسين بالمقارنة مع KA1. **الاستنتاج**: أظهرت هذه الدراسة أن الافراط في تعبير جين الإيلاستيز (*lasB*) له تأثيرات كبيرة على تكوين الغشاء الحيوي ومقاومة المضادات الحيوية في الكلبسيلا الرئوية .

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INTRODUCTION

Animal mucosa may harbor the gram-negative bacteria *Klebsiella pneumoniae*, which can also be present in the environment in places like soil and water. This infection is primarily located in the gastrointestinal tract and, rarely, in the nasopharynx, from which it can spread to other tissues or the bloodstream and cause health complications [1]. It is known to cause numerous illnesses in hospitals, such as pneumonia, septicemia, and urinary tract infections [2]. Hospitals all across the world are facing an increasing problem with antimicrobial resistance. One of the bacteria that can quickly evolve into strains resistant to drugs is *K. pneumoniae*, which can often be quite dangerous for people. This is because the current treatments are not as effective, which is leading to a higher death rate [3]. Research has shown that eliminating biofilm-forming illnesses with a single antibiotic treatment is ineffective. Therefore, to evaluate the outcome of novel approaches for the treatment of biofilm-associated illnesses, it is crucial to manage infections using the current antibiotics available. Researchers have suggested adding erythromycin and azithromycin, two macrolides considered important antibiotics, to antibiotic therapy for the treatment of infections caused by biofilm-producing bacteria [4]. In *K. pneumoniae*, biofilm development and antibiotic resistance are correlated; biofilm-producing bacteria are more resistant to antibiotics than planktonic germs [5,6]. The lasB gene produces Elastase lasB, also known as "pseudolysin," a zinc-dependent metalloprotease that belongs to the thermolysin family [7]. Through the bacterial type II secretion system, the pathogenic *Pseudomonas aeruginosa* releases an extracellular enzyme that is toxic. It is the main extracellular virulence factor and the most prevalent protease. In addition, it injures tissue, invades it, produces immunomodulation by processing immune system components, and acts intracellularly by controlling rhamnolipids to initiate the creation of bacterial biofilms [8,9]. This work aimed to investigate the impact of the cloned lasB gene from P. aeruginosa on biofilm formation and antibiotic resistance in K. pneumoniae.

METHODS

Bacterial isolates and plasmid

A total of 25 clinical isolates of *K. pneumoniae* were obtained from the Chemistry Analysis Center (CAC), Baghdad, Iraq and re-diagnosed by using Vitek-2 system. The *lasB* gene (1497 pb) of *P. aeruginosa* was cloned on pMG-Kan plasmid (Macrogen) to produce plasB plasmid.

Biofilm formation assay

We assessed the isolates' ability to produce biofilm using a microtiter plate assay [10]. We grew the bacterial isolates on nutrient agar and kept them at 37 °C for a full day of incubation. We suspended a few colonies in Muller-Hinton broth, enhanced with 1% glucose, in a test tube and vortexed them. Afterwards, a 96-well microtiter plate with a flat bottom was filled with 20 µl of the bacterial suspension per well. There was already 200µl of uninoculated broth and 180µl of Muller-Hinton broth with 1% glucose in the dish. We followed this specific procedure for the control wells. For twenty-four hours, the plate was in an incubator with the temperature adjusted to 37 °C. We removed the contents of each well and rinsed them three times with PBS. We left the plate to air-dry at room temperature after an hour. The wells were filled with 150 µl of crystal violet and allowed to stand for 15 minutes. After removing the crystal violet, we rinsed the wells three times with PBS to eliminate any remaining dye. We then allowed the wells to air dry at ambient temperature. A solution of 150 µl of 95% ethanol was used to remove the dye biofilm. We measured the absorbance at 570 nm for each well using the spectrophotometer reader. The bacterial adhesion capabilities were determined by the following methods: non-biofilm producer ($OD_s \leq OD_C$), weak biofilm producer ($O.D_C < O.D_S \leq 2* O.D_C$), moderate biofilm producer ($2*O.D_C < O.D_S \leq 4* O.D_C$) and strong biofilm producer ($4*O.D_C < O.D_S$).

Antibiotic sensitivity test

The sensitivity test was carried out by using the disk diffusion method [11]. All strong and moderate biofilm producers were tested against kanamycin (30 µg) as a selectable marker, and then one of the strong biofilm producers, which is kanamycin sensitive (KA1), was selected for further experiments. The study included the following antimicrobial agents: ampicillin (10 µg), amoxicillin-clavulanic acid (30 µg), amikacin (30 µg), cefotaxime (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), piperacillin-tazobactam (100/10 µg), levofloxacin (5 µg), and tetracycline (30 µg). A sterile cotton swab was used to mix a number of colonies of the isolate into 5 ml of normal saline until the bacterial suspension reached turbidity of 0.5 McFarland standards, which is $1.5x10^8$ CFU/ml. The mixture was then spread out on nutrient agar medium and allowed to dry for a few minutes. To ensure that the antibiotic discs made contact with the agar, they were carefully pressed onto the plates of agar using sterile forceps. After that, the plates were incubated at 37 °C for 24 hours. After incubation, the inhibition zone around discs was measured, and the results were interpreted according to CLSI, 2022 [12].

Preparation of electro-competent cells

Fifty ml of nutrient broth was prepared in a flask, and 500µl from an overnight culture of KA1 isolate was added and grown at 37 °C and 200 rpm in the shaker incubator for 2 hours, until it reached a cell density of $O.D_{600}$ (0.4–0.6). After that, the culture was placed on ice for 15-20 minutes, then centrifuged at 3500 rpm for 10 minutes at 4 °C. The cell pellet was mixed with 50 ml of ice-cold sterile distilled water and then centrifuged at 3500 rpm for 10 minutes at 4 °C. This

wash step was repeated one more time, but added 25 ml instead of 50 ml of ice-cold sterile distilled water. The cell pellet was mixed with 1 ml of sterile 10% glycerol and centrifuged as mentioned above. The pellet was mixed with 120 µl of sterile 10% glycerol. Cells were separated into 40µl aliquots in sterile Eppendorf tubes and stored at -20 °C [13].

Transformation of electro-competent cells

3.0 µl of (40 ng) plasB was added to 40 µl of the competent KA1 into a pre-chilled electroporation cuvette (Eppendorf, Germany). The cuvette was placed into the electroporator (Eppendorf, Germany) adjusted to 2500-volt electric current with a resistance of 200 Ω [14]. One ml of fresh nutrient broth medium was immediately added into the electroporation cuvette, incubated for 1 hour at 37 °C with shaking, and plated on nutrient agar medium with kanamycin (50 µg) and incubated for 24 hours at 37 °C. A few kanamycin-resistant colonies were selected to identify the presence of plasB plasmids carrying *the lasB* gene.

Primers used in this study

The following primers were designed (NCBI) for detection of *lasB* gene in plasB plasmid: F: 5'-CATTTCGTCGCCAACAT CGC-3' and R: 5'-TGCTTGTAGGTG TTGGTCGG-3'. The product size is 470 bp.

PCR mixture

To make the mixture up to 20µl, 16µl of the nuclease-free water was added, and 2µl of the forward primer and 2µl of the reverse primer were added to the PCR premix tube. Colonies from the KA1 (plasB) agar plate were picked and added separately to the PCR tubes. The PCR tubes were placed in a thermal cycler, and the PCR assay was performed in the following manner: The process begins with a 5-minute denaturation at 95°C for one cycle, followed by 30 cycles of 1-minute denaturation at 95°C, 30-second annealing at 58°C, 35-second extension at 72°C, and a 5-minute final extension at 72°C. After completion of the PCR, the PCR tubes were run on 1% agarose gel [15].

RESULTS

Out of 25 isolates of *K. pneumoniae*, 11 (44%) were strong, 11 (44%) were moderate, and 3 (12%) were weak biofilm producers (our published research) [16]. A kanamycin sensitivity test was performed for strong and moderate biofilm producers (22 isolates) in order to select the sensitive isolates. 17 (77%) of the 22 isolates were sensitive to kanamycin, divided into 11 isolates of strong biofilm producers and 6 isolates of moderate biofilm producers (Figure 1). While only 5 isolates (23%) were kanamycin-resistant, we selected a kanamycin-sensitive isolate (KA1), known for its high biofilm production. Although *K. pneumoniae* doesn't have *the lasB* gene, KA1 tested for *lasB* gene presence, and *P. aeruginosa* was used as a positive control.

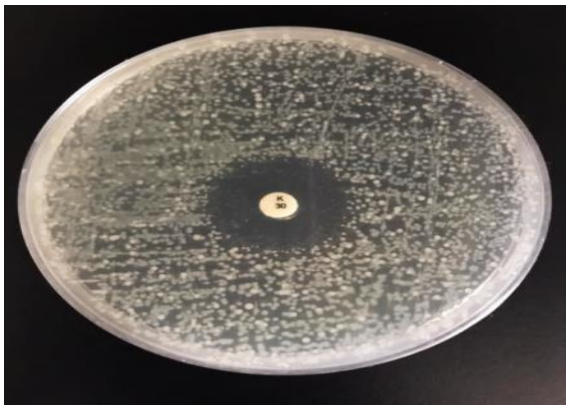

Figure 1: Kanamycin sensitivity test.

Figure 2 shows that KA1 was negative for this gene. The transformation results indicated the presence of KA1 (plasB) colonies on the nutrient agar media containing kanamycin compared to the KA1 result (as a negative control).

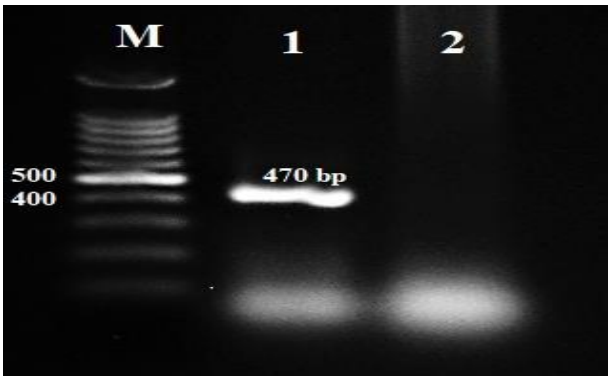

Figure 2: Detection of *lasB* gene. M: DNA marker. Lane 1: *P. aeruginosa* (+ve control). Lane 2: KA1.

Four suspected colonies, KA1 (plasB), were screened for the presence of *the lasB* gene, and the results show that all of them were carrying the *lasB* gene compared to KA1 as a negative control (Figure 3).

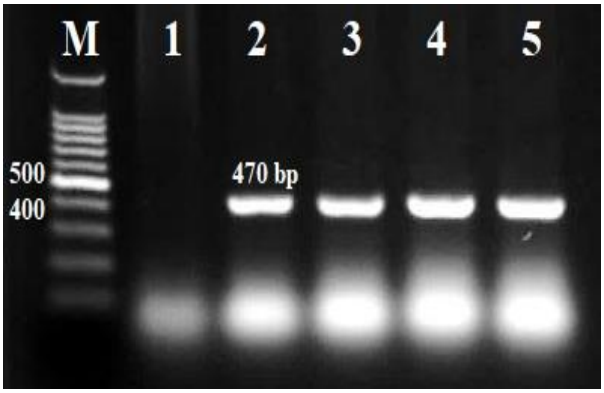

Figure 3: Screening of *lasB* gene. M: DNA marker. Lane 1: KA1 (-ve control). Lane 2-5: KA1(plasB) tested colonies.

One KA1 (plasB) isolate was selected for further experiments. The results showed a significant decrease in biofilm formation in KA1 (plasB) in comparison to KA1 (Table 1).

Table 1: Biofilm formation (OD values) of KA1 and KA1(plasB)

Isolate	Optical Density (n=3)	Results	*p*-value
Control	0.06±0.00	-----	-----
KA1	0.53±0.031	Strong	0.0001
KA1(plas)	0.23±0.02	Moderate	

KA1 and KA1 (plasB) exhibit different levels of sensitivity and resistance to the selected antibiotics. KA1 (plasB) was sensitive to amoxicillin-clavulanic acid, doxycycline and tetracycline in comparison to KA1, which was resistant to these antibiotics. In addition, KA1 (plasB) showed a significant decrease in resistance to ampicillin and a significant increase in sensitivity to ciprofloxacin. However, there were no observed alterations in susceptibility to piperacillin-tazobactam, levofloxacin, erythromycin, cefotaxime or amikacin when compared to KA1 (Table 2).

Table 2: Antibiotic resistance of KA1 and KA1(plasB)

Antibiotic	KA1		KA1(plasB)		*p*-value
	Inhibition zone (mm) (n=3)	Sensitivity	Inhibition zone (mm) (n=3)	Sensitivity	
Levofloxacin	28.33±2.08	S	31.66±1.52	S	0.21
Ampicillin	0.33± 0.57	R	11.00±3.0	I	0.03
Cefotaxime	0.00	R	0.00	R	-
Tetracycline	9.00 ±1.0	R	15.00±2.0	S	0.01
Piperacillin-tazobactam	30.00±2.0	S	30.33±1.52	S	0.87
Ciprofloxacin	32.66±1.52	S	38.66±1.52	S	0.04
Amikacin	25.66±1.52	S	26.66±2.37	S	0.88
Doxycycline	7.00 ±2.0	R	22.33±2.51	S	0.01
Amoxicillin-Clavulanic acid	11.33±1.52	R	22.33±3.79	S	0.01
Erythromycin	3.00±2.65	R	3.33±3.06	R	0.90

DISCUSSION

The growth of KA1 (plasB) on agar treated with kanamycin indicated the effective transformation of plasB into KA1, and the appearance of the lasB band on an agarose gel proved that the lasB gene could be changed into *K. pneumoniae*.

This was confirmed by an Iraqi study by Al-Rubii (2017), who effectively transformed *E. coli* BL21 and *E. coli* DH5α strains using the PGEXKG-lasB vector when they were cultivated on LB agar with ampicillin. The ability to insert the plasmid containing the lasB gene into both bacterial strains was demonstrated by an agarose gel analysis [17]. The effect of overexpressing the lasB gene on KA1 (plasB) biofilm development is inconsistent with findings from another study conducted by Roshani *et al*. (2018), which showed that *P. aeruginosa* strains harboring the lasB gene did not create as much biofilm [18]. Mahdavi *et al*. (2020) found that isolates of *P. aeruginosa* that were lasB-positive had a strong propensity to produce biofilms [19]. The present study's findings were consistent with those of Tielen *et al*. (2010), who documented the impact of lasB overexpression on *P. aeruginosa* biofilm development [20]. It was found that this overexpression of elastase increased the levels of extracellular rhamnolipids and mono-rhamnolipids. This suggests that overexpression of elastase modifies the physical-chemical properties and composition of extracellular phospholipid (EPS), which in turn modifies the production of *P. aeruginosa* biofilms. Conversely, the variations in antibiotic sensitivity between KA1 and KA1 (plasB) show how elastase overexpression influences *K. pneumoniae's* resistance to antimicrobial drugs. It matched the findings of Abd al-Rahman and Al-Aubaydi (2015), who reported that amikacin sensitivity was present in all of their *K. pneumoniae* isolates [21]. Furthermore, Jabar and Abid (2021) showed that amikacin was effective against 83% of K. pneumoniae isolates [22]. In line with the KA1 result [23], Al-Sheboul *et al*. (2023) reported that 98% of the isolates were ampicillin-resistant. Furthermore, according to Abbas (2023) [24], 89% of *K. pneumoniae* isolates had ampicillin resistance. Cefotaxime's KA1 result matched the findings of Raouf *et al*. (2022), who discovered that 97.6% of the samples were resistant [25]. Cefotaxime resistance is caused by extended beta-lactamase enzymes [26]. The erythromycin result for KA1 was in line with findings from Bokaeian *et al*. (2014) and Safika *et al*. (2022), which reported that erythromycin resistance was present in 70% and 100%, respectively, of 30 and 45 isolates [27,28]. According to research by AlAnsary and Al-Saryi (2023), 72% of 100 isolates of *K. pneumoniae* had tetracycline resistance, which is consistent with the KA1 result [29]. According to Sweedan *et al*. (2022), doxycycline resistance was seen in 67.7% of the 15 isolates of *K. pneumoniae* [30]. When bacteria pick up genes that make proteins that shield ribosomes from antimicrobial drugs or that function as an efflux mechanism, they develop resistance to antibiotics [4]. The results for levofloxacin and ciprofloxacin were in line with those of Ghanem *et al*. (2017), who found that 96.1% of *K. pneumoniae* isolates in Saudi Arabia were susceptible to ciprofloxacin [31]. According to a different study by Akter *et al*. (2019), 80.3% of *K. pneumoniae* isolates were sensitive to ciprofloxacin, and 84.3% were susceptible to levofloxacin [32]. Ahmadi *et al*. (2021) reported that 50% of isolates were resistant to piperacillin-tazobactam; this finding was consistent with the findings of the present investigation [33]. One major factor thought to be responsible for the emergence of antibiotic resistance in bacteria is the overuse of antibiotics. Therefore, there is a strong need to look for novel therapeutic approaches [34].

Conclusion

This study revealed that the KA1 (plasB) strain formed biofilms at a significantly lower rate than the KA1 strain due to the overexpression of the initial

strain's lasB gene. Because KA1 (plasB) reduces the production of biofilms, the KA1 and KA1 (plasB) strains show varying degrees of resistance to the chosen antibiotics.

Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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