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Study the relation between *luxS* gene and forming biofilm and non-forming isolates of *Klebsiella pneumonia*

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Abstract

Klebsiella pneumoniae is a notorious pathogenic bacterium, armed with multiple virulence factors, and is commonly associated with pneumonia infections. One of the virulence factors helps *K. pneumonia* to cause many diseases and escape immune system and antimicrobial effect is biofilm construction. Therefore, the overall aim was to investigate the genic profile for *K. pneumonia* that formed and non-formed biofilm. The result revealed that around half of the isolates did not form biofilm and this may because *luxS* gene was present in only two isolates forming biofilm and absent in others while *fimH* genes were absent in all other isolates. *K. pneumonia* isolates were sensitive to green tea, Imipenem, and Amikacin antibiotics. The findings suggest that these compounds may have therapeutic potential against the specific strain of bacterium. Finally, genic variation was identified in *the luxS* gene between two isolates forming biofilm however, this variation was synonymous and no variation was detected in the amino acid sequence. This may indicate that *the luxS* gene is highly conserved among *K. pneumonia* isolates. The absence of *luxS* and *fimH* genes in most isolates appears to hinder their ability to form biofilms, making them susceptible to antibiotics such as green tea, Imipenem, and Amikacin.

Keyword: *Klebsiella pneumonia, luxS*, *fimH* genes, biofilm formation

دراسة العلاقة بين جين luxS للعزلات المكونة والغير المكونة للاغشية الحيوية لبكتريا *Klebsiella pneumonia*

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الخلاصة

الكَلِبْسية الرئوية هي بكتيريا مسببة للأمراض سيئة السمعة، مسلحة بعوامل ضراوة متعددة، وترتبط عادة بعدوى الالتهاب الرئوي. أحد عوامل الضراوة تساعد الكَلِبْسية الرئوية على التسبب في العديد من الأمراض والهروب من الجهاز المناعي والتأثير المضاد للميكروبات هو بناء الأغشية الحيوية. لذلك، كان الهدف العام هو التحقيق في الملف الجيني لـ الكَلِبْسية الرئوية التي شكلت الأغشية الحيوية وغير المشكل لاغشية الحيوية. كشفت النتيجة أن حوالي نصف العزلات لم تشكل أغشية حيوية وقد يكون هذا لأن جين **luxS** كان موجودًا في عزلتين فقط تشكلان أغشية حيوية وغائبًا في عزلات أخرى بينما كانت جينات **fimH** غائبة في جميع العزلات الأخرى. كانت عزلات الكَلِبْسية الرئوية حساسة للشاي الأخضر والمضادات الحيوية Amikacin و Imipenem. تشير النتائج إلى أن هذه المركبات قد يكون لها إمكانات علاجية ضد سلالة معينة من البكتيريا. أخيرًا، تم التعرف على التباين الجيني في جين **luxS** بين عزلتين تشكلان غشاءً حيويًا، إلا أن هذا التباين كان

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صامتا ولم يتم الكشف عن أي تباين في تسلسل الأحماض الأمينية. قد يشير هذا إلى أن جين **luxS** محفوظ بدرجة عالية بين عزلات الكَلِبْسية الرئوية. يبدو أن غياب جينات **luxS** و **fimH**في معظم العزلات يعيق قدرتها على تكوين أغشية حيوية، مما يجعلها عرضة للمضادات الحيوية مثل الشاي الأخضر وImipenem و Amikacin.

1.Introduction

One of the opportunistic pathogen that infects skin, urinary tract and respiratory tracts and causes pneumonia and sepsis is *K. pneumonia* [1, 2]. Klebsiella pneumoniae is a highly virulent bacterium, renowned for its capacity to produce a plethora of virulence factors, including enterotoxin and capsule antigens, which enables it to thrive and cause infection, further complicated by its ability to exhibit resistance to multiple antibiotics [3-5]. Quorum sensing play a crucial role in regulation of many functions in the bacterial cells through changing in population density that serves as a tool in bacterial adaptation for stressful environmental agents. Through quorum sensing, signaling molecules are produced by converting S-ribosylhomocysteine to compound that is finally converted to AI-2 molecule, these molecules are called autoinducers and help in interspecies communication among community of bacteria [6-8]. Many virulence factors in *K. pneumonia* that represented by secretion systems and motility are regulated by the action of AI-2 [9-11]. *K. pneumonia* can resist several antibiotics such carbapenems, fluoroquinolones and cephalosporins. The mechanisms of antibiotics resistance in *K. pneumonia* include decreased in permeability of outer membrane, alteration the proteins that are responsible on binding in penicillin and overexpression of efflux pump [12-14]. *K. pneumonia* mediating resistance in carbapenems through their ability to form biofilm has mortality rate of 42% [15-17]. Many virulence factors play significant role in evading immune system of the host and in forming of biofilm, these factors are outer membrane proteins, capsule polysaccharide and all types of fimbriae [18-20]. Biofilm formation is controlled by many genetic factors, including the *luxS* gene which is responsible for quorum sensing activity, the pgaA gene, which produces polysaccharides production, and *fimA* and *fimH* for fimbriae formation [21-23]. It has been observed that *luxS* producing quorum sensing signaling molecules participate in construction of biofilm in *K. pneumonia* [9, 24, 25]. Biofilm is a sheath of extracellular polymeric substances that consists mainly of polysaccharides and has a role in protecting the community of bacteria from antimicrobial agents and immune system [26-28]. *FimH* gene encode for Type 1 fimbriae that correlated with UTI infection [29-31]. Type 3 fimbria help *K. pneumonia* to bind with epithelial cells that found in urinary tract and respiratory tract and to construct biofilm [32, 33]. Binding to extracellular matrix is achieved by the action of MrkA protein encoded to Type 3 fimbriae and this accelerate biofilm formation in human endothelial and bladder cells [34, 35]. Initiation and maturation of biofilm mediated by the action of Type 3 fimbriae[36]. The study aimed to investigate the genic variation in quorum sensing *luxS* gene and type 3 fimbriae *fimH* gene and their correlation with biofilm construction in *K. pneumonia*.

2. Materials and methods

2.1 Strains collection

The 20 *K. pneumonia* strains were taken from different specimens (urine, sputum, wound, burn, vagina and ear) from patients in hospitals in Bagdad city. The strains were identified using morphological and biochemical approaches. The appearance of the isolates on MacConkey agar seems to be pink mucoid colonies with large size, while it showed non-hemolytic characterization growing on blood agar. Hydrogen sulfide production, urease test, indole production, motility and other chemical tests were achieved to help in diagnosis the *K. pneumonia* isolates. 16sRNA sequencing was carried out for determination of all strains of *K. pneumonia*.

2.2 Susceptibility testing for antimicrobial agents

Regarding with CLSI; M100-S14 protocol, the disk agar diffusion approach was carried out using Mueller-Hinton agar plates for testing susceptibility of antibiotic for four different types of antibiotics which are gentamicin (10 µg), colistin (COL:10 µg), ceftazidime (CAZ: 30 µg), and tobramycin (10 µg) antibiotic. Multidrug resistance (MDR) isolates are defined at those resistant to three or more different antibiotics [20].

2.3 Susceptibility testing for green tea against isolates

Antimicrobial susceptibility testing of green tea was performed for the isolates as follows; Pour was created in plates containing nutrient agar and 100 µl of planktonic cells mixed with 50 µl of green tea and added to the pour in the plates and kept at 37 ◦C for 24 h. Measuring the zone of inhibition was carried out and compared with standard using CLSI; M100-S14 protocol [4].

2.4 Biofilm formation protocol

The inoculum of *K. pneumonia* in tryptic soy broth was incubated, in addition to 1 % glucose at 37 °C for 24 h. Later, 200 µl of inoculum for each type of *K. pneumonia* among 20 isolates was poured into 96 well of microtitre plate and kept at 37 °C for 24 h. Washing the wells with 0.2 ml of distilled water (DW) was conducted and subsequently the microtitre plate was inverted on filter paper for drying the wells. Later, the staining of the well with 0.1% crystal violet with 180 µl for 15 min was carried out and destained with acetic acid. 570 nm is the wavelength for reading all absorbance for 20 isolates on well and this was carried out in triplicate for each assay while the negative control represented by absorbance of the media without the inoculum. ODc represented by taking the mean of OD of negative control plus three of standard deviations while ODi indicated by subtracting OD of the control from ODs of each sample after taking their average from the triplicate. The detection of different schemes of biofilm construction was as (non_biofilm_construction: ODi < ODc), (weak_biofilm_ construction: ODc < ODi < or = 2*ODc), (moderate_biofilm_ construction: 2*ODc < ODi < or = 4*ODc), and (strong_biofilm_ construction: 4*ODc < ODi) [4].

2.5 Molecular detection and sequencing

The PCR was performed to detect the presence of *luxS* gene in six isolates thereby three isolates were forming biofilm and three non-forming biofilms. The DNA was extracted using kit for extraction the DNA (Bioneer kit, Korea). The PCR reaction mixture had a total volume of 25 µL, containing 12.5 µL of master mix, distilled water of 10.4 µL, primer of 0.8 µL of forward and reverse primers, and finally 1 µL of extracted DNA. The PCR run with initiation cycle for denaturation with 94 °C and 5 min, then denaturation, annealing and extension was achieved with for 30 s at 95 °C, 53 °C for 60 s, and at 72 °C for 60 s respectively. The forward primer was GCCGTTGTTAGATAGTTTCACAG and reverse primer was CAGTTCGTCGTTGCTGTTGATG(luxS-4). The product of PCR was run on gel electrophoresis using 1% agarose gel and stained using ethidium bromide pigment [20]. The product of PCR was also sent for sequencing using ABI 3730 DNA Sequencer with reaction of sequencer that consists of PCR product, primer, D. W, sequencing mix that contains Big dye (4.5 µl) and sequencing buffer (10 µl) with 0.5 µl, 1 µl and 4.5 µl respectively.

2.6 Statistical analysis

The statistical analysis was carried out on data under the study by calculating P-value using Chi-squared test (Campbell, 2007)

3. Results

3.1: Concentration of biofilm

A significant proportion of isolates, specifically 55%, were found to be biofilm producers, with 30% of these classified as weak biofilm formers and 25% as moderate biofilm formers. However, biofilm non-producer isolates were 45% Figure 1. The result was not significant through comparison among biofilm and non-biofilm constructor with P = 0.6645.

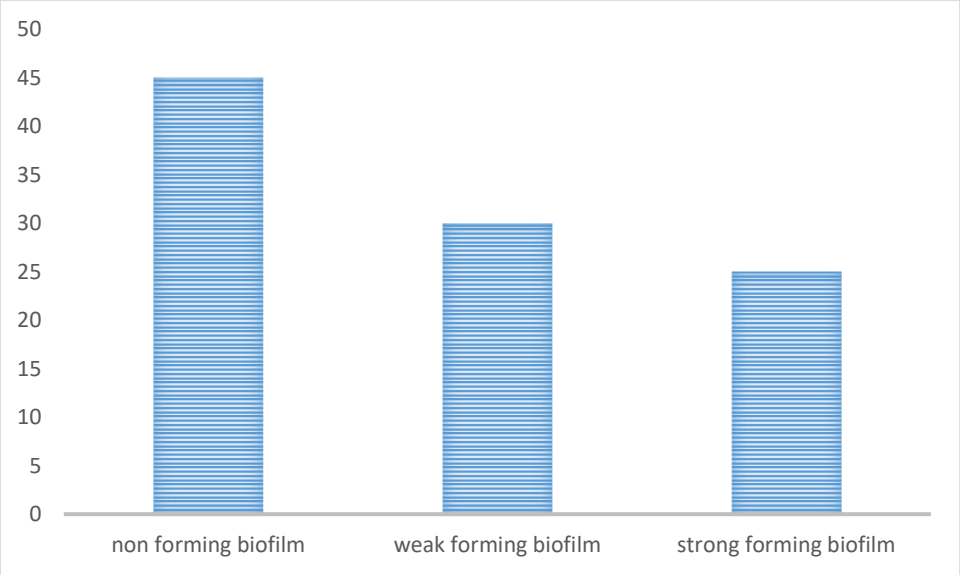

Figure 1: Percentage of isolates constructed biofilm for weak and moderate scheme and isolates non-constructed biofilm.

3.2: Antimicrobial susceptibility testing

The result of antimicrobial sensitivity test revealed that all strains were sensitive to Imipenem and most of them were also sensitive to Amikacin with 90%. However, treating the bacterial cells with Colistin showed that most of isolates were resistance with 95%. Moreover, Ceftazidime sensitivity testing appeared that the percentage of isolates were resistance were 60% while only 30% of isolates were sensitive. In summary, the percentages of MDR were remarkably low, at just 5%. Further statistical analysis revealed significant differences in the resistance and sensitive isolates of Colistin and Ceftazidime. Specifically, P-values were significantly higher in resistance isolates with 0.0001 and 0.0597 compared with percentage of sensitive isolates for Colistin and Ceftazidime respectively. However, the percentages of sensitive isolates were higher with P-values 0.0001 and 0.0958 for Imipenem and Amikacin Table 1.

Table 1: Percentage of resistance and sensitive isolates for antibiotics susceptibility

Type of antibiotics	Percentage of isolates %		
	Resistance	**Intermediate**	**Sensitive**
Colistin	19 (95%)	1(5%)	non
Ceftazidime	12(60%)	2(10%)	6(30%)
Imipenem	-	-	20(100%)
Amikacin	2(10%)	-	18(90%)

The effect of green tea of *K. pneumonia* isolates showed that most of isolates were sensitive with 60% while both resistance and intermediate isolates were 20% for each one. The percent

of sensitive isolates were significantly higher than resistance isolates with P-value 0.0108. This indicates that the green tea is a good therapeutic agent against *K. pneumonia* isolates Figure 2.

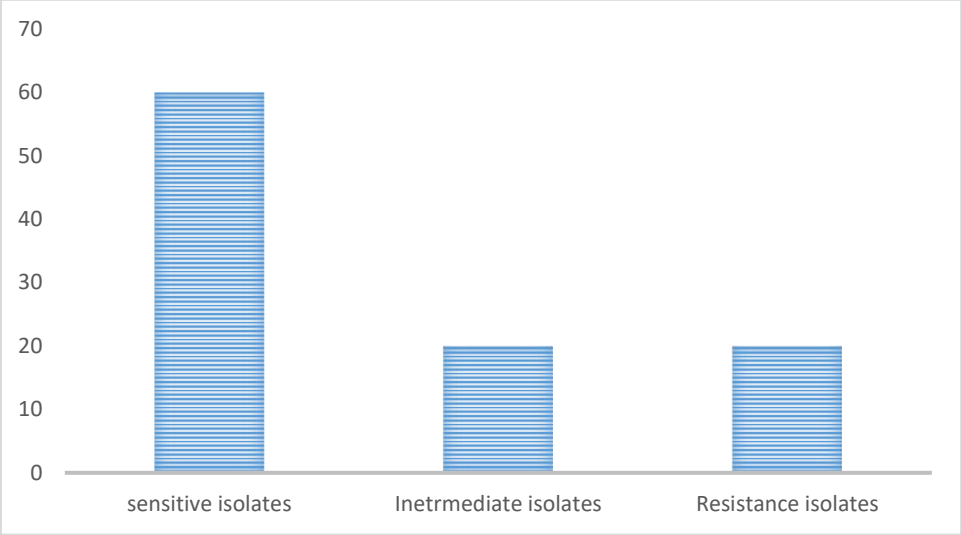

Figure 2: Schematic representation for the effect of green tea on *K. pneumonia* isolates

3.3: Molecular detection and sequencing

Detection the presence and absence of *luxS* and *fimH* genes in three isolates non-forming biofilm (Isolates NO 1, 2, and 3) and three isolates forming biofilm (Isolates NO 4, 5, and 6) using PCR technique revealed that the availability of the *luxS* gene was detected in strains number 5 and 6, these are biofilm-forming isolates, while they are absent in other isolates (see Figure 3). However, fimH was not detected in any of the isolates, both biofilm-forming and non-forming (see Figure 4).

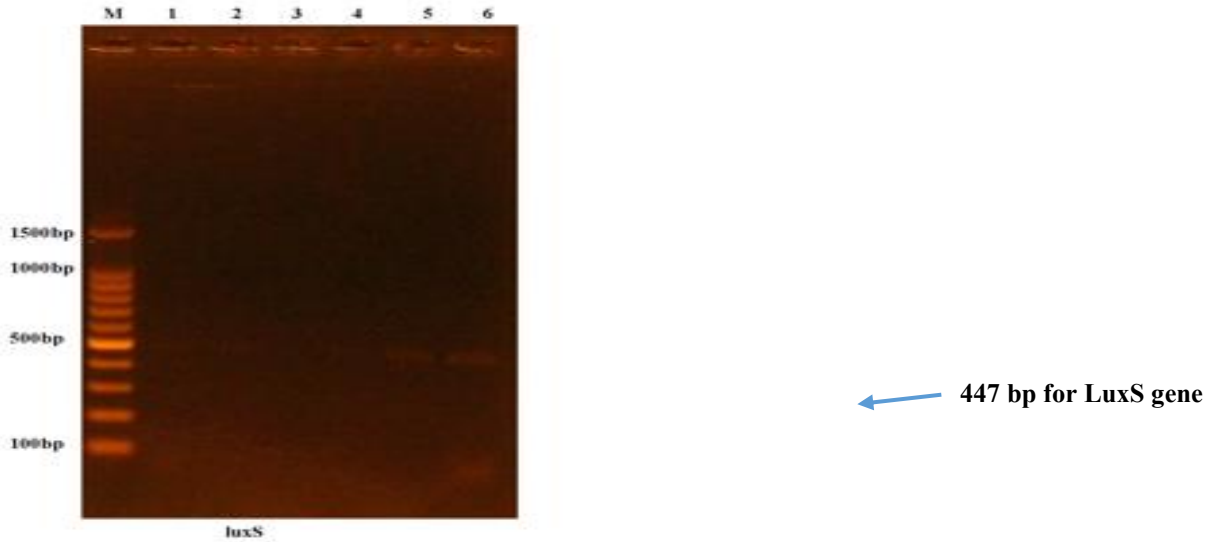

Figure 3: Visualization the bands of *LuxS* gene using gel documentation. Line 1: the ladder, Lines with numbers 1, 2, 3, 4, 5 and 6 are the isolates. The size of *LuxS* band was 447 bp.

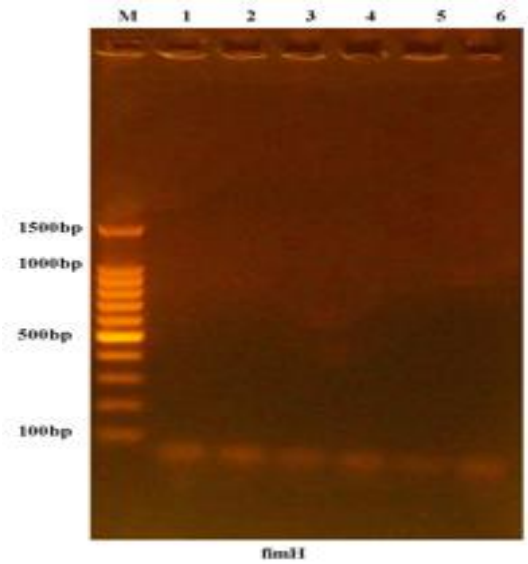

Figure 4: Visualization the bands of *fimH* gene using gel documentation. Line 1: the ladder, Lines with numbers 1, 2, 3, 4, 5 and 6 are the isolates. The size of MrkA band is 615 bp. No bands were detected

The sequencing results also confirmed that *luxS* gene was only presence in biofilm forming isolates No 5 and 6 and there was a difference in sequencing pattern in position 262 by which T replaced by G and in position 358 by which (C) replaced to (T) as shown in Figure 5. However, both mutations in position 262 and 358 where synonymous mutation therefore amino acids pattern were not changed Figure 6.

Figure 5: Alignment pattern between isolate No 5 and 6 that showed the difference in position 262 and 358.

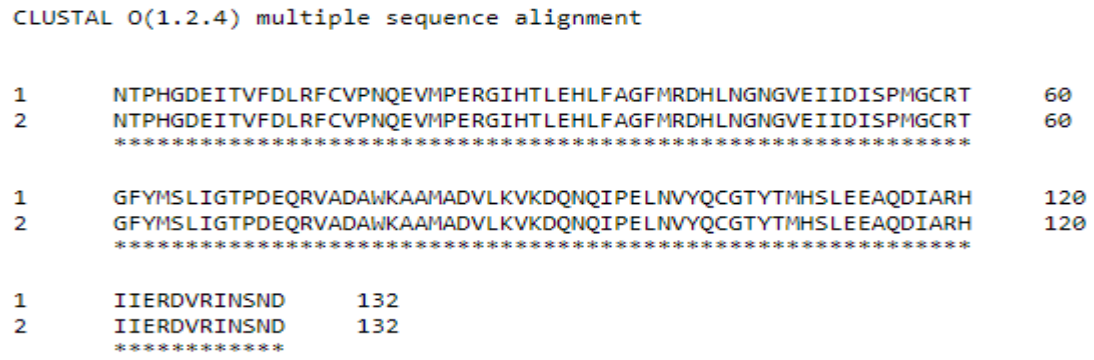

Figure 6: Alignment pattern for amino acids that showed all mutation were synonymous and no difference in amino acids

Aligning the sequence of *luxS* gene in isolate under study with *luxS* gene of reference genome using BLAST tool showed that there was no any difference in sequencing pattern Figure 7.

Score	Expect	Identities	Gaps	Strand
721 bits(390)	0.0	390/390(100%)	0/390(0%)	Plus/Plus

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Query  1        GAACACGCCGCATGGCGATGAAATCACCGTTTTCGATCTGCGCTTCTGCGTACCGAACCA  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150445  GAACACGCCGCATGGCGATGAAATCACCGTTTTCGATCTGCGCTTCTGCGTACCGAACCA  1150504

Query  61       GGAAGTGATGCCGGAACGCGGTATCCACACCCTGGAGCATCTGTTCGCGGGCTTTATGCG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150505  GGAAGTGATGCCGGAACGCGGTATCCACACCCTGGAGCATCTGTTCGCGGGCTTTATGCG  1150564

Query  121      CGATCATCTGAACGGGAATGGCGTGGAAATTATCGACATTTCGCCAATGGGCTGCCGCAC  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150565  CGATCATCTGAACGGGAATGGCGTGGAAATTATCGACATTTCGCCAATGGGCTGCCGCAC  1150624

Query  181      CGGCTTCTATATGAGCCTGATTGGTACGCCGGACGAGCAGCGCGTCGCTGACGCCTGGAA  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150625  CGGCTTCTATATGAGCCTGATTGGTACGCCGGACGAGCAGCGCGTCGCTGACGCCTGGAA  1150684

Query  241      AGCGGCGATGGCCGATGTGCTTAAGGTGAAAGATCAGAACCAGATCCCGGAGCTCAACGT  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150685  AGCGGCGATGGCCGATGTGCTTAAGGTGAAAGATCAGAACCAGATCCCGGAGCTCAACGT  1150744

Query  301      CTACCAGTGCGGGACTTACACCATGCACTCGCTGGAAGAGGCCCAGGACATCGCTCGCCA  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150745  CTACCAGTGCGGGACTTACACCATGCACTCGCTGGAAGAGGCCCAGGACATCGCTCGCCA  1150804

Query  361      TATCATTGAGCGCGATGTGCGCATCAACAG  390
               ||||||||||||||||||||||||||||||
Sbjct  1150805  TATCATTGAGCGCGATGTGCGCATCAACAG  1150834
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Figure 7: Alignment of luxS gene of isolate under study with reference genome

Discussion

Biofilm is the famous strategy constructed by the bacterial cells to face the killing effect of antimicrobial agents. The percentage of biofilm producers were 93.6% and 75% as reported by Shadkam *et al.* (2021) and Seifi *et.al* (2016) [20,51] respectively. However, in current study, low percentage of isolates was formed biofilm with 55%. The controversy between current study and previous studies in percentage of isolates forming biofilm return to many factors. Availability of *luxS* and *fimH* genes is considered as a one of the important factors that contributes in formation of biofilm. *luxS* is a quorum sensing gene that help in formation signal molecules engaged in formation of biofilm, in addition *fimH* encode for type 1 fimbriae that contributes in biofilm formation through binding with epithelial cells in lung and urinary tract system in our body. It has been reported that absence of *luxS* gene in isolates lead to reduce in colonization of bacterial cells hence reduce the possibility of strains to construct biofilm [52]. Furthermore, the absence of the fimH gene in the isolates was found to significantly impede the binding of bacterial cells to epithelial cells in both the lung and urinary tract systems, thereby compromising the isolates' ability to form biofilms [46]. In current study, low percentage of isolates form biofilm because *luxS* and *fimH* genes were absence in 70% and 100% of strains respectively while Shadkam *et al.,* (2021) showed that 98% of isolates harbored *luxS* gene therefore most of isolates form biofilm [20]. Hammad *et al.* (2020) showed that most of isolates with 75% harbored *luxS* gene were able to form biofilm compared with 25% of isolates that were missing to *luxS* gene and were unable to form biofilm [4]. Shivaee *et al.* (2019) showed that most of isolates formed biofilm with 80% due the prevalence of fimH gene with 92% [53]. Shadkam *et al.,* (2021) and Subramanian et al, (2008) revealed that most of isolates were resistance to antimicrobial agents were able to form biofilm [20,54]. However, in current study most of isolates were resistance to antimicrobial agents were unable to form biofilm, this resistance to antimicrobial in our isolates due to other mechanisms but does not relate with formation of biofilm. Shadkam *et al.,* (2021) showed that resistance to antibiotics IPM, AN and CAZ were 34%, 10.4% and 43% while Moghadas *et al.* (2018) showed that

resistance to antibiotics IPM, AN and CAZ were 7.5%, 36% and 42% [20,55]. Resistance to antibiotics IPM, AN and CAZ were 0%, 10% and 60% in current study which was compatible with results of [20,55]. However, P-values were significantly higher in resistance isolates with 0.0001 and 0.0597 compared with percentage of sensitive isolates for Colistin and Ceftazidime respectively and this due to other mechanisms but does not relate with construction of biofilm. The effect of green tea on biofilm formation showed that all isolates resistance to green tea were able to form biofilm however, percent of sensitive isolates were significantly higher than resistance isolates with P-value 0.0108. This indicates that the green tea is a good therapeutic agent against *K. pneumonia* isolates. However, Aponte (2018) showed that green tea has no significant effect on *K. pneumonia* isolates. Sequencing pattern for *luxS* gene showed that difference in DNA sequence between isolate No 5 and 6 were synonymous changes and does not lead to amino acid changes and we thing this was because both isolates were biofilm producer and we did not compare between sequencing of *luxS* gene in isolate forming and non-forming biofilm.

Conclusion

In the present study, it was concluded that the possibility of *K. pneumonia* strains to construct biofilm and resist different antimicrobial agents correlated with the availability of *luxS* and *fimH* genes. The *luxS* gene was highly conserved among *K. pneumonia* isolates when we compared their DNA sequence with sequencing of reference genome available database of NCBI.

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