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Study on *tssC1* Gene Mediating Biofilm Antibiotics Resistance of *Pseudomonas aeruginosa*

Shaymaa Fouad Rasheed Al-Khazraji
Biology department, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

P. aeruginosa is a famous bacterium that causes several diseases and has a high ability to be a multidrug resistant organism that is linked with the formation of biofilm. This study aimed to investigate *tssC1* gene role in the resistance of different antibiotics in the presence of biofilm. We constructed biofilm for the isolates under the study and showed the effect of different antibiotics on biofilm formation and maturation. The presence of the gene was detected through achieving PCR reaction. Finally, *tssC1* gene variation was determined through sequencing and aligning the sequencing products. The results showed that most of the isolates (80%) formed biofilm that played a role in the resistance of different antibiotics which could be due to the presence of *tssC1* gene. However, the genic variation of *tssC1* gene showed that no variation was detected. Therefore, we think this gene has no a role in the resistance of antibiotics and that the resistance may have been raised by other mechanisms found in *P. aeruginosa* isolates. This led us to conclude that the *tssC1* gene does not contribute to the resistance of antibiotics through biofilm.

Key word: *tssC1* gene, *P. aeruginosa*, Biofilm, Antibiotics resistance

دراسة جين *tssC1* لمتوسط مقاومة الغشاء الحيوي للمضادات لبكتريا الزائفة الزنجارية في العراق

الوسيط في مقاومة الغشاء الحيوي للمضادات في بكتريا الزائفة الزنجارية *tss C1* دراسة جين

شيماء فؤاد رشيد الخزرجي
قسم علوم الحياة ، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة :

الزائفة الزنجارية هي بكتيريا شهيرة تسبب العديد من الأمراض ولها قدرة عالية على أن تكون كائنًا مقاومًا للأدوية المتعددة عبر تكوين الأغشية الحيوية. هدفت هذه الدراسة إلى معرفة دور جين *tssC1* في مقاومة المضادات الحيوية المختلفة في وجود الأغشية الحيوية. تم تكوين غشاء حيوي للعزلات قيد الدراسة وأظهرنا تأثير المضادات الحيوية المختلفة على تكوين ونضج الاغشية الحيوية .تم الكشف عن وجود الجين من خلال تفاعل البلمرة. اخيرا أظهرت النتائج أن معظم العزلات التي تحتوي على 80٪ غشاء حيوي كان لها دور في مقاومة المضادات الحيوية المختلفة وقد تكون هذه المقاومة المقاومة بسبب وجود *tssC1*. مع ذلك ، لم يظهر التباين الجيني لجين *tssC1* اي اختلاف، لذلك يعتقد أن هذا الجين ليس له دور في مقاومة المضادات الحيوية

*Email: sh_frbio@yahoo.com

التي من الممكن ان تعزى الى اليات اخرى موجودة في عزلات *P. aeruginosa* وان جين*tssC1* لا يساهم في مقاومة المضادات الحيوية عبر تكوين الأغشية الحيوية.

Introduction

P. aeruginosa is a major causative agent for many diseases in immunocompromised patients; it is an opportunistic pathogen with multiple mechanisms for antibiotic resistance and biofilm formation [1]. *P. aeruginosa* causes morbidity and mortality which is related with many diseases such as respiratory and urinary tracts and wounds infection [2]. The persistent success of bacteria for long time in host relies on the its ability to evolve to resist the stress condition [3]. The behavior of this bacterium is different in early stages and is comparable with chronic infection. In the beginning of infection *P. aeruginosa* is coded for high level of virulence factors whereas in chronic infection it reduces the expression for the virulence factor [4]. The chronic infection caused by *P. aeruginosa* is correlated with their ability to form biofilm and the presence of different mechanisms for antibiotic resistance [5]. Due to *P. aeruginosa* presence, antibiotic resistance forms major problem for people suffering from cystic fibrosis (CF). This resistance can be acquired through mutation or acted by drug efflux pumps and low outer membrane permeability [6]. It has been observed that hyper-mutation isolates of *P. aeruginosa* are found in CF patients and are highly resistance to different antibiotics which suggests that there is relation between hyper-mutation and antibiotic resistance [7]. In CF patients, the antibiotic resistance is raised due to the ability of *P. aeruginosa* to form biofilm [8]. Biofilm of *P. aeruginosa* isolates can provide good example of within-population diversification that leads to acquiring antibiotic resistance and resist the stress condition [9]. *P. aeruginosa* biofilm shows resistance for immune system attack as well as third and fourth generation of antibiotics such as cephalosporins and carbapenems.

This biofilm is mainly formed by different exopolysaccharides which are alginate *Psl* and *Pel* genes that help in making scaffolds of biofilm [10, 11]. Biofilm is associated with urinary catheters and 449 patients were affected by this phenomenon while biofilm correlated with60–70% of nosocomial infections [12]. Different genes in *P. aeruginosa* play an important role in making exopolysaccharide for biofilm, like *Psl* and *Pel* [13]. Understanding how this bacterium evolves in CF and how the genome is changed over time may help in finding a good remedy for this disease [14]. However, due to the availability of adaptive and acquired resistance mechanisms and biofilm formation in *P. aeruginosa* pathogen, the treatment of the disease correlated with this bacterium becomes hard to achieve [15, 16]. It is not clear whether the *tssC1* gene plays an essential role in the resistance of antibiotics in the presence of biofilm. Hence, this study aimed to evaluate the direct effect of *tssC1* gene on resistance to antimicrobials through biofilm which could be achieved through the study of the correlation between the *tssC1* gene presence and the bacteria ability to form biofilm. In addition, study of the variation in *tssC1* gene sequence also gives an indication of the antimicrobial resistance in biofilm formation.

Materials and Methods

Isolates Under the Study

Different samples were collected from various hospital located in Baghdad city. Relying on morphological and biochemical tests, 20 strains of *P. aeruginosa* were isolated from the samples. MacConkey, blood and cetrimide agars were used to culture the isolates from the collected samples. Further identification was carried out using biochemical tests with indol, motility, oxidase, catalase and fermentative tests [25].

Biofilm Construction Assay

Bacterial suspension was prepared by mixing 20 µl (Bacterial culture was adjusted to the turbidity of a 1 McFarland standard) of each 20 *P. aeruginosa* isolates with 180 µl tryptic soy broth. Later 0.25% glucose was added to the suspension and was then kept rest for 24 h at 37^0C [17]. The suspension was then loaded into 96 wells of microtitre plate and incubated at 37^0C for 48 h. Control was also loaded in the wells which contained broth without inoculation. Unbound cells were removed by inverting the plate and washing it with distilled water. After that crystal violet with 0.1% and 125 µl was used to stain fixed cells on the wells for 5 min. Distilled water was used to wash the stain then the wells were left to dry. The optical density (OD) was measured for the bound cells in each well by ELISA reader with 630 nm wavelength before strong, moderate and weak biofilm producing isolates were detected relying on [18] as follows: three standard deviations plus mean O.D. to the negative control represented the O.D.c while O.D of samples of each isolate averaged and subtracted from O.D of control and represented as ODi. (ODi<ODc) referred to non-biofilm producer, (ODc<ODi_2_ODc) referred to weak biofilm producer, (2_ODc<ODi _4_ODc) referred to moderate biofilm producer and (4_ODc<ODi) referred to strong biofilm producer.

Effect of Antibiotic on Biofilm Maturation

Microbial bactericidal concentration (MBC) was determined for bound cells in biofilm construction using the following [19, 20]. Six *P. aeruginosa* isolates (2 non-biofilm producers, 2 weak-biofilm producers and 2 moderate-biofilm producers) were used to construct biofilm as mentioned previously. Then, the antibiotic was diluted as serial dilutions as following: gentamicin started at 12.5 mg/ml till 800 mg/ml, ciprofloxacin started at 2.5 mg/ml till 160 mg/ml and tobramycin started at 6.25 mg/ml till 400 mg/ml. The diluted antibiotics with different concentrations were later added to bound cells as biofilm in wells and were later kept aside for 24 h. Finally, live bacteria were determined by culturing a small amount of bound cells in biofilm on the nutrient agar plates.

Effect of Antibiotic on Biofilm Production

Determining the effects of different antibiotic concentrations on biofilm production was achieved [21] as following: Tryptic soy broth with 100 µl was mixed with bacterial suspension of bacterial cells with 20 µl for 6 *P. aeruginosa* isolates (2 non-biofilm producers, 2 weak-biofilm producers and 2 moderate-biofilm producers) and were then added to 96 wells of microtitre plates. Next100 µl of each antibiotic with serial dilution (2.5 to 160 mg/ml for ciprofloxacin, 12.5 to 800 mg/ml for gentamicin and 6.25 to 400 mg/ml for tobramycin) was also added to 96 wells of microtitre plates. After incubation at 37^0C for 24 h, the content of wells was removed and stained with crystal violet with a volume of 125 µl and concentration of 0.1% for 10–15 min. The OD was taken at 573 nm using ELISA reader for each well, and then strong, moderate and weak biofilm producers were detected as mentioned previously.

This work was carried out to check if 6 *P. aeruginosa* isolates (2 non-biofilm producers, 2 weak-biofilm producers and 2 moderate-biofilm producers) were able to produce biofilm in presence of different concentrations of different antibiotics.

Detection and Sequencing of *tssC1* Gene and Bioinformatics Analysis:

The purpose of achieving tssC1 gene sequencing was to detect the genic variation for the non, weak and moderate biofilm producers so that we could have profile for each sequence related with non, weak and moderate biofilm producers. DNA was extracted from *P. aeruginosa* isolates. The extraction was achieved on 2 moderate-biofilm producers, 2 weak-biofilm producers and 2 non-biofilm producers by genomic DNA mini extraction kit. The primers used

in PCR reaction (designed using primer quest tools) were forward primer: CGAATTGAGCACCGAGAA and reverse primer: TTGAAGGAGCGGTTGATG for the purpose of amplification of *tssC1* gene. In the PCR reaction, the following substances were added with final volume of 50 µl which were 18µl distilled water, 25 master mix, 5µl with a concentration of 10ng bacterial DNA template, 1 µl with concentration of 0.5 µM forward primer and 1 µl reverse primer. PCR was carried out with one cycle of initial denaturation at 95^0C for 2 min, followed by 30 cycles with denaturation at 95^0C for 1 min, annealing at 60^0C for 1 min and extension at 72^0C for 90 s. The final extension was achieved at 72^0C for 10 min and then the PCR product was visualized on gel electrophoresis with 1% concentration of agarose and ethidium bromide was used for staining. ABI 3730 DNA Sequencer was used to sequence all the DNA extracted from 6 *P. aeruginosa* isolates. BLAST tool was used for searching the database and achieving alignment with reference genome, while alignment of the isolates under the study was carried out using BioEdit program.

Statistical Test

The analysis of the categorical data was achieved using chi-square test to compare the different proportions [22].

Results

The biofilm formation results showed that 20% isolates do not produce biofilm while 80% were biofilm producers with 45% weak-biofilm producers and 35% moderate biofilm producers (Figure1). The biofilm producing isolates were highly significant with P-value=P = 0.0002 compared with non-biofilm producing isolates.

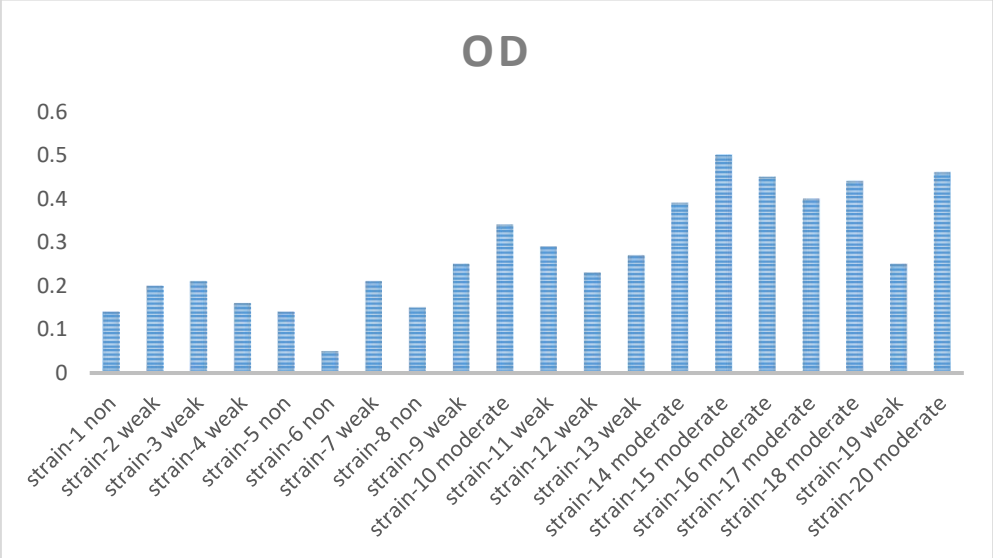

Figure1: Optical density for isolates formed biofilm and isolates did not form biofilm with 630 nm wavelength

Effects of the different antibiotics on biofilm production showed that all three antibiotics induced non-biofilm producing isolates to form weak biofilm with different concentrations of antibiotics. However, different antibiotics inhibit biofilm formation in weak and moderate biofilm producers. There was no significant difference with P-value=0.2 for comparing proportion of isolates with inhibition of their biofilms with proportion of isolates with induction of their biofilms after exposure to antibiotics treatment. The results of MBC for biofilm maturation revealed that MBC was the highest in strain – 6 non biofilm producers with 400 comparable with other strains using tobramycin antibiotic. On the other hand, the highest MBC

was detected against strain-15 moderate biofilm producers with 400 using gentamycin antibiotic. Furthermore, highest MBC was detected against strain-12 as being weak biofilm producer and strain-15 moderate as biofilm producer with 80 using ciprofloxacin antibiotic (Table1).

Table 1: The effect of different concentrations of antibiotics on production and maturation of biofilm: (↓): inhibition, (↑): induction

	Effected Concentration of Tobramycin	Effected Concentration of Tobramycin	Effected Concentration of Gentamicin	Effected Concentration of Gentamicin	Effected Concentration ofCiprofloxacin	Effected Concentration of Ciprofloxacin
Strains	**Biofilm production**	**MBC-biofilm maturation**	**Biofilm production**	**MBC-biofilm maturation**	**Biofilm production**	**MBC-biofilm maturation**
Strain-8 (non-biofilm producer)	**6.25 ↑**	**25**	**50 ↑**	**12.5**	**5 ↑**	**40**
Strain-6 (non-biofilm producer)	**12.5 ↑**	**400**	**100 ↑**	**200**	**10 ↑**	**10**
Strain-12 (weak-biofilm producer)	**6.25 ↓**	**100**	**12.5 ↓**	**50**	**5 ↓**	**80**
Strain-19 (weak-biofilm producer)	**6.25 ↓**	**25**	**12.5 ↓**	**200**	**2.5 ↓**	**40**
Strain-15 (moderate-biofilm producer)	**6.25 ↓**	**100**	**12.5 ↓**	**400**	**2.5 ↓**	**80**
Strain-16 (moderate-biofilm producer)	**6.25 ↓**	**200**	**12.5 ↓**	**200**	**5 ↓**	**10**

None, weak and moderate biofilm producing isolates of 6 *P. aeruginosa* were subjected to tobramycin, gentamycin and ciprofloxacin antibiotics, and then the extracted DNA from 6 *P. aeruginosa* was amplified by PCR. The bands of DNA for *tssC1* gene were detected in most of the isolates (Figure2).

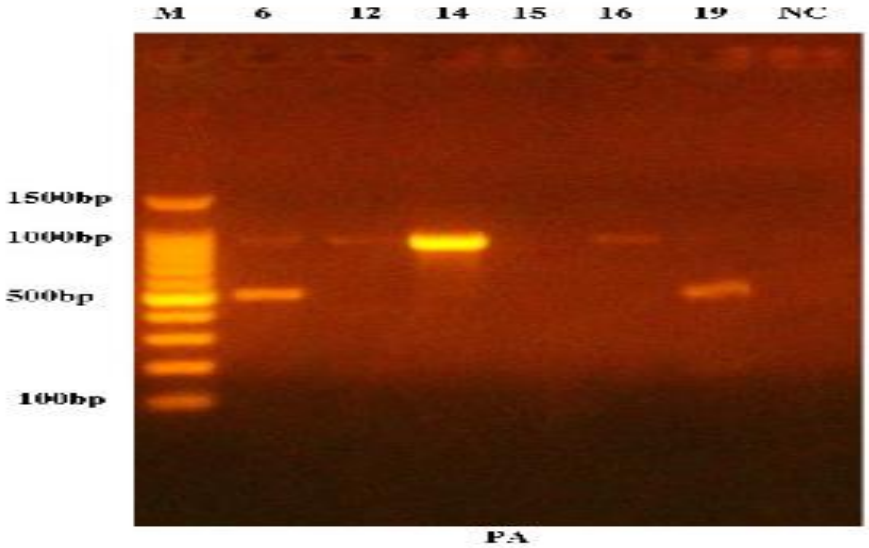

Figure 2: Gel image for *tssC1* gene with size of 879 bp. Lane 1 the ladder, Lanes numbers 6, 12, 8, 15, 16 and 19 show bands for different isolates, last lane: Negative control.

To confirm if the deletion occurred within the *tssC1* gene due to the exposure of the isolates to different concentrations of antibiotics, the *tssC1* gene sequencing was achieved. The results revealed that *tssC1* gene was not detected in isolates 15 and 19.

The genic variation of *tssC1* gene showed that our isolate did not vary in their genomic sequence (Figure3).However, comparison of the genic sequence of our isolates with reference genome which was taken from database showed that there were variations in three positions. In positions 804 and 805 GC converted to CG and there was deletion in one base pair at position 808 (Figure 4).

Figure 3: Alignment pattern between isolates 14 and 16 under study. No genomic variation was detected among the genic of our isolates

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Query  1       GCCTGCTGCTGCAGGAGTTCAAGCCCAAGACCGAGCGCGCCCGCGAAGCGGTGGAGACCG  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99731   GCCTGCTGCTGCAGGAGTTCAAGCCCAAGACCGAGCGCGCCCGCGAAGCGGTGGAGACCG  99790

Query  61      CCGTGCGGACCCTCGCCGAGCATGCCCTGGAGCAGACCAGCCTGATCTCCAACGACGCGA  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99791   CCGTGCGGACCCTCGCCGAGCATGCCCTGGAGCAGACCAGCCTGATCTCCAACGACGCGA  99850

Query  121     TCAAGTCGATCGAGTCGATCATCGCGGCGATCGACGCCAAGCTCACCGCGCAGGTCAACC  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99851   TCAAGTCGATCGAGTCGATCATCGCGGCGATCGACGCCAAGCTCACCGCGCAGGTCAACC  99910

Query  181     TGATCATGCACCACGCCGACTTCCAGCAACTGGAAAGCGCCTGGCGCGGCCTGCACTACC  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99911   TGATCATGCACCACGCCGACTTCCAGCAACTGGAAAGCGCCTGGCGCGGCCTGCACTACC  99970

Query  241     TGGTCAACAACACCGAGACCGACGAGCAACTGAAGATCCGCGTGCTGAACATCTCCAAGC  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99971   TGGTCAACAACACCGAGACCGACGAGCAACTGAAGATCCGCGTGCTGAACATCTCCAAGC  100030

Query  301     CGGAGCTGCACAAGACCCTGAAGAAATTCAAGGGCACCACCTGGGACCAGAGCCCGATCT  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100031  CGGAGCTGCACAAGACCCTGAAGAAATTCAAGGGCACCACCTGGGACCAGAGCCCGATCT  100090

Query  361     TCAAGAAGCTCTACGAAGAGGAATACGGCCAGTTCGGCGGCGAGCCCTATGGCTGCCTGG  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100091  TCAAGAAGCTCTACGAAGAGGAATACGGCCAGTTCGGCGGCGAGCCCTATGGCTGCCTGG  100150

Query  421     TCGGCGACTACTACTTCGACCAGTCGCCGCCGGACGTCGAGCTGCTCGGCGAGATGGCGA  480
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100151  TCGGCGACTACTACTTCGACCAGTCGCCGCCGGACGTCGAGCTGCTCGGCGAGATGGCGA  100210

Query  481     AGATCTCCGCCGCCATGCACGCGCCGTTCATTTCCGCCGCCTCGCCGACGGTGATGGGCA  540
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100211  AGATCTCCGCCGCCATGCACGCGCCGTTCATTTCCGCCGCCTCGCCGACGGTGATGGGCA  100270

Query  541     TGGGTTCCTGGCAGGAACTGTCCAACCCGCGCGACCTGACCAAGATCTTCACCACCCCGG  600
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100271  TGGGTTCCTGGCAGGAACTGTCCAACCCGCGCGACCTGACCAAGATCTTCACCACCCCGG  100330

Query  601     AATACGCCGGCTGGCGTTCGCTGCGCGAGTCCGAGGACTCCCGCTACATCGGCCTGACCA  660
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100331  AATACGCCGGCTGGCGTTCGCTGCGCGAGTCCGAGGACTCCCGCTACATCGGCCTGACCA  100390

Query  661     TGCCGCGCTTCCTGGCGCGCCTGCCCTACGGGGCGAAGACCGATCCGGTGGAAGAGTTCG  720
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100391  TGCCGCGCTTCCTGGCGCGCCTGCCCTACGGGGCGAAGACCGATCCGGTGGAAGAGTTCG  100450

Query  721     CCTTCGAGGAAGAAACCGACGGCGCCGACAGCAGCAAGTACGCCTGGGCCAACTCGGCCT  780
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100451  CCTTCGAGGAAGAAACCGACGGCGCCGACAGCAGCAAGTACGCCTGGGCCAACTCGGCCT  100510

Query  781     ACGCGATGGCGGTCAACATCAACGCCTTCCTTCAA  815
                |||||||||||||||||||||||  || |||||||
Sbjct  100511  ACGCGATGGCGGTCAACATCAACCGCT-CCTTCAA  100544
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Figure 4: Alignment pattern between isolate 16 under study with reference genome which was taken from database. Genic variation was detected.

Discussion

80% of the 20 *P. aeruginosa* isolates formed biofilm and this result agrees with Kamali e*t al.*[23] who showed that 83.75% of the isolates formed biofilm. Therefore, we think that these isolates have high pathogenicity. As far as the effect of three antibiotics on biofilm production is concerned, the three antibiotics inhibited formation of the biofilm with very low doses for biofilm producing isolates. This result indicated that the three antibiotics are effective against the bacterial cell and inhibit formation of biofilm. However, the three antibiotics induced the formation of biofilm for non-biofilm producing isolates which may indicate that the non-biofilm producing isolates formed biofilm to resist the effects of antibiotics. Similar results were observed by Uzunbayir-Akel *et al.*[21]who showed that the ciprofloxacin effect on biofilm production inhibited 70% from isolates and induced 30% from isolates to form biofilm. For the effect of three antibiotics on biofilm maturation, in general, MBC for the gentamycin and ciprofloxacin antibiotics for the isolates forming biofilm under the study was very high. This means that high doses of antibiotic are required to destroyed the biofilm and kill the bacteria. Therefore, biofilm has essential role in survival of the isolates. Similar result showed that the biofilm played a role in resistance to antimicrobial and survival of the isolates [24]. The result of PCR analysis showed that the prevalence of *tssC1* gene was 66.6% in biofilm and non-biofilm producing isolates in spite of we subjected the isolates to different concentrations of antibiotics before detecting the presence of the gene. However, Saffari *et al.*[25] showed that 90.2% of the isolates carried *tssC1* gene. We thought that MBC for the gentamycin and ciprofloxacin antibiotics for the isolates forming biofilm was very high due to the presence of *tssC1* gene that has a role in antibiotic resistance mediating biofilm formation. However, the genic variation of *tssC1* gene showed there was not any variation through comparing isolates highly resistant to antibiotic and moderate biofilm producers (ID 16) with isolates low in resistance to antibiotic with non-biofilm producers (ID 14) which may imply there was no role for *tssC1* gene in antibiotic resistance mediating biofilm formation. Nevertheless, the variation may be in the promoter region of *tssC1* gene which may result in increased expression for isolate with ID 16 comparable with isolate with ID 14. Zhang *et al.* [26] showed that the level of expression for tssC1 gene was high in biofilm forming isolates comparable with planktonic cell. In addition, the results showed that there was genetic variation in *tssC1* gene through comparing isolate (ID 16) with the database. This may indicate this variation is important in antibiotic resistance mediating biofilm formation for *tssC1* gene.

Conclusion

This study indicated that despite most biofilm forming isolates were able to resist different antibiotics under the study. However, we think that the resistance in *P. aeruginosa* is due to the presence of many mechanisms and can rarely be correlated with the presence of *tssC1* gene that introduces the resistance to the antibiotic through biofilm formation.

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