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Effect of prodigiosin in biofilm formation of its producing bacterial isolates

Nagham Shakir Alattar, Heba Khaleel Tawfeeq, Jinan Mohammed Hasan*
Department of Biology, College of science, University of Baghdad, Baghdad, Iraq

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Abstract

Serratia marcescens is gram negative bacteria, an opportunistic and nosocomial pathogen. Some strains possess the ability to produce a pigment called prodigiosin. While numerous studies have explored the antimicrobial properties of prodigiosin produced by *S. marcescens* against other microorganisms, the effect of prodigiosin on the producing *S. marcescens* isolates themselves remains unclear and warrants further investigation. Ten isolates of *S. marcescens* were isolated from 135 wound samples collected from some hospitals in Baghdad city. The isolates were cultured on selective media, identified by further biochemical tests and confirmed by VITEK2 compact system in (7.4%) isolation percentage. Four from the ten isolates were pigment producers. The ability of the bacterial isolates to form biofilm was assessed to the bacterial isolates by microtiter plate method; all isolates were biofilm producers. Prodigiosin's effect on the biofilm development of the producer isolates of *S. marcescens* was examined depending on incubation in different temperature (37 and 30ºC) the results revealed reducing in their biofilm layers thickness. DNA was extracted from the four isolates to investigate the presence of four genes (*flhD*, *fimC*, *bsmA* and *bsmB*) related to the formation of biofilms, the results showed that the four isolates had all four genes (*flhD*, *fimC*, *bsmA* and *bsmB*). Prodigiosin effect on the expression of four genes was examined and the results showed an up regulation in gene expression in all isolates which indicates a relation between prodigiosin production and biofilm formation in *S. marcescens*.

Key words: Biofilm, Prodigiosin, *Serratia marcescens*, Wound infections, *flhD* gene

تأثير البروديجيوسين على تكوين الاغشية الحيوية في عزلاته المنتجة

نغم شاكر العطار, هبة خليل توفيق , جنان محمد حسن*
قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد, العراق

الخلاصة

Serratia marcescens هي بكتيريا سالبة لصبغة الجرام، وهي من مسببات الأمراض الانتهازية والامراض المكتسبة من المستشفيات. تمتلك بعض السلالات القدرة على إنتاج صبغة تسمى البروديجيوسين. فيما تبحث العديد من الدراسات في الفعاليه الضد مايكروبية للبروديجيوسين من *S. marcescens* وخاصة نشاطها المضاد للميكروبات تجاه الكائنات الحية الدقيقة الأخرى، لكن تأثير البروديجيوسين في العزلات المنتجة لها لا يزال غير محدد ويتطلب اجراء بحوث اضافية . تم عزل عشر عزلات من *S. marcescens* من 135 عينة جروح تم جمعها من بعض المستشفيات في مدينة بغداد بعد زراعتها على أوساط انتقائية وتم تشخيصها من خلال اختبارات كيميائية حيوية إضافية وتم تأكيدها بواسطة نظام VITEK2 بنسبة عزل (7.4٪). أربع من

*Email: Ganan.hasan@sc.uobaghdad.edu.iq

العزلات العشر كانت منتجة للصبغة. تم تقييم تكوين الأغشية الحيوية للعزلات البكتيرية باستعمال طريقه لوحة المايكروتيتر.
وكانت جميع العزلات منتجة للأغشية الحيوية. تم دراسة تأثير البروديجيوسين على تطور الأغشية الحيوية لعزلات منتجه من *S. marcescens* اعتمادا على درجات حضانة مختلفة (37 و 30 درجة مئوية) وأظهرت النتائج انخفاض في سمك طبقات الأغشية الحيوية الخاصة بها. تم استخلاص الحمض النووي من العزلات الأربع للتحقق من وجود أربعة جينات (*flhD*، *fimC*، *bsmA* و *bsmB*) المتعلقة بتكوين الأغشية الحيوية، أظهرت النتائج أن العزلات الأربع تحتوي على جميع الجينات الأربعة (*flhD*، *fimC*، *bsmA* و *bsmB*). تم فحص تأثير البروديجيوسين على التعبير الجيني لأربعة جينات وأظهرت النتائج زيادة في التعبير الجيني في جميع العزلات مما يدل على وجود علاقة بين إنتاج البروديجيوسين وتكوين الأغشية الحيوية في *S. marcescens*.

Introduction

Over the past four decades, *Serratia marcescens* has emerged as a notable healthcare-associated pathogen. There is a real cost associated with this organism incurs significant costs in terms of patient morbidity and antibiotic usage. This naturally occurring gram-negative bacillus can be found in water, soil, and at room temperature it produces a red pigment known as prodigiosin [1, 2]. This bacterium belongs to the family Enterobacteriaceae and is frequently linked to serious drug resistance. Since 1960, the number of *S. marcescens* infections has been rising, and the bacteria have been linked to a broad range of diseases, comprising sepsis and skin/soft tissue infections [3, 4], septicemia, wound, osteomyelitis, ocular, respiratory infections and endocarditis, as well as meningitis. Transmission occurs by direct contact with infected person [5, 6].

This pathogen is a DNase-producing, oxidase-negative bacterium capable of thrive in harsh environments [7, 8]. The bacteria secrete several enzymes, including hemolysin, proteases, nucleases, and lecithinases, which may are virulence factors contributing to its pathogenicity [9, 10]. A wider range of species are experiencing an increase in antibiotic resistance [11]. It has been documented to be effective against multiple classes of antimicrobials, such as aminoglycosides, polymyxins, and penicillins (first- and second-generation cephalosporins, carbapenem resistance mediated by β-lactamase). Due to its resistance to various antibiotics, treating infections caused *S. marcescens* can be challenging [12, 13]. Colored *S. marcescens* are strains that produce prodigiosin, which turns the bacteria red. Non-pigmented *S. marcescens*, on the other hand, do not have this characteristic [7, 14].
S. marcescens, a human pathogen, normally secretes prodigiosin, a red tripyrrole bacterial pigment, as a secondary metabolic product in the idiophase of bacteria. The three pyrrole rings that make up prodigiosin are A, B, and C. While the B and C rings are joined in a dipyrrin, the A and B rings are both bridged in a bipyrrole unit. A methylene bridge joins the monopyrrole moiety (C ring) to the methoxy bipyrrole moiety (A and B rings) [15, 16]. The bioactivity and color make this pigment a promising food colorant, antibacterial, and antifungal agent for use in textiles. Specifically, prodigiosin has demonstrated high apoptotic activity in many cancer cell lines both in vivo and in vitro, and low cytotoxicity in noncancerous cells; further investigation into this pigment may lead to the discovery of new anticancer medications [17, 18].

"A community of microorganisms attached to a suitable surface" is the definition of biofilm [19]. The extracellular polymeric substances (EPS), also known as the biofilm matrix, which make up roughly 90% of the biofilm's dry mass, regulates the formation and persistence of the biofilm. Microorganisms are shielded by their matrices from host immune defenses, chemical toxins, and protozoan grazers. Additionally, biofilm matrix, primarily composed of

polysaccharides, lipids, and nucleic acids, permits the diffusion of oxygen, nutrients, and waste products [20, 21]. *S. marcescens* depends on the quorum-sensing system to create biofilms by going through a series of unique steps that produce a highly porous, filamentous biofilm composed of clusters, filaments, and chains of cells [22]. An increase in population density activates a gene regulatory system called quorum sensing. *S. marcescens* produced quorum sensing via acyl homoserine lactone (AHL), which controls the formation of biofilms, swarming motility, and prodigiosin production [23]. It is common knowledge that quorum sensing (QS) contributes to bacterial pathogenicity, antibiotic resistance, and biofilm formation. Thus, QS inhibition may lower the chance of microbial pathogenicity in both local and systemic infections [24].

This study aims to: isolation and identify of *S. marcescens* from wound; detection the ability of isolates to produce prodigiosin, detection the ability of isolates to biofilm formation, and examined the effect of prodigiosin on *S. marcescens* biofilm.

Materials and Methods

1- **Bacterial isolates**: About 135 specimens were collected from wound infection by sterile cotton swab from different hospitals Al- Yarmouk Teaching Hospital, Al- Kindi Teaching Hospital, and Baghdad Teaching Hospitals at Baghdad city, Iraq. These specimens were cultured on MacConkey agar and Nutrient agar for colonial morphology at 37 ºC for 18- 24 hrs. [25]. Further biochemical tests were performed and the diagnosis of the isolates was confirmed by Vitek-2 system.

2- **Prodigiosin production**: The non-lactose fermenter isolates, initially grown on MacConkey agar were sub-cultured on nutrient agar and incubated at 30 ºC (the optimum temperature for prodigiosin production) for 18- 24 hrs in order to detect their ability to produce pigment. The pigmented isolates were used in the further tests [26].

3- Effect of prodigiosin on biofilm formation: The capacity of the pigmented bacterial isolates to produce biofilm was determined using a microtiter plate assay [27]. Additionally, the effect of prodigiosin on biofilm formation was assessed depending on incubation in different temperatures (37 and 30 ºC).

The bacterial broth culture was diluted 1:100 in Tryptic soy broth (TSB) and added with 1% glucose before being placed into the 200 µl well, with the exception of the negative control well, which contains only TBS broth, in order to prepare the inoculum for biofilm formation. 200 µl of TSB supplemented with 1% glucose and at least three copies of each strain were added to each well. The inoculation plate lid was placed, and the culture was grown aerobically for 24 to 30 hours at 37 ºC in a static state. After three washes with sterile phosphate-buffered saline (PBS; pH 7.2), 150 µl of 1% crystal violet was added and allowed to sit at room temperature for 15 minutes. The excess stain should then be rinsed off with tap water. To re-solubilize the dye, 150 µl of (95%) ethanol was added to each well. Each well that has been dyed crystal violet is measured for optical density (OD) at 630 nm using a microtiter-plate reader (GloMax, Promega, USA). The following categories can be used to group the strains: When $OD \leq ODc$, no biofilm is formed; when $ODc < OD \leq 2\times ODc$, mild biofilm is formed; when $2\times ODc < OD \leq 4\times ODc$; and when $4\times ODc < OD$, strong biofilm is formed.

To assess the effect of prodigiosin on biofilm formation, the same protocol was followed. Two plates were incubated at 30ºC and 37 ºC for 24 hrs (taking into account providing the same conditions and additives for both plates so that the only variable is temperature). After incubation, all wells were washed, stained, and read.

4- **Molecular methods:**

1- Extraction of genomic DNA: DNA was isolated from *S. marcescence*, employing EasyPure® Genomic DNA Kit (Transgene®/China), OneTaq® 2X Master Mix (NEB® (England) and Qubit™ dsDNA HS Assay Kit (Thermo Fisher®/USA) as directed by the manufacturer.

2- PCR Amplification: The primers names (the source of all primers used in this study was Macrogen® (Korea)) for the four genes were detected *flhD*, *fimC*, *bsmA*, *bsmB* and *rpiU* as housekeeping gene and the sequence according to [28]. After being thawed at 4°C and vortexed to guarantee uniform contents, for a 100-mL PCR mixture, distributed maxima PCR premix (Intron, Korea) was made according to Table 1.

Table 1: PCR reaction tubes were used to amplify DNA in a thermocycler PCR. Gradient PCR was used to achieve the optimal PCR temperature and duration.

Name of primer	Sequence	Product size (pb)
flhD	**F:**TTGCCACTTCCGCTTTAACG **R:** TCTTTTCTTCGTCGGGCTAG	**155**
fimC	**F:**ACCAGCCGTTTCAACAACAA **R:** GTTTGTACGGTGCGATCTT	**138**
bsmA	**F:**TAGTCCGCACACTCATCGC **R:** GATCTCCTGCGCCTGTGC	**110**
bsmB	**F:**GCGGATGTGTATGCCTTCG **R:** GCCACGCATTTCTTCACTCA	**180**
rpiU	**F:**AAATCGGCGTTCCTTTCGTC **R:** GCTTACGGTGGTGTTTACG	**115**

3- Electrophoresis Following PCR: Utilizing agarose gel electrophoresis, the outcomes were confirmed. PCR was used to extract all of the DNA parameters. One gram of agarose was added to a 100 ml beaker containing 1X TBE buffer in order to make a 2% agarose gel. The agarose was stained with Red Safe Nucleic Acid Stain (10 mg/ml). After mixing the agarose to avoid bubbles, the mixture was left to cool to a temperature of 50 to 60°C.

4- RNA purification protocol method: The GENEzolTM TriRNA pure Kit from Bioer/ Japan was employed to extract the RNA from the *S. marcescence* isolates.

5- QRT-PCR, or quantitative real-time PCR: RT-PCR should be performed in a nuclease-free environment. The steps of RNA sample preparation, reaction mixture assembly, PCR, and subsequent reaction analysis should be completed using RealMODTM Green qRT-PCR Mix in different locations.

The levels of expression were measured using relative quantitation. The difference in cycle thresholds (ΔCT) and fold changes between the treated groups and each gene's calibrators were calculated [29]. The expression of 16SrRNA was used to standardize the results as shown below:

Folding =2-ΔΔCT

ΔΔCT =ΔCT Treated - ΔCT Control

ΔCT =CT gene - CT House Keeping gene

Results

1- **Bacterial isolates:** Ten isolates of *S. marcescens* were isolated from 135 wound samples collected from some hospitals (Al- Yarmouk Teaching Hospital, Al- Kindi Teaching Hospital, and Baghdad Teaching Hospitals) at Baghdad city, Iraq in Baghdad city after culturing on selective media and identified by further biochemical tests and confirmed by VITEK2 compact system in (7.4%) isolation percentage.

2- Prodigiosin production: Four of the ten isolates were pigment producers (S6, S7, S8 and S9) at 30°C. (Optimum temperature for prodigiosin production).

3- **Effect of prodigiosin on biofilm formation:** Four of *S. marcescence* isolates were detected for biofilm formation (S6, S7, S8 and S9) by using two microtiter plates the first one incubated in 37°C (no pigment was produced) Table 2.

Table 2: The biofilm development optical density (OD) value for *S. marcescence* isolates without prodigiosin production

No. of isolates	OD1	OD2	OD3	Average	Result
S6	**227**	**222**	**226**	**225**	**Moderate**
S7	**289**	**280**	**288**	**285**	**Strong**
S8	**257**	**259**	**258**	**258**	**Strong**
S9	**159**	**155**	**135**	**149**	**Moderate**

Table 3: The biofilm development optical density (OD) value for *S. marcescence* isolates with prodigiosin production

No. of isolates	OD1	OD2	OD3	Average	Result
S6	373	370	369	370	Strong
S7	400	399	397	398	Very Strong
S8	418	422	425	421	Very Strong
S9	290	287	285	287	Very Strong

4- **Detection of biofilm genes:** five genes were detected *flhD*, *fimC*, *bsmA*, *bsmB* and *rpiU* for the four isolates of prodigiosin producing *S. marcescens* (S6, S7, S8 and S9). The results show that these isolates had the four genes, figure (1) and figure (2).

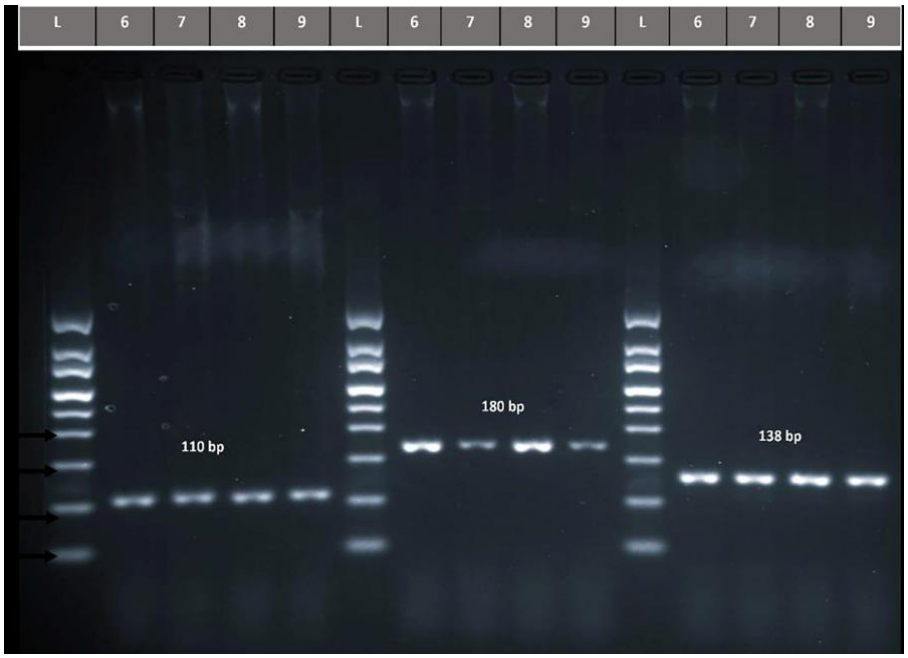

Figure 1: Gel electrophoresis of amplified PCR product of *fimC*, *bsmA*, *bsmB* for *Serratia marcescens*. 1.5% agarose gel electrophoresis stained with (Red Safe Nucleic Acid Stain (10 mg/ml), 100 v/m Amp for 75 min. TBE buffer (1X).

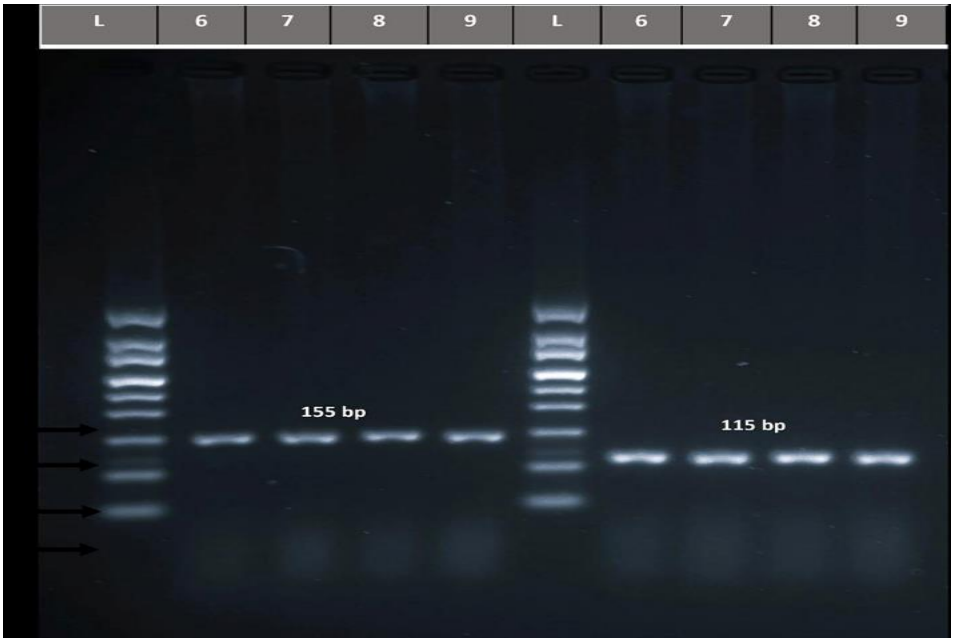

Figure 2: Gel electrophoresis of amplified PCR product of *flhD* and *rpiU* for *Serratia marcescens*. 1.5% agarose gel electrophoresis stained with (Red Safe Nucleic Acid Stain (10 mg/ml), 100 v/m Amp for 75 min. TBE buffer (1X).

5-Effect of prodigiosin on gene expression: To evaluate the effect of prodigiosin produced by of *S. marcescens* isolates on biofilm formation, gene expression of biofilm genes was detected with absence and presence of prodigiosin production, for *all* four genes. The results showed that an up regulation in gene expression of most genes in all isolates while there was a slight down regulation of *bsmB* and *fimC* in S9 and S8 isolates respectively (table 4).

Table 4: The effect of prodigiosin on gene expression of *flhD*, *fimC*, *bsmA*, *bsmB* genes in four isolates S6, S7, S8, S9.

Isolate code	pre			Post			ΔΔCt	Fold change	Result
	rplU	bsmB	ΔCt	rplU	bsmB	ΔCt			
S6	23.64	25.57	1.93	24.6	26.45	1.85	-0.08	1.057018	Increase
S7	23.65	25.83	2.18	24.3	26.44	2.14	-0.04	1.0281138	Increase
S8	23.03	34.56	11.53	22.79	30.53	7.74	-3.79	13.832596	Increase
S9	23.11	31.6	8.49	22.96	31.57	8.61	0.12	0.9201877	Decrease
								4.2094788	

Isolate code	pre			Post			ΔΔCt	Fold change	Result
	rplU	flhD	ΔCt	rplU	flhD	ΔCt			
S6	23.64	24.48	0.84	24.6	24.81	0.21	-0.63	1.547565	Increase
S7	23.65	24.73	1.08	24.3	25.1	0.8	-0.28	1.2141949	Increase
S8	23.03	24.03	1	22.79	23.77	0.98	-0.02	1.0139595	Increase
S9	23.11	24	0.89	22.96	23.2	0.24	-0.65	1.5691682	Increase
								1.3362219	

Isolate code	pre			Post			ΔΔCt	Fold change	Result
	rplU	fimC	ΔCt	rplU	fimC	ΔCt			
S6	23.64	25.06	1.42	24.6	25.83	1.23	-0.19	1.1407637	Increase
S7	23.65	25.39	1.74	24.3	25.8	1.5	-0.24	1.1809927	Increase
S8	23.03	24.68	1.65	22.79	24.5	1.71	0.06	0.9592641	Decrease
S9	23.11	24.76	1.65	22.96	24.5	1.54	-0.11	1.0792282	Increase
								1.0900622	

Discussion

Prodigiosin ($C_{20}H_{25}N_3O$) is a water- insoluble red pigment produced by *Serratia marcescens*, its name derived from "prodigiosine" a red extracted from *Bacillus prodigiosus* [30, 31]. Because the relevant puplication based on individual case report, guidelines for treatment of infection caused by *Serratia marcescens* do not exist [32]. One of the most urgent issues facing public health is bacterial resistance to antibiotics. Biofilms are hotspots to evolution which strong preference favors resistance to microbes, requiring new strategies for biofilm control [33, 34]. This sensitivity may be attributed to the structure of exopolysaccharides and a decrease in metabolic activity [35].

Both pigmented and non-pigmented strains of *S. marcescens* are extensively dispersed throughout environment. A number of non-pigmented varieties not possess any *pig* genes and in some cases, the entire gene cluster, according to the GeneBank databases sequence data [36, 37]. The findings suggest that *S. marcescens* inhibits high-cost processes, such as prodigiosin biosynthesis, but initiates amino acid metabolite and transport systems for additional resources, which support growth advantage in lab settings and the non-pigmented morphotypes exhibited a competitive growth advantage. .

A viable strategy to increase the dissemination of antibiotics and other antimicrobial agents into the biofilm's base, encourage the removal of bacterial cells, and ultimately control infections is to disintegrate the biofilm matrices [38, 39]. Prodigiosin and related artificial substances, such as electron-rich pyrrolylpyrromethene structures, can be included in the group of substances that may disrupt biofilms [40, 41]. On a range of clinical (biomaterials, medical devices) and non-clinical (water membrane filters) related substrata, prodigiosin could be used as an antibacterial coating agent. As a potential treatment, prodigiosin aerosols may help break down eDNA and reduce the viscosity of phlegm in cystic fibrosis patients' lungs. One main offender that makes sputum more viscous and, as a result, seriously obstructs airways and inhibits easy breathing in cystic fibrosis patients is eDNA [20]. While numerous studies have explored the impact of prodigiosin as antimicrobial agent and alternative to antibiotics, but its role within the bacterial cell that produces it is still not completely known.

Mun'im et al. (2008) found that 398 swab samples from hospitals in Baghdad identified as *S. marcescens*. The detection was made based on bacterial cell culture, microscopical features, biochemical testing, and confirmation with api20E. In the nutrient broth, prodigiosin production was observed at 28°C. *S. marcescens* produces optimal pigments in peptone-glycerol broth at 30°C. It also forms colonies by producing prodigiosin on various culture media. [10]. The function of red pigment prodigiosin in the pathogenesis of *S. marcescens* is unclear, despite the fact that pigments produced by pathogenic microbes are typically considered to be significant virulence factors [42]. According to earlier reports, bacterial pigments are most likely connected to the pathogenic process [43].

In this study, we investigated whether prodigiosin pigment affects virulence factors or not. The formation of biofilm was chosen, and comparison was made between the thickness of the biofilm if it was produced or not depending on the temperature, as 30°C is considered the optimum temperature for prodigiosn production by bacteria [44]. The results revealed a significant difference in biofilm production between isolates incubated at 37°C and isolates incubated at 30°C, as biofilm production was greater at 30°C (optimum for prodigiosin production) than at 37°C (prodigiosin is not produced). The effect of the pigment on the expression of genes responsible for biofilm production in bacterial isolates was also studied. The results showed that the four bacterial isolates possessed all four genes (*flhD*, *fimC*, *bsmA*, *bsmB*). The expression of these genes was increased in all four isolates incubated in 30°C

except *bsmB* and *fimC* in S9 and S8 isolates respectively were slightly decreased. There are no previous studies that have investigated this aspect for comparison.

Conclusions

This study investigates the role of prodiogiocin on biofilm formation of *S. marcescence* isolated from wound infection. It was concluded that prodigiosin has an effect on the expression of the *flhD*, *fimC*, *bsmA*, *bsmB* genes involved in biofilm formation in *S. marcescence* which obviously increased i.e. an indication of the existence of a correlation between pigment production in bacteria and the thickness of their biofilm, and that means prodigiosin may has a role in *S. marcescence* pathogenicity by affecting biofilm, one of the important virulence factors in this bacterium. Further research is necessary to explore and clarify the role of prodigiosin in *Serratia marcescens* pathogenicity.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The research's concept and design were aided by the authors, NSA and HKT. Every author who contributed to the study's methodology. HKT and NSA made contributions to the study's data collection and analysis. The manuscript was written in collaboration with JMH and NSA. After reading the final manuscript in its published form, each author gave their approval.

Ethics approval and consent to participate

The Iraqi Ministry of Health (Reference: CSEC/0823/0104) and the Ethics Committee of the University of Baghdad's Department of Biology approved the study's protocol. For each patient, written informed consent was obtained. The work was completed in compliance with the World Medical Association's code of ethics (Declaration of Helsinki).

Patient consent for publication

All of the study participants gave their written informed consent in order for their data to be published.

Competing interests

The authors declare that they have no competing interests.

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