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Effect of pyocyanin as secondary metabolite on pseudomonal biofilm and in increasing the resistance degree to antipseudomonal agents

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ABSTRACT

Background: *Pseudomonas aeruginosa* produced the maximum amount of pyocyanin pigment as a secondary metabolite as a virulence factor that responsible for increase pathogenesis. The study aims to improve our understanding of the emergence of pseudomonal biofilm formation by pyocyanin production. The correlation between minimal inhibitory concentration (MICs) values of ceftazidime, piperacillin, and ciprofloxacin with pyocyanin production is also targeting in this study. **Materials and Methods:** A total of 96 isolates from patients with catheter-associated urinary tract and wound infections were included in this study. Pyocyanin production was determined. The antimicrobial susceptibility tests including, Kirby–Bauer and E-test, were estimated. Finally, the detection of biofilm production, which includes microtiter plate assay and biofilm formation on FBCL, was done. **Results:** One hundred thirty-eight isolates of *P. aeruginosa*, isolates that produced biofilm were 96 (69.5%). There is a progressive relationship between pyocyanin production level and biofilm production. Furthermore, there is a relationship between MICs values of ceftazidime and pyocyanin production level. **Conclusion:** Our suggestion showed that pyocyanin could be one of the factors that help to induce biofilm formation. Wherefore, sessile cells were showed an increase in isolates that have higher pyocyanin levels more than others. Furthermore, pyocyanin increases the resistance against ceftazidime, which could be of clinical significance.

KEY WORDS: Minimal inhibitory concentration, Pseudomonal biofilm, Pyocyanin

INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative bacterium, is responsible for opportunistic infections and is capable of causing both acute and chronic infections. This bacterium is capable of surviving in human-associated environments with minimal nutritional availability. Importantly, over the last few decades, there has been a steady increase in the number of drug-resistant *P. aeruginosa* strains. In the hospital setting, patients with surgical wounds or burns, patients fitted with catheters are potentially at risk for life-threatening untreatable infections. The persistence of chronic *P. aeruginosa* infections, especially with immunocompromised patients, is attributed to biofilm formation, which enhances its adhesion to cell walls and enables it to evade host immune functions. Biofilm formation also facilitates antibiotic tolerance relative to free-living planktonic cells and accordingly limits eradication.[1,2] *P. aeruginosa* synthesizes a variety of phenazines; however, the most abundantly produced is pyocyanin, and up to 95% of *P. aeruginosa* isolates preferentially produce this pigment. Pyocyanin was formerly disregarded as a bacterial secondary metabolite but has recently been described a variety of roles in microbial ecology, and importantly a relationship with the severity of *P. aeruginosa* infections. A biofilm is a community of microbes that typically inhabit on surfaces and are encasing in an extracellular matrix, and quorum sensing (QS) plays an essential role in bacterial biofilm.[3] The steps process of pyocyanin produced by *P. aeruginosa*, beginning with the synthesis of the QS molecule (acylated homoserine lactone [AHL]) during the exponential growth phase followed by the secondary QS molecule *Pseudomonas* quinolone signaling (PQS) during the late exponential phase.[4] PQS directly controls the expression of

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Received on: 12-01-2020; Revised on: 18-02-2020; Accepted on: 25-03-2020

phzA-G operons resulting in the production of phenazine-1-carboxylic acid (PCA) from its precursor chorismic acid. PCA is then modified to produce three metabolites during the early stationary phase, of which pyocyanin is the predominant product and is regulating by the *phzM* gene.[5] The role of pyocyanin is intensively studying due to its importance as a virulence factor facilitating the formation of biofilm, which increases the pathogenicity. eDNA was well previously recognized as a biofilm-promoting factor.

In contrast, pyocyanin is mainly considering as a secondary metabolite essential for the persistence of *P. aeruginosa* cells in highly dense biofilm by enabling maintenance of a basal rate of respiration for energy harvesting and further maintaining cytoplasmic redox homeostasis.[6] Pyocyanin-eDNA binding influences essential physicochemical interactions that drive bacterial cell-to-cell interactions.[7] Pyocyanin is essential for *P. aeruginosa* biofilm formation. Thus, we are focusing on detecting a relationship between pyocyanin production and the formation of the pseudomonal biofilm. Furthermore, pyocyanin could play a role in resistance to some antibiotics. Thus, the relationship between pyocyanin and antibiotic resistance can be studied in this research.

MATERIALS AND METHODS

Bacterial Isolates

All bacterial specimens have been collected from patients in the Urology Department of the Al-Ramadi Teaching Hospital (Ramadi, Iraq) (from both sexes, mean age, 31 ± 18.8 years) and outpatients visiting Private Clinics in Ramadi city between October 2019 and February 2020. One hundred and thirty-eight isolates bacteriologically identified as *P. aeruginosa*. Of these, 96 (69.5%) isolates produced pseudomonal biofilm.

Diagnosis of *P. aeruginosa*

Microbiology culture

P. aeruginosa was cultured on selective media (2.2% of nalidixic acid was added to cetrimide agar). Furthermore, the morphology of the colony was confirmed: Pigment formation and the findings of specific growth tests (indole, methyl red, Voges–Proskauer, citrate utilization, gelatin liquefaction, and growth at 42°C).[8]

Reading of Pyocyanin Production

Three microliters were taken from the bacterial suspensions which were prepared by a 100 ml of the fresh culture of each bacterial isolate (containing 1×10^8 colony-forming unit [CFU]/ml of bacterial growth) that was used to inoculate the production medium (Brain-heart infusion [BHI]) and incubated in a shaker incubator (150 rpm) at 30°C for overnight. The growth culture was filtrated by 0.20 µm pore size. Then absorbance was measured at 400 nm in a spectrophotometer. The results were calculated according to the following equation: Pyocyanin production = Absorption of the sample test at 400 nm - Absorption of control (broth only) at 400 nm.[9]

Antimicrobial Susceptibility Method

Standard Kirby–Bauer

Antimicrobial activity was performed as per Kirby–Bauer method and Clinical and Laboratory Standards Institute (CLSI) (2019),[10] using an international quality isolate of *P. aeruginosa* American Type Culture Collection 27,853 using following technique.[11] The antibiotic susceptibility test was carried out using the following technique: Two to four colonies were transporting to 2 ml of nutrient broth and incubated at 37°C overnight to make a bacterial suspension adapted to 0.5 McFarland turbidity equivalents to 10^8 CFU/ml. Then, a sterile swab was inserting in a bacterial suspension. The plate was inoculating by swab, the entire surface of the plate was streaking with the swab, then also the plate was rotating at an angle of 45° and streaked the entire surface again; at last, the plate rotated another 90° and streaked again. Then, it was left to dry the plates. The chosen antibiotics disks were softly pressed on the agar surface to achieve complete contact (three disks in each plate) using sterile pliers. Then, the plates were incubating at 37°C for 24 h. The findings were interpreting by reference to the CLSI recommendation (2019) after the incubation; inhibition area diameter was recording and measuring in mm of each antibiotic disk.

Epsilometer test

Mueller-Hinton agar plates had prepared. Then, a sterile swab was placed in the standardized inoculum (McFarland standard 0.5). The entire agar surface of the plate was spreading. After that, the Ezy MIC TM strip container was lifted from the cold and kept at room temperature for 15 min before opening. One strip was put in the center and softly pressed its more full sticky side to the center of the Ezy MICTM strip. The strip was lifting with the Ezy MICTM strip attaches, and the strip was putting on an agar plate swabbed with test culture in the correct place. The strip was softly switching with fingers in the clockwise direction. The strip will detach from the stripe with this action; after that, the Ezy MICTM strip will be adsorbed and adhered firmly to the agar surface within 1 min. Once positioned, under appropriate conditions, plates had transferred to the incubator. The minimal inhibitory concentration (MIC) value was interpreting where the MIC scale on the panel was intersecting by the ellipse. The MIC was reading at the point of completion, suppressing all growth, such as hazes, microcolonies, and isolated colonies. It is possible to

obtain MIC values in-between "two-fold dilutions." These values should always be rounded up to the next two-fold dilution before categorization.[12]

Detection of Biofilm Productions By

Microtiter plate assay

A spectrophotometric technique had used to determine adhesion essay as follows: Stock culture had prepared using Columbia agar supplemented with 3% blood. After inoculation, they incubated aerobic incubation at 35°C for 24 h. After that, the stock culture used to prepare standardized bacterial suspension in sterile distilled water (SDW) and adjusted to a 0.5 McFarland turbidity standard of up to 10^8 CFU/ml. The acquired suspension was inoculating with glucose in the BHI broth. After that, 200 µl of standardized cultures were supplying for each well of a sterile microtiter plate and incubated overnight at 37°C. After incubation, each well washed 3–4 times with SDW. The attached bacteria were solving in 200 µl of methanol per well. After 15 min, the wells of the plate were drained and the left to dry in air. After that, the sheets were staining for 5 min, with 160 µL per crystal purple well (0.25%). Then, the stain was rinsing by putting the microtiter plate under running water. The plate was air-drying, and the dye bound to the adhesive cells was re-solubilized by 160 µl of 33% glacial acetic acid per well. Finally, the optical density (OD) for each well was evaluated at 570 and 630 nm, respectively, using the Stat Fax 3200 ELISA Reader. The isolates were classified as follows: A strong producer with OD more than 2.156; intermediate producer between 2.156 and 1.078 and a non-producer <1.078.[13]

Biofilm formation on Foley balloon latex catheter (FBLC) pieces

Twenty-four hours before each experiment, a sterile disposable FBLC (ENTEPLIN size G16) was cut under a biological safety cabinet (to maintain sterile conditions), in pieces of 0.5 cm length. To allow biofilm formation on FBLC pieces, 600 µl was taken after diluted ($1/10^4$) of overnight cultures, this 600 µl of *P. aeruginosa* put in each well of 48 well-plates, containing FBLC one piece/well, the plates were then incubated at 37°C for 24 h (adhesion period). After incubation, the FBLC pieces were washed with SDW at room temperature, transferred to new wells and incubated for 24 h in fresh BHI broth at 37°C. Unless otherwise specified, After 24 h of incubation, FBLC pieces were washed with SDW at room temperature, and each piece of FBLC put in 2 ml of BHI broth.[14]

Dislodging Sessile Cells from Beads by Sonicating Water Bath

Using the sonicating water bath (Grant, Ultrasonic Ltd.) [Figure 1], following the end of the experiment. In terms of preventing leakage and contamination, the tubs have been coated with parafilm. The tubs were placed under water, but not completely submerged. The pieces of FBLC were transferred to a 2 ml of BHI broth after washing by SDW, falcon containing 2 ml broth following the required sonicating time. It was vortexing and tested, sonicated for 6 min at 30,000 Hz in a water bath sonicator, vortexed for 20 s. Subsequently, each microbial suspension, appropriately diluted, was plated onto Tryptic Soy Agar and incubated for overnight at 37°C. After incubation, the CFU was counted, and data were expressed as CFU/ml by Miles Misra method.[14]

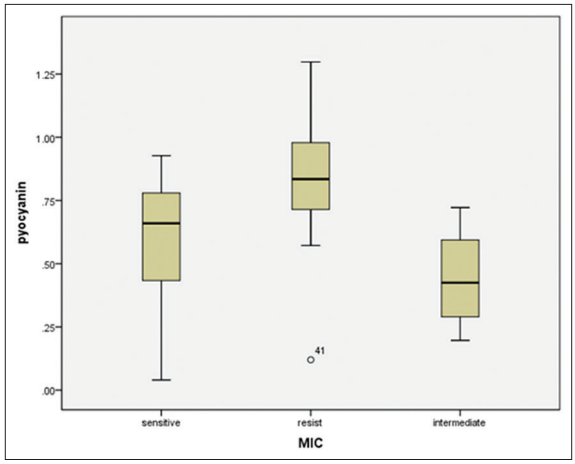

Figure 1: Relationship between minimal inhibitory concentration of ceftazidime and pyocyanin production level

Counting of Sessile Cells

Bacterial suspensions were serially diluted and plated on BHI agar. Following overnight growth, at 37° C, the resultant colonies were enumerated, and the density of the original bacterial suspension was calculated regarding the Miles Misra method. The sample was treated for every piece. The CFU/ml number of bacteria was calculated according to criteria laid down by Irwin and associates.[15]

Statistical Analysis

The experiments were repeated at least 3 times on separate dates. Mean, and standard deviation calculations were achieved using SPSS (version-23). Data were evaluated using one-way ANOVA. The Pearson correlation was used in a significant effect. Student's *t*-test was used to determine the significance. $P \leq 0.05$ was considered significant.[16]

RESULTS

Pyocyanin Production

Production of pyocyanin was examined for *P. aeruginosa* 96 isolates, where the absorption of pyocyanin-containing broth media was calculated at a wavelength of 400 nm. The results demonstrate that most isolates produce pyocyanin. However, even at

a variable and gradual level, the highest production rate of the isolate was 1.298 nm, whereas a certain number of isolate did not produce precise amounts of pyocyanin as in other isolates, absorption at 400 nm was 0.04 nm. The production values of pyocyanin for other isolates varied according to their development frequency between these values.

Biofilm Production on FBLC Pieces

P. aeruginosa cultured onto pieces of FBCL. Isolates of viable counts were taking in 24 h. Furthermore, plating serial dilutions reach ten-fold. Growth on the matrix was monitoring for a period of 24 h. Under these conditions, *P. aeruginosa* showed a growth pattern on pieces of FBCL. The lowest CFU was 2.5×10^6, while the highest CFU was 8.75×10^7. Furthermore, the average was 1.5×10^7 and std. deviation was 1.9×10^7, and the median was 5.25×10^6.

The Correlation between Pyocyanin Production Level and Resistance to Antibiotics

In this study, ceftazidime, piperacillin, and ciprofloxacin were tested with pyocyanin levels for 96 isolates that produced biofilm. The isolates showed the highest resistance (21.9%) to ciprofloxacin, followed by ceftazidime (19.8%) [Table 1]. With statistical analysis, the positive correlation was observed with resistance to ceftazidime ($P < 0.05$) [Figure 1]. Furthermore, no correlation observed with piperacillin and ciprofloxacin [Figure 2 and Table 1].

The Correlation between Pyocyanin Production Level and Biofilm Production

Pyocyanin was mainly classified as a secondary metabolite that is necessary for the survival of *P. aeruginosa* cells in highly dense biofilms. It was revealed that pyocyanin and OD of biofilm were a progressive relationship; the correlation was significant differences at the 0.01 level [Figure 3].

According to the CFU numbers for bacteria, which dislodged from pieces of Foley catheter and OD of pyocyanin pigment produced from clinical isolates. This research was finding that the correlation between the CFU of sessile cells and pyocyanin production level was a progressive relationship ($P = 0.01$) [Figure 4].

DISCUSSION

The widespread resistance of bacteria to antibiotics is currently a pressing public health concern. Bacterial evolution within biofilms resulting in antimicrobial resistance to antibiotics has led to the requirement for the development of novel approaches through which biofilms can be controlled. One of the significant challenges in the disruption of biofilms is the penetration of antimicrobial agents into the biofilm

Table 1: The antimicrobial susceptibility test of *Pseudomonas aeruginosa* against piperacillin, ciprofloxacin, and ceftazidime

Antibiotics	*Pseudomonas aeruginosa* isolates		
	Resistant	Intermediate	Sensitive
	No. (%)	No. (%)	No. (%)
Piperacillin	6 (6.3)	7 (7.2)	83 (86.5)
Ciprofloxacin	21 (21.9)	14 (14.6)	61 (63.5)
Ceftazidime	19 (19.8)	7 (7.2)	70 (73)

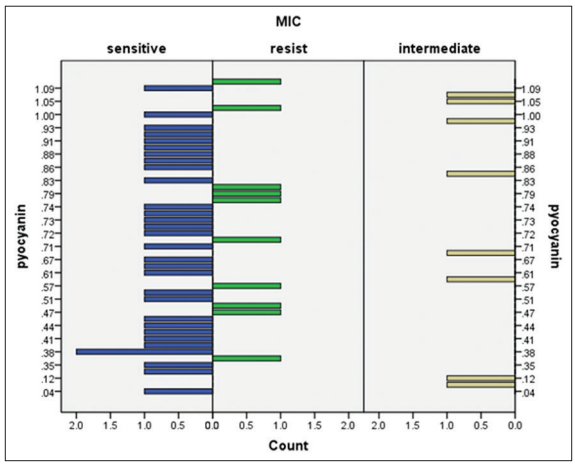

Figure 2: Distribution of isolates according to the susceptibility test and pyocyanin production

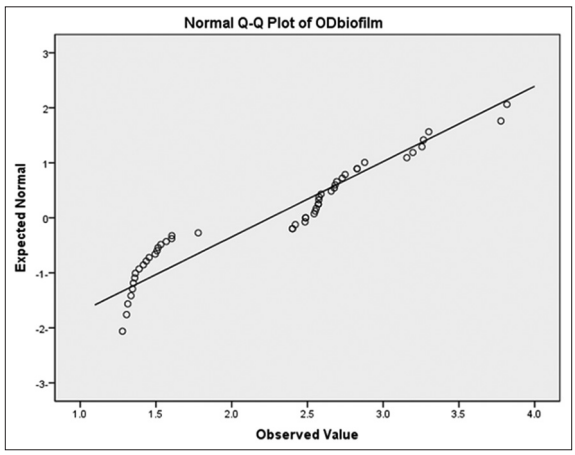

Figure 3: Relationship between the optical density of biofilm and pyocyanin level production

to kill bacterial cells. Pyocyanin pigment induces to a rise of eDNA in most bacterial biofilms, including *P. aeruginosa* makes penetration of antimicrobials extremely difficult, as it binds to biomolecules, cations, and antibiotics, in addition to increasing bacterial adhesion and aggregation.[17] From this standpoint, it could highlight the pyocyanin production level.

The Relationship between Pyocyanin Production Level and Resistance to Antibiotics

We hypothesized based on our investigation that the pyocyanin is playing a role with resistance

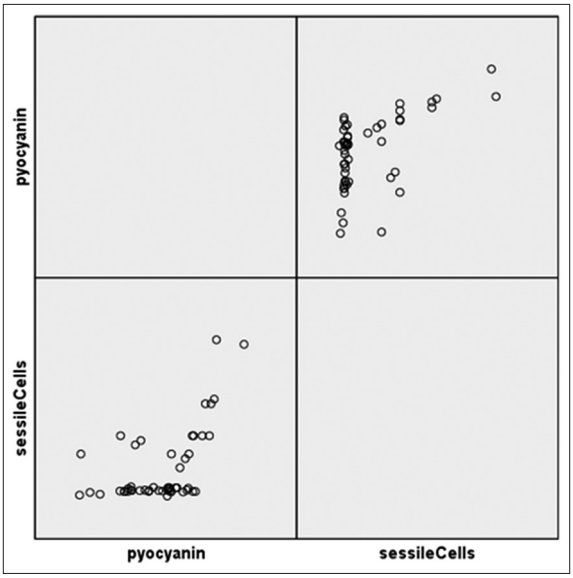

Figure 4: Relationship between pseudomonal sessile cells and pyocyanin production level

to ceftazidime. This result is the agreement with those observed with Khadim and ALmarjani[18] who concluded that the resistance of some isolates to antibiotics, *P. aeruginosa* and in protecting the bacterial cells by inducing drug resistance by pyocyanin pigment. Further, Fothergill *et al*.[19] were going to say that the strategy of ceftazidime therapy may have contributed to the emergence of the pyocyanin phenotype exhibited. However, it suggests pyocyanin itself may play a direct role in the observed link between antimicrobial susceptibilities and the phenotype. Furthermore, Sweedan[20] suggested that the pyocyanin production was relatively affected by antibiotic challenge. The antibiotic at low concentration can upregulates virulence factors and influence bacterial pathogenesis. Pyocyanin production was relatively low and inducible on an antibiotic challenge. The interpretation is may be due to the metabolic versatility in redox balancing that contributes to tolerance to ciprofloxacin. It proposed that mediated phenazine reduction constitutes a redox-balancing pathway that confers a physiological condition of enhanced ciprofloxacin tolerance. For aminoglycoside antibiotics, also observed an antagonistic effect of phenazines.[21]

The Correlation between Pyocyanin Production Level and Biofilm Production

The study suggested that the higher the pyocyanin production, the more biofilm production. This result is in line with Das and Manefield,[22] who suggested that the pyocyanin is impacting on biofilm formation in *P. aeruginosa*. Perhaps this is explained by the following; in *P. aeruginosa*, pyocyanin production involves a stepwise process, beginning with the synthesis of the primary QS molecule N-acyl-L-homoserine lactone (AHL) during the exponential growth phase followed by the secondary QS molecule PQS during the late exponential phase. The release of pyocyanin in *P. aeruginosa* laboratory strains induces a significant rise of eDNA as a biofilm promoter, the release of eDNA, which is an improvement in biofilm virulence.[4,23] Furthermore, the *P. aeruginosa* cells in the presence of eDNA have favorable acid-base interactions, which aid in biofilm production.[24] Further, pyocyanin influences essential physicochemical interactions that drive bacterial cell-to-cell interactions. Pyocyanin is essential in the ability to foster aggregation. Such findings prompted the hypothesis that pyocyanin is essential for *P. aeruginosa* biofilm formation, the pyocyanin, characterized by deregulation of the QS system leading to increased production of exo-products.[25]

CONCLUSION

The study suggested that the ability of *P. aeruginosa* isolates for biofilm production, sessile cells, MICs, and pyocyanin production. Overall, it seems that at least the pigments synthesized by *P. aeruginosa* may contribute to protecting bacteria from stress. In particular, pyocyanin appeared to be particularly efficient in deficiency of sensitive to ceftazidime, and pyocyanin production can spontaneously arise in resistance to ceftazidime. Independently of the cell's physiological status, pyocyanin pigment also provides a certain degree of protection by enhancing biofilm production; this suggested that effect on the pyocyanin production could be a useful means of disrupting pseudomonal biofilms.

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Source of support: Nil; Conflicts of interest: None Declared