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EFFECT OF PRODIGIOSIN ON BIOFILM FORMATION IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

From the duration between August to December 2019, a total of 120 burn and wound specimens were collected from Baghdad hospitals. The obtained specimens were streaked directly on macConkey and cetramide agar plates, followed by further biochemical testing; 46 isolates were identified as *Pseudomonas aeruginosa.* Furthermore, the identification was confirmed using Vitek-2 compact system. Moreover; from the 120 obtained specimens, four isolates were identified as *Serratia marcescens* (prodigiosin producers) using biochemical tests and Vitek-2 compact system. Mineral salt broth with pepton (0.5%) was used, followed by prodigiosin pigment extraction, purification and determination the concentration of the prodigiosin pigment, During prigment extraction, the four isolates of *S. marcescens* (S1, S2, S3, S4) were cultured singly and also cultured by mixing them randomly. The results showed that mixing *Serratia* isolates led to further results, the production of pigment was better by mixing two isolates (S1+S2). Antibiotic susceptibility testing of all (46) *P. aeruginosa* isolates to Ciprofloxacin, Amikacin, Tobramycin, Cefotaxime, Azteronam and Meropenem was assessed using Kirby-Bauer disk diffusion assay. The result revealed that 46 (100%) isolates were resistant to Amikacin; additionally, 50%, 48%, 43% and 28% isolates were resistant to Tobramycin, Cefotaxime, Ciprofloxacin and Azteronam respectively. On the other hand, Meropenem revealed to be most effective drug since it recorded the highest sensitivity percentage of 98%. Moreover, the bacterial ability for the formation of biofilm was assessed for 30 selected *P. aeruginosa* isolates (multi drug resistant isolates) using microtiter plate assay, the result indicated that only 5 strong isolates were biofilm producer whereas 18 and 7 isolates were moderate and weak biofilm producers respectively. DNA of *P. aeruginosa* were successfully extracted from overnight cultures, and PCR was conducted to amplify constitutional genes *16srRNA, AlgD*, the bands were confirmed with gel electrophoresis, the results showed that genes *16srRNA* (956 bp) and *AlgD* (313bp) were detected. The presence of *AlgD* gene was tested in 17 selected *P. aeruginosa* isolates (strong and moderate biofilm producers) using polymerase chain reaction technique; however, only 2 isolates (P9 and P21) harbored *AlgD* gene. The minimal inhibitory concentrations of prodigiosin and Ciprofloxacin were assessed for *P. aeruginosa* isolates (P9 and P21) using broth microdilution method; even more, the synergistic effect of prodigiosin combined with the Ciprofloxacin was also assessed for the same tested isolates. The result revealed that Ciprofloxacin minimum inhibitory concentration values were 31.2 µg/ml and 15.6 µg/ml for *P. aeruginosa*(P9 and P21) isolates respectively, Additionally, the minimum inhibitory concentration value for prodigiosin pigment were 87.5 µg/ml and 175 µg/ml for *P. aeruginosa* (P9 and P21) isolates respectively; regarding the synergistic effect the Ciprofloxacin combined with the prodigiosin, the result revealed that their minimum inhibitory concentration values of Ciprofloxacin were 15.6 µg/ml and 7.8 µg/ml for *P. aeruginosa* (P9 and P21) isolates respectively while the values of prodigiosin were 2.7 µg/ml and 1.36 µg/ml for *P. aeruginosa* (P9 and P21) isolates respectively. To perform the reduction biofilm assay, *P.*aeruginosa (P9 and P21) isolates were treated with two sub MICs of (prodigiosin, ciprofloxacin and synergisim between them). The results showed that the biofilm inhibition of P9 isolates was (84%, 82% and 85% under the effect of sub MIC 1, and were 81%, 80% and 83% under the effect of sub MIC 2) and for P21 isolates was (81%, 79% and 82% under the effect of sub MIC1, and were 80%, 77% and 81% under the effect of sub MIC2)

Keywords **:** Prodigiosin, peptone, *Pseudomonas aeruginosa*, *AlgD* gene, 16srRNA.

Introduction

The *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen and leading cause of diverse nosocomial infections and it is commonly difficult to eradicate with conventional antibiotic therapy, particularly when established as biofilms (Soukarieh *et al*., 2018). While *P. aeruginosa* infrequently infects healthy individuals those persons whose skin mucous membranes or immune system are affected, are additional liable to becoming infected by this organism; for instance, burn fatalities, patients with cystic fibrosis or cancer patients treated with chemotherapy (Markou *et al*., 2014).

The misuse and over employment of antibiotics is a rising concern about public health, which competence consequence in development of bacterial strains resistant to drugs and unnecessary, side effects (Ventola, 2015). Moreover, the progression of new antibiotics is consuming long time and very limited. Therefore, the expansion of novel therapeutic means to *Pseudomonas aeruginosa* infections treatment is highly necessary and has increased attention in the past years (Chatterjee *et al.,* 2016).

The handling of infections of *P. aeruginosa* keep to be an important challenge. Resistance of *P. aeruginosa*is to antibiotic is multi-factorial, in that it can occur through acquired, innate or adaptive mechanisms (Poole *et al*., 2016).

However, *P. aeruginosa* has an obvious capacity to acquire or develop new technique of resistance to these new antibiotics so that the misuse of antibiotics lead to serious

impact used for public health (Pang *et al*., 2018). The therapeutic with non-antibiotic approaches have shown significant antimicrobial effects against *P. aeruginosa* that represent as antibiotic-resistant strains in animal models or *in vitro*, and they are being considered as alternatives or adjuncts to conventional antibiotics (Pal *et al*., 2018).

The Biofilms, are surface-associated bacterial communities embedded in an extracellular matrix, that are considered to be a major problem in the context of chronic infections, because biofilm dwelling cells have greater than before antibiotic resistance compared to their planktonic counterparts (Flemming *et al*., 2016). The dangerous characters of the matrix into microbial relations and virulence, in addition to for antimicrobial tolerance are existence progressively recognized. The matrix making increases bacterial cell adhesion and consistency (resulting in thickly crowded cell aggregates), providing mechanical stability (Liu *et al*., 2018).

The production of Prodigiosin, by *S. marcescens* occurs together under aerobic and anaerobic situations and be determined by various factors such as, temperature, bacterial strain, and time of incubation, aeration, and agitation of cultures, and sources of nitrogen and carbon, concentration of inorganic salts, and pH of culture medium (Su *et al*., 2011; Suryawanshi *et al*., 2014).

Prodigiosin have a wide diversity of biological properties as well as, antibiotic, antibacterial, antimalarial, antifungal, immunosuppressive, and anticancer (Matsuya *et al*., 2006). Antibacterial effect against *P. aeruginosa*, *E. coli*, and *S. aureus* is in comparison with previous reports (Chandni *et al*., 2012). Prodigiosin affect intracellular pH gradient of the organism (Francisco *et al*., 2007)

In this study we aimed to isolate *S. marcescens* which produce prodigiosin pigment, then extra it study its antibacterial and antibiofilm effect as an alternative to antibiotics against *P. aeruginosa* isolates.

Materials and Methods

Isolates Collection of *Pseudomonas aeruginosa* and *Serratia marcescens*

Between August and December 2019, 150 burn wounds swab specimens were collected by laboratories at Al-Kindi hospital, Al-Yarmook hospital, and Teaching Hospital of the Baghdad Medical city and cultured on MacConkey agar. Isolates were obtained from these laboratories by sub culturing on MacConkey agar, subsequently incubated at 37 ºC for 18-24 hours.

Identification of Bacteria

Isolates were inoculated on various culture media including MacConkey agar, Cetrimide agar, and Nutrient agar; the media were incubated at 37˚C for 18-24 hrs. Suspicious colonies were identified by morphologically and biochemically.

Microscopic Characteristics

The Suspicious colonies were examined by Gram staining, to identify the characteristic morphology of bacteria under the light microscope.

Catalase Production Test

Catalase Production Test (Benson, 2002), growth at 4 ºC and 42 ºC (Holt *et al*., 1994) ,oxidase Test (Benson, 2002) and motility test (Collee *et al*., 1996) were performed.

Identification of bacteria by Vitek -2 system.

Vitek -2- was applied to identify the bacterial isolates, This device is used to diagnose bacterial isolates and confirm that is *Pseudomonas areginosa* and *Serratia marcescens,* after being confirmed by first biochemical test as well as testing their sensitivity.

Screening of *Serratia* spp. isolates for pigment prodigiosin production

Serratia isolates which confirmed through the identification process were screened into the production of the prodigiosin in liquid culture in order to select the higher prodigiosin producing isolate as described in the following sections:

Preparation of inoculums

Inoculum of *S. marcescens* isolates was prepared as follows: a few loopfuls of *Serratia* spp. growth of an overnight culture on nutrient agar was inoculated to a 150 ml Erlenmeyer flask containing 20 ml of Brain heart broth. such culture was incubated for 24 hrs in an incubator at 30˚C.

Cultivation method

Erlenmeyer flasks (250 ml) each contained 50 ml of minerals salt broth medium was prepared and autoclaved at 121˚C, for 15 min. 1 ml (2% inocula) inoculum of each *Serratia* isolate was inoculated in each flask. Then flasks were incubated in an orbital shaker at 30˚C and 200 rpm for 48hrs. After the incubation, samples were taken for the analyses of prodigiosin.

Determination of prodigiosin concentration

The method for prodigiosin concentration described by (Venil and Lakshmanaperumalsamy, 2009).

Prodigiosin pigment production and its estimation by UV-Vis spectroscopy

The quantitative determination from the red pigment was done by measuring the absorbance at 530 nm using double beam of UV-Visible spectrophotometer as propose by Wei and Chen, (2005).

Partial purification of prodigiosin pigment

Prodigiosin was partially purified according to Chen and co workers with modification (Chen *et al*., 2013).

Antibiotic Susceptibility Testing

The modified Kirby-Bauer method (Vandeppitte *et al*., 2003) was used.

Biofilm formation assay

The quantification of biofilm formation by the more antibiotic resistant isolates of *P. aeruginosa* was assessed as mentioned by Atshan *et al*. (2012).

Molecular Assay

Extraction of Genomic DNA

DNA was extracted from *P. aeruginosa* isolates using a commercial extraction kit (G-spin extraction kit), following the manufacturer's instructions, for DNA purification from gram negative bacteria.

PCR Amplification

The extracted DNA, primers (table 1), and distributed maxime PCR premix (intron, Korea), were thawed at 4ºC,

vortexed to have homogenous contents, a PCR mixture was made in a total volume as described in table 2.

Table 1 : Primers sequences

Gene	Primer	Sequences ´5 →´3
AlgD	F R	CGTCTGCCGCGAGATCGGCT GACCTCGACGGTCTTGCGGA
16srRNA For detection *P.aeruginosa*	F R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG
16srRNA For RT-PCR	F R	ACCTGGACTGATACTGACACTGA GTGGACTACCAGGGTATCTAATCCT

Table 2: Reaction mixture .

Component	Volume	Final concentration
FIREPol® Master Mix, 5X	5 µl	1X
Forward primer	1µl	1 µM
Reverse primer	1µl	1 µM
DNA template	2µl	25 ng
Nuclease free dH_2O	11 µl	
Final volume	**20µl**	

PCR reaction tubes were placed in a thermo-cycler PCR instrument, DNA was amplified as in the conditions indicated in Table 2-8. The temperature and time of PCR program were optimized by using gradient PCR.

Electrophoresis

After PCR, agarose gel electrophoresis was adopted for confirmation. PCR was completely dependable on the extracted DNA criteria. An amount of 100 ml of 1X TBE was taken in a beaker, agarose gel (2%) was prepared by adding one gram of agarose to the buffer, the solution was heated to boiling (using microwave) until all the gel particles were dissolved, 1 µl of Red Safe Nucleic Acid Stain (10 mg/ml) was added to the agarose, the agarose was stirred in order to be mixed and to avoid bubbles, the solution was allowed to cool down at 50-60°C.

Determination of minimum inhibitory concentration(MIC)

The minimum inhibitory concentration of the antibiotic and prodigiosin was determined against *P. aeruginosa* isolates which had the AlgD gene.

The Broth microdilution method

Broth microdilution method was performed for ciprofloxacin, prodigiosin, synergism between of them MIC determination as follows (Andrews, 2001).

Estimation effect of sub MIC of Ciprofloxacin, prodigiosin, synergism between prodigiosin and ciprofloxacin on biofilm formation

The same protocol of the biofilm formation assay was used. However, tryptic soy broth contained ciprofloxacin, prodigiosin, synergism between them at sub MIC. The plates were incubated at 37ºC for 24h. Thereafter, all wells were washed, stained, and read at 630 nm. The positive controls were performed as well by adding 200 µl of ciprofloxacin-free fresh bacterial suspension and prodigiosin- free freshbacterial suspension,also prodigiosin and ciprofloxacin-free fresh bacterial suspension (compatible to 0.5McFarland standard).

Estimation biofilm inhibition

The prodigiosin pigment, ciprofloxacin, synergistic between prodigiosin and ciprofloxacin as antibiofilm activity were tested on *P. aeruginosa* biofilm using 96 well microtitere plate as mentioned by (Selim *et al*., 2014)

Results and discussion

Sample Collection, Isolation, Identification, Cultural Characteristics and biochemical tests

Eighty three gram negative Bacterial isolates were obtained from 120 burn wounds specimens after culturing on MacConkey's agar medium. MacConkey agar, is a differential and selective culture medium used for bacteria, considered to selectively isolate gram-negative and enteric (usually found in the intestinal tract) bacilli and distinguish them depending on lactose fermentation (Anderson and Cindy, 2013), a crystal violet and bile salts prevent the growth of gram-positive organisms (Anderson *et al*., 2013). The results presented that 65 isolates (78.3%) could not ferment lactose (lactose non fermenter), 46 (55.4%) of them characterized by grape-like odor, and were re-cultured on cetramide agar medium, this medium was used to regulate the capability of an organism to grow in the presence of the 0.03% cetrimide that acts as a quaternary ammonium cationic detergent (acetyl trimethyl ammonium bromide) and inhibits the development of other microorganisms (Tang and Stratton, 2006). Further most non-*Pseudomonas* species, are inhibited on cetrimide agar and species of *Pseudomonas* also can be inhibited, cetrimide also increases the pigments production by *Pseudomonas* such as pyocyanin and pyoverdine, which show a characteristic blue-green and yellow-green colour, respectively (Mahmudullah *et al*., 2018).

All bacterial isolates which cultivated on this medium were tested for their oxidase and catalase activity, the results presented that, all bacterial isolates were oxidase and catalase positive, then tested for their capability to grow at 42 ºC, all isolates were capable to grow at 42 ºC but not at 4°C. This is a very important character to distinguish *P. aeruginosa* from other *Pseudomonas* spp. like *P. putida* and *P. fluoresences* that grow at 4°C, but not 42°C (Govan, 2005). Also tested for motility, the results revealed that all isolates were motile. The forty six bacterial isolates which were obtained are expected to be *P. aeruginosa*.

Identification of bacteria by Vitek -2 compact system

The fourty six isolates which grow on cetramide agar were identified by Vitek-2 compact device, the results displayed that all the isolates were *P.aeruginosa.*

Isolation and identification of the *Serratia marceacens*

Four Bacterial isolates (red pigment producers) were isolated from 120 burn wounds specimens after culturing on macConkey agar medium, they were pale colonies (L.N.F). And then recultured on the nutrient agar for prodigiosin production, by incubating for 18-24 hrs. at 30˚C.

The major identifier that differentiates *S. marcescens* from other enteric bacteria is the cell associated prodigiosin production (Mahlen, 2011). But, many species of the genus *Serratia,* are non-pigmented or vary widely in pigmentation (Winshell and Neu, 1974).

Morphological identification, of the *Serratia* isolates was mostly completed by examining the presence of the intracellular bright red pigmentation which obviously

presented in the interior the colonies. In addition to the red pigmentation, other remarkable indications were considered such as the fishery - urinary culture specific smell and rod shape of cells under microscopic observation as well as the Gram negative staining. The optimal temperature to produce prodigiosin at 30˚C that showed a normal growth with significant indicating of the prodigiosin production (Wang *et al*., 2013).

Identification of the *Serratia marcescens* by Vitek -2 compact system

The four isolates that grew on nutrient ager and produced red pigment were identified by Vitek -2 compact device, the results exposed that all the isolates were *Serratia marcescens.*

Screening of *Serratia marcescens* isolates for prodigiosin production

The four isolates (red pigment producers) S1, S2, S3, S4 were subjected for screening process that select the higher prodigiosin producing isolate to be used for further experiments in this study. The screening was achieved in minerals salt medium, with pepton (0.5%) and, for more reliability all isolates were cultivated under the same situations in terms of inoculum size, pH, cells number, incubation period and shaking speed. During prigment extraction, the four isolates of *S. marcescens* (S1, S2, S3, S4) were cultured singly and also cultured by mixing them randomly. The results exhibited that mixing *Serratia* isolates led to further results, the releasing of pigment was better by mixing two isolates (the colour was darker) than one isolates and even better than mixing three isolates. S1 and S2 combination was the best of all in prodigiosin production. It was not found if both isolates were equal in pigment production activity, or may be one of them was more efficient than the other. Dengo and shiao (2015) reported the bacterial synergism stimulated the production of B-1, 4-glucosidase (BG), they found that the mean BG activity in synergistic mixed culture, (133.5 nmol/h/ml) was over three times that of pure cultures (43.7 nmol/h/ml).

The combination of (S1+S2) isolates was choosen to produce and extract pigment so, it was cultured in mineral salt broth with different concentration of pepton (0.5%, 1%, 1.5%, 2%, 2.5%), the results indicated that (0.5%, 1%) of pepton were the best for prodigiosin production.

Giri *et al.* (2004) reported that during extraction prodigiosin from the *S. marcescens* in peptone glycerol broth, maximum pigment production was seen only at 30°C, while Demain, (1986) mentioned that *Aspergillus nidulans* showed higher pigment production when peptone was used like the nitrogen source.

Determination of prodigiosin concentration

Concentration, of the prodigiosin was determined using the colorimetric method described by Venil and Lakshmanaperumalsamy (2009), and measured by O.D530, the results shown in table (3).

Table 3 : Determination of prodigiosin concentration

Isolates	$O.D_{530}$
S_1	2.841
S_2	2.172
S3	2.204
S_4	2.841
S_1, S_2	3.318
S_3, S_4	2.363
S_2, S_4	2.039
S_2, S_3	1.684
S_1, S_3	3.017
S_1, S_4	3.052
S_1, S_2, S_3	1.826
S_1, S_2, S_3, S_4	1.112

And also determination the prodigiosin at $O.D_{530}$ treated with different concentration of pepton during extraction of isolates mixing (S1, S2), the result showed that the concentrations (0.5% and 1%) were the best concentrations for producing prodigiosin during extraction in mineral salt broth presented in Table (4). Several studies have revealed that prodigiosin production, can be stimulated in culture that contains peptone (Su *et al*., 2011; Chen *et al*., 2013).

Table 4 : Determination the prodigiosin concentration for two isolates (S1+S2) treated with different pepton concentration

Pepton concentration	O.D 530
0.5% pepton	3.017
1% pepton	2.539
1.5% pepton	1.995
2% pepton	1.021
2.5% pepton	1.020

Determination prodigiosin pigment by UV-visible by spectroscopy

The outcome indicated that the absorbance of prodigiosin pigment in UV visible was at about OD529, the absorbance value of prodigiosin was determined by running an UV-wavelength scan. Prodigiosin pigment has a characteristic absorbance at 530 nm. sample extracts each remained dissolved in methanol, methanol extract showed maximum absorbance at 538 nm, Figure (1). These results were similar to Song *et al*., (2006) stated that prodigiosin improved from *Serratia* spp. KH-95 had a maximum absorption spectrum at 535 nm.

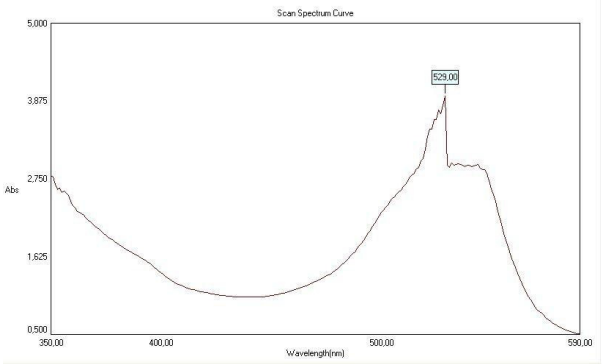

Fig. 1 : Prodigiosin pigment in UV-Visible about OD530

Partial purification of prodigiosin

It was take out from the cell pellets only. Dark red powder of the extracted prodigiosin was obtained which then stored in the fridge in a dark glass plate due to its lower stability in the light (Someya *et al*., 2004).

The Antibiotic Susceptibility Testing for *P. aeruginosa*

Forty six isolates went through the susceptibility test, for six different antibiotics (Tobramycin, Amikacin, Meropenem, Ciprofloxacin, Cefotaxime, Azteronam) by the disc diffusion method recommended by the medical and laboratory standards association (CLSI, 2013) guidelines, the results revealed that (98%, 70%, 57%, 52% and 50%) of the isolates were sensitive toward (Meropenem, Azetronam, Ciprofloxacin, Cefotaxime and tobramycin) respectively, while 100% of isolates were resistant to Amikacin.

Biofilm assay

Thirty Bacterial isolates, were selected for biofilm assay which were more resistant to antibiotic, biofilm production is considered as a marker of virulence; numerous new methodologies must been lately developed for, or modified to biofilm studies that have contributed to profounder information on biofilm physiology, structure and composition (Azeredo *et al*., 2017). In this study, the capability of *P. aeruginosa* biofilm producing isolates was estimated by means of pre-sterilized 96-well polystyrene microtiter plates, which considered as a standard test for the exposure of biofilm biomass (Gad *et al*., 2009; Atshan *et al*., 2012; Azeredo *et al*., 2017).

For the reason that this method is more sensitive than the other methods causes to the crystal violet stained only the cells, not the slime materials and the cell, which are not in the biofilm structure are rinsed –off by washing steps (Stepanovic *et al*., 2007).

In order to estimate biofilm intensity, absorbance was determined at 630nm by a microplate reader. Given that, absorbance values represented the intensity of biofilm thickness that formed by the studied isolates at the surface of the microtiter well. The obtained results were considered to three groups (weak, moderate, and strong) based on limits summarized in Table (5).

Table 5 : Biofilm forming capacity of *P.aeruginosa* isolates

Strong biofilm isolates	Mean OD630	Moderate biofilm isolates	Mean OD630	Weak biofilm isolates	Mean OD630
P1	0.227	P2	0.151	P18	0.088
P9	0.314	P3	0.149	P23	0.076
P20	0.495	P5	0.157	P24	0.102
P21	0.379	P6	0.139	P27	0.092
P35	0.228	P7	0.124	P30	0.088
C	0.048	P8	0.131	P32	0.082
		P10	0.137	P34	0.09
		P11	0.125		
		P12	0.201		
		P13	0.112		
		P14	0.131		
		P15	0.136		
		P16	0.142		
		P17	0.16		
		P19	0.182		
		P22	0.133		
		P28	0.191		
		P31	0.129		

P: *P.aeruginosa* ; C: control ; cut off value 0.05324924

Upon the criteria listed in (Table 5), the current study declared that out of, 5 (16.70%) of *P. aeruginosa* isolates existed strong biofilm producers; while 18 (60%) and 7 (23.30%) of the isolates existed moderate and weak producers respectively. The changes in biofilm thickness might be due to several reasons; differences of isolates ability to procedure biofilm or perhaps differences in primary amount of cells that prospered in adherence and differences of quality and quantity of the quorum sensing, signaling molecules (autoinducer) that produced from each isolate play important roles (Brady *et al*., 2008; Beenken *et al*., 2010).

Molecular Analysis

DNA Extraction

DNA of seventeen isolates (5 strong and 12 moderate) of *P. aeruginosa* were successfully extracted from overnight cultures of isolates. Concentration were confirmed with biodrop which was 105 ng/µL, and the intact DNA bands were confirmed through gel electrophoresis.

Detection of *16srRNA* gene

One of the most, attractive possible employ of *16srRNA* gene sequence informatics is to supply genus then species or taxa identification used for isolates (Janda and Abbott, 2007). Although *16srRNA* gene sequencing is highly valuable in respects to bacterial classification (Bosshard *et al.,* 2006), the result indicated that the tested isolates harbored this gene (956 bp).

Detection of *AlgD* gene

PCR was conducted for two isolates (P9 and P21), using the, *AlgD* primer to amplify the constitutional genes *AlgD*, and bands were confirmed with gel electrophoresis. The results revealed that *AlgD*gene (313 bp) was detected in two *P. aeruginosa* isolates (P9 and P21). Figure (2)

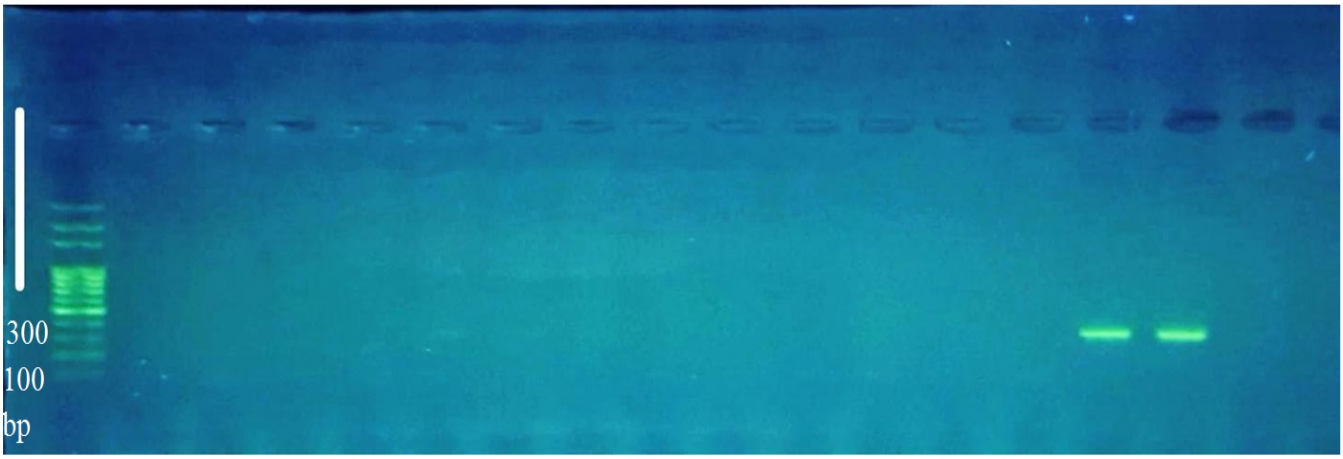

Fig. 2 : Agarose gel electrophoresis, of *AlgD* gene of *P. aeruginosa* responsible for the biofilm formation, AlgD amplicon to (313bp).

Strohl *et al.,* (2013) reported that *P. aeruginosa* produces an exopolysaccharide named alginate, protect the bacteria for changed environmental conditions and encourages bond to solid surfaces.

Minimal Inhibitory Concentration Test of (ciprofloxacin, prodigiosin, synergistic between prodigiosin and ciprofloxacin) for *P. aeruginosa*

Two *Pseudomonas aeruginosa* isolates (P9, P21) were tested to regulate the minimal inhibitory concentration for ciprofloxacin, prodigiosin and synergistic between prodigiosin and ciprofloxacin against *P. aeruginosa*, by the microdilution method recommended by the clinical trial and laboratory values found (CLSI, 2013) guidelines, the results presented that the MICs of ciprofloxacin were (31.2 and 15.6µg/ml) and MIC of prodigiosin (87.5 and 175 µg/ml) and MIC of synergistic between prodigiosin and ciprofloxacin (2.7 and 1.36 µg/ml for prodigioin and 15.6 and 7.8 µg/ml for ciprofloxacin) against two isolates (P9, P21) respectively. Obviously it was clear that prodigiosin decreased the MIC of ciprofloxacin against the two isolates by the synergistic effect, it decreased from 31.2, 15.6 to 15.6, 7.8 against the two isolates (P9, P21) respectively as given in Table (6).

Table 6 : Shows MIC of (Ciprofloxacin, prodigiosin, synergistic between prodigiosin and ciprofloxacin) against two isolates of *P.aeruginosa* (P9, P21).

Isolates	Ciprofloxacin µg/ml	Prodigiosin µg/ml	Synergistic between prodigiosin and ciprofloxacin µg/ml	
P9	31.2	87.5	Pro.	2.7
			Cipro.	15.6
P21	15.6	175	Pro.	1.36
			Cipro.	7.8

Pro: prodigiosin, Cipro: Ciprofloxacin

Chin and neu (1984) mentioned that the MIC of ciprofloxacin against *P. aeruginosa* was 6.3 µg /ml, while Forrest *et al*. (1993) stated that the MIC of ciprofloxacin against the pathogen where distributed between 0.008 and 4.0 µg/ml. Ni *et al*., (2013) mentioned that the studies of combination treatments reported empirically inferred that synergistic validity was probable a key to avoid antimicrobial resistance then balance these factors during combination therapy

Vora*et al*., (2014) reported that extracted prodigiosin of *S. marcescens* strain and then investigated its anti-microbial and antioxidant property, This pigment has displayed an anti-bacterial activity against *B. cereus, S.aureus and E.coli*within an inhibition zone of 12 and 7 and 6 mm respectively.

3.13 Effect of sub MIC of prodigiosin, Ciprofloxacin and synergism between prodigiosin and ciprofloxacin on biofilm formation by *P. aeruginosa*.

Treating the two isolates P9 and P21 (strong biofilm formers), with two sub MICs of prodigiosin (sub MIC 1: 43.7 and 87.5 µg/ml, sub MIC 2: 21.9 and 43.7 µg/ml), two sub MICs of Ciprofloxacin (sub MIC 1: 15.6 and 7.8 µg/ml, sub MIC 2: 7.8 and 3.9 µg/ml), and synergism between prodigiosin and Ciprofloxacin (sub MIC 1: 1.36 and 0.68 µg/ml for prodigiosin and 7.8 and 3.9 µg/ml for Ciprofloxacin, sub MIC 2: 0.68 and 0.34 µg/ml for prodigiosin and 3.9 and 1.95 µg/ml for ciprofloxacin) affected the thickness of biofilm formed by the two isolates, and the results exhibited that the biofilm formed by them became weak and therefore was a reduction in biofilm formation as presented in Table (3-4), the biofilm inhibition of P9 isolate was (under effect of sub MIC 1: 84%, 82% and 85%) and (under effect of sub MIC 2: 81% , 80% and 83%), and for P21 isolate was (under effect of sub MIC 1: 81% , 79% and 82%) and (under effect of sub MIC 2: 80% , 77% and 81%) after treating with sub MICs of (prodigiosin, ciprofloxacin and synergism between them) respectively, Table (7).

Table 7 : Shows the reduction biofilm formation by *P. aeruginosa*

Isolates (Biofilm formaers)	Biofilm reduction% after treated with prodigiosin		Biofilm reduction% after treated with ciprofloxacin		Biofilm reduction% after treated with synergisim between prodigiosin and ciprofloxacin	
P9	sub MIC 1 (43.7 µg/ml)	84%	sub MIC 1 (15.6 µg/ml)	82%	sub MIC 1 (Pro..:1.36, Cipro.:7.8 µg/ml)	85%
	sub MIC 2 (21.9 µg/ml)	81%	sub MIC 2 (7.8 µg/ml)	80%	sub MIC 2 (Pro..:0.68, Cipro.: 3.9 µg/ml)	83%
P21	sub MIC 1 (87.5 µg/ml)	81%	sub MIC 1 (7.8 µg/ml)	79%	sub MIC 1 (Pro..:0.68, Cipro.: 3.9 µg/ml)	82%
	sub MIC 2 (43.7µg/ml)	80%	sub MIC 2 (3.9 µg/ml)	77%	sub MIC 2 (Pro..:0.34, Cipro.: 1.95 µg/ml)	81%

Pro.: prodigiosin, Cipro.: ciprofloxacin

So, the results showed that the influence of prodigiosin, was more than of ciprofloxacin in biofilm inhibition on both isolates, while synergism between them gave results better than each one of them alone. Also there was a difference in their impact between the two isolates P9 and P21, the biofilm formation reduction of P9 was more than P21 under three treatments (i.e. the effect of treatments was greater on P9 than P21), since P21 was stronger than P9 in biofilm formation.

Gupta *et al.* (2016) reported that ciprofloxacin at subinhibitory concentration, inhibited biofilm formation by *P. aeruginosa,* thereby decreasing its pathogenicity. Reduction in production of virulence factors reduces the organism defenseless which might let greater action of immune response and bactericidal agents against *P. aeruginosa.* Another study by Aleksandra *et al.* (2012) who reported that a bacterial biofilm is known as bacterial community surrounded in a self-produced polymeric matrix that adherent into an inert or living surface. Also, Hoiby *et al.* (2010) reported that biofilm-forming bacteria have lower sensitivity to antibiotics in comparison with planktonic cells. Such sensitivity could be attributed to the exopolysaccharide structure and reduced metabolic activity.

Another study by Hemati *et al.* (2016) mentioned that antibiotics reduced biofilm formation; however, several studies indicated that the antibiotics could significantly stimulate biofilm formation depending on antibiotics class and the bacterial strain. Also, Vuotto *et al*. (2014) mentioned that the high amount of biofilm formation might be related to phenotypes and genes complex in biofilm such as capsule, lipopolysaccharide and fimbriae.

Additionally, Magesh *et al*. (2013) reported that the reason for inhibiting biofilm at low concentration of natural compounds because of the inhibition of receptors and molecules involved in quorum sensing pathway which is required for biofilm formation.

Conclusions

We conclude from our study that there is a necessity of using another antibiotics other than ciprofloxacin as drug of choice for *P. aeruginosa,* also mixing *S. marcescens* isolates inhances prodigiosin production efficiently, and high concentration of pepton is not necessary for prodigiosin production, while low concentration of it give good results. Prodigiosin is better than ciprofloxacin against *P. aeruginosa*, it reduces the biofilm formation in *P. aeruginosa* more than ciprofloxacin. Additionally, synergism between prodigiosin and ciprofloxacin reduces biofilm formation efficiently more than each one of them alone and leads to decrease the MIC of ciprofloxacin (i.e. decrease the antibiotic dose). In AlgD expression, one of the genes responsible of biofilm formation in *P. aeruginosa*, both prodigiosin and ciprofloxacin stopped its expression at their sub MICs. So, *in vitro* prodigiosin was a good alternative to ciprofloxacin.

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