ORIGINAL ARTICLE

EVALUATION THE EFFECT OF PRODIGIOSIN ON ALGD EXPRESSION IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The aim of this study is to investigate the role of prodigiosin on *P. aeruginosa' s* biofilm genes involved in the pathogenicity and persistency of the bacteria;

Materials and methods: Gram negative bacterial isolates were taken from burn and wounds specimen obtained from some of Baghdad hospitals. Forty six isolates were identified as *Pseudomonas aeruginosa* and four isolates as *Serratia marcescens* by using biochemical tests and VITEK 2 compact system. Susceptibility test was performed for all *P. aeruginosa* isolates, the results showed that 100% were resistant to Amikacin and 98% were sensitive to Meropenem. Resistant isolates were tested for biofilm formation; the strong and moderate isolates (17) were detected by PCR for *AlgD* gene presence. From 17 isolates only two had *AlgD* gene. All *serratia* isolates were screened for prodigiosin production, which were extracted from the best producer isolate. Minimal inhibitory concentration was assessed for prodigiosin and ciprofloxacin and synergism between them against the two isolates of *P. aeruginosa*.

Results and conclusions: The results showed that the synergistic effect decreased MIC of both prodigiosin and ciprofloxacin by combination, and reduction of biofilm formation was detected. RNA was extracted from the two selected isolates as control in addition to three treatments. The result of quantitative real time PCR showed down regulation in the *AlgD* gene expression level under some treatments, while there was no gene expression in most treatments with both sub-MICs treatment

KEY WORDS: ciprofloxacin, prodigiosin, *Pseudomonas aeruginosa*, *AlgD* gene, 16srRNA

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INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen in humans, animals, and plants [1]. It is an aerobic gram-negative rod, possessing a strictly respiratory metabolism. The organisms are usually 1.5-5 μm in length and 0.5-1.0 μm in width, and are motile due to the presence of flagella [2]. *P. aeruginosa* develops antimicrobial resistance rapidly, which complicates medical treatment of infections. It is frequently isolated from patients and hospital environments and has been implicated as the cause of nosocomial infections in burn patients [3]. Resistance to antibiotics is, thus, a serious public health problem worldwide, which translates into an inevitable increase in morbidity and mortality caused by infectious diseases, resulting in a reduction in the quality of life and an increase of pressure in health and care sectors [4]. *P. aeruginosa* has a strong potential to gain or establish new resistance techniques to new antibiotics, meaning that the misuse of antibiotics has a significant effect on public health [5]. In addition to antimicrobial resistance, the dangerous characters of the matrix are increasingly recognized in microbial relationships and virulence. Matrix development increases the adhesion and cohesion of bacterial cells (resulting in thickly crowded cell aggregates), providing mechanical stability [6]. Biofilms are surface-related bacterial communities, fixed in an extracellular matrix that are thought to be a major problem in the case of chronic infections, since biofilm-dwelling cells have higher antibiotic resistance than planktonic counterparts [7]. Prodigiosin has a broad variety of biological and antibiotic, antibacterial, antimalarial, antifungal, immunosuppressive and anticancer properties [8]. In contrast to previous studies, the antibacterial activity against *P. aeruginosa*, *Escherichia coli*, and then *Staphylococcus aureus* was observed. [9]. Prodigiosin affects intracellular pH gradient of the organism [10].

THE AIM

The aim of this study is to investigate the role of prodigiosin on *P. aeruginosa' s* biofilm genes involved in the pathogenicity and persistency of the bacteria; to study the effect of prodigiosin, antibiotic and synergism between them on the expression of *AlgD* gene, responsible for biofilm formation by *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

ISOLATES COLLECTION OF *SERRATIA MARCESCENS* AND *PSEUDOMONAS AERUGINOSA*

The specimens were obtained from hospitals in Baghdad. Burn and wound swab specimens were collected and cultured on

MacConkey agar. The standard microbiological tests such as oxidase test, catalase test, growth on MacConkey agar, growth on cetrimide agar, motility, growth at 42°C and at 4°C, pigment production [11], were conducted. Then identification was confirmed by using Vitek-2 compact system according to the manufacturer instructions. The isolates were maintained in nutrient broth medium containing 40% glycerol at 20°C [12,13].

PRODIGIOSIN PIGMENT PRODUCTION

Serratia marcescense isolates that were validated through the identification process were screened into the development of prodigiosin in liquid culture (brain-heart broth) as stated in the following sections in order to choose the higher prodigiosin generating isolate.

CULTIVATION METHOD

50 mL minerals salt broth medium was made and autoclaved at 121°C for 15 minutes (250 mL) in Erlenmeyer flasks. Each flask received a 1 mL (2 percent inoculum) Serratia isolate inoculum. The flasks were then incubated on an orbital shaker for 48 hours at 30°C and 200 rpm. Following the incubation time, samples for prodigiosin assay were obtained.

PRODIGIOSIN PIGMENT PARTIAL PURIFICATION

Prodigiosin was partially purified with modification according to Chen et al. [14].

ANTIBIOTIC SUSCEPTIBILITY TESTING

The Antimicrobial susceptibility profiles of *P. aeruginosa* isolates were determined by the disk diffusion test (DDT) according to Kirby Bauer's method, depending on the recommendations made by the Clinical and Laboratory Standards Institute [15].

BIOFILM FORMATION ASSAY

Biofilm production by higher antibiotic resistant *P. aeruginosa* strains was quantified [16].

MOLECULAR ASSAY

EXTRACTION OF GENOMIC DNA

DNA was isolated from *P. aeruginosa*, using a commercial extraction kit (G-spin extraction kit) as directed by the manufacturer.

PCR AMPLIFICATION

The extracted DNA, primers (table I), and distributed maxime PCR premix (intron, Korea) were thawed at 4°C, vortexed to ensure uniform contents, and a 100-mL PCR mixture was made as stated in table I.

DNA was amplified using PCR reaction tubes in a thermo-cycler PCR instrument under conditions described in Table II. Gradient PCR was used to optimize the temperature and time of the PCR.

ELECTROPHORESIS

Following PCR, the results were validated using agarose gel electrophoresis. PCR was used to extract all of the DNA parameters. A 100 ml beaker of 1X TBE was filled, and one gram of agarose was added to the buffer to make an agarose gel

Table I. Primers sequences

Gene	Primer	Sequences ´5 ´3	Refrence
AlgD	F R	CGTCTGCCGCGAGATCGGCT GACCTCGACGGTCTTGCGGA	Asmaa *et al.,* 2019
16srRNA For detection *P.aeruginosa*	F R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	Nowroozi *et al.,* 2012
16srRNA For qRT-PCR	F R	ACCTGGACTGATACTGACACTGA GTGGACTACCAGGGTATCTAATCCT	Nowroozi *et al.,* 2012

Table II. 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 dH2O	11 µl	
Final volume	20µl	

Table III. Reaction mixture of QRT-PCR

Component	Volume
5x HOT FIREPol® EvaGreen® qPCR Supermix	4 µl
Primer Forward (10 pmol/µl)	0.5 µl
Primer Reverse (10 pmol/µl)	0.5 µl
DNA template2	2 µl
H2O PCR grade	up to 20 µl

Table IV. Program of QRT-PCR

Cycle step	Temp.	Time	Cycles
Initial activation	95oC	12 min	1
Denaturation	95oC	15 s	40
Annealing			
Elongation	72oC	20 – 30 s	

(2%). The agarose was stained with 1 l Red Safe Nucleic Acid Stain (10 mg/ml), the agarose was swirled to combine and prevent bubbles, and the solution was left to cool till 50-60°C.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The antibiotic's and prodigiosin's minimum inhibitory concentrations were evaluated against *P. aeruginosa* isolates with the *AlgD* gene.

THE BROTH MICRODILUTION METHOD

Broth microdilution method was performed for ciprofloxacin, prodigiosin, synergism with MIC determination for their combination as follows [17].

ESTIMATION EFFECT OF SUB-MIC OF CIPROFLOXACIN, PRODIGIOSIN AND SYNERGISM BETWEEN THEM ON BIOFILM FORMATION

The same methodology was used for the biofilm development experiment. Tryptic soy broth, on the other hand, contained sub-MIC synergy between ciprofloxacin and prodigiosin. The plates were kept at 37°C for 24 hours. After that, all of the wells were cleaned, stained, and measured at 630 nm. 200 µl of ciprofloxacin-free fresh bacterial suspension, prodigiosin-free fresh bacterial suspension, and prodigiosin and ciprofloxacin-free fresh bacterial suspension were used as positive controls (compatible to 0.5McFarland standard).

ESTIMATION BIOFILM INHIBITION

The 96-well microtiter plate was used to assess the antibiofilm activity of the prodigiosin pigment, ciprofloxacin, and a synergistic combination of prodigiosin and ciprofloxacin against P. a*eruginosa* biofilm [18].

RNA PURIFICATION PROTOCOL PROCEDURE

GENEzolTM TriRNA pure Kit, Japan, was used to extract RNA from P. aeruginosa isolates.

QUANTITATIVE REAL TIME – POLYMERASE CHAIN REACTION TECHNIQUE (QRT-PCR)

RT-PCR should be assembled in a nuclease-free environment. RNA sample preparation, reaction mixture assembly, PCR and subsequent reaction analysis should be performed in separate areas using RealMODTM Green qRT-PCR Mix (Table III, IV).

RESULTS AND DISCUSSION

SAMPLES ISOLATION AND IDENTIFICATION

Eighty-three gram-negative bacterial isolates were obtained from 120 burn wound specimens after cultivation on MacConkey's agar medium (a differential and selective bacterial culture medium). The results revealed that 65 isolates (78.3%) could not ferment lactose (lactose non-fermenter), 46 (55.4%) had a grape-like odor, and were re-cultured on cetrimide agar medium. This medium was used to monitor an organism's ability to grow in the presence of 0.03% cetrimide [19]. Furthermore, most non-*Pseudomonas* species, as well as *Pseudomonas* species, are suppressed on cetrimide agar. *Pseudomonas* pigments like as pyocyanin and pyoverdine, which have typical blue-green and yellow-green colors, respectively, are similarly promoted by cetrimide [20]. The oxidase and catalase activity of both bacterial isolates cultured on this medium was examined, and all bacterial isolates tested positive for oxidase and catalase. They were then put through a series of tests to see if they could grow at 42 degrees Celsius. All isolates were able to expand at 42°C, but not at 4°C. This is an important distinction between P. aeruginosa and other Pseudomonas species, such as *P. putida* and *P. fluoresences,* which grow at 4°C but not 42°C [21]. The isolates were also tested for motility, and all of them were shown to be motile. The 46 bacterial isolates obtained were all categorized using the VITEK 2 compact method and are all likely to be *P. aeruginosa*. *P.aeruginosa* was detected in all isolates.

ISOLATION AND IDENTIFICATION OF SERRATIA *MARCEACENS*

Four bacterial isolates (red pigment producers) were isolated from 120 burn wound specimens after cultivation on MacConkey agar medium; they were light-colored colonies (L.N.F). The bacteria were then re-cultured for 18-24 hours on nutrient agar for prodigiosin development. At 30 °C. *S. marcescens* is distinguished from other enteric bacteria by its ability to produce prodigiosin in the cell [22]. However, many *Serratia* species are non-pigmented or have a wide range of pigmentation [23]. The presence of bright red intracellular pigmentation, which

was clearly present in the colonies' interior, was used to complete the morphological identification of the *Serratia* isolates. Apart from the red pigmentation, other important indicators were taken into account, such as the distinct odor and rod form of cells under microscopic inspection of the urinary culture; gram-negative staining is also used. The ideal temperature for producing prodigiosin was 30°C, which resulted in normal development and large amounts of prodigiosin [24]. The Vitek -2 compact system was then used to identify the four isolates. All of the isolates were found to be *Serratia marcescens*, according to the results.

SCREENING OF *SERRATIA MARCESCENS* ISOLATES FOR PRODIGIOSIN PRODUCTION

The four isolates (red pigment producers) S1, S2, S3, S4 were screened and the isolate with the highest prodigiosin output was chosen for the study's subsequent experiments. All isolates were cultured in minerals salt medium with peptone under the identical circumstances in terms of inoculum size, pH, cell number, incubation period, and shaking speed for improved dependability (0.5 %). The four *S.marcescens* isolates (S1, S2, S3, S4) were cultivated individually and then mixed randomly during pigment extraction. The findings showed that combining *Serratia* isolates yielded better results; pigment release was better when two isolates were mixed (the color was darker), and even better when three isolates were mixed. In terms of prodigiosin output, the combination of S1 and S2 was the most efficient. It was unclear whether both isolates produced the same amount of pigment or if one was more efficient than the other [25]. They showed that bacterial synergism increased the development of B-1, 4-glucosidase (BG), with average BG activity in synergistic mixed cultures (133.5 nmol/h/ml) more than three times higher than in pure cultures (43.7 nmol/h/ml). To produce and extract pigment, a combination of (S1+S2) isolates was chosen, and it was cultured in mineral salt broth with various concentrations of peptone (0.5%, 1 %, 1.5 %, 2 %, 2.5 %), with the results indicating that (0.5 %, 1 %) of peptone were the best for prodigiosin production [26]. Maximum pigment production from *S. marcescens* was seen only at 30°C during extraction prodigiosin in peptone glycerol broth, [27] and *Aspergillus nidulans* demonstrated increased pigment production when peptone was utilized as the nitrogen source.

PRODIGIOSIN PIGMENT PARTIAL PURIFICATION

Prodigiosin was isolated only from cell pellets. Due to its lower stability in the light, extracted prodigiosin was ground into a dark red powder and stored in the fridge in dark glass containers [28].

THE ANTIBIOTIC SUSCEPTIBILITY TESTING FOR *P. AERUGINOSA*

Forty-six isolates were tested for their susceptibility towards six different antibiotics (Tobramycin, Amikacin, Meropenem, Ciprofloxacin, Cefotaxime, Aztreonam) by the disc diffusion method recommended by the medical and laboratory standards association [29] guidelines. The results demonstrated that (98 %, 70 %, 57%, 52 %, and 50 %) of the isolates were sensitive to (Meropenem, Aztreonam, Ciprofloxacin, Cefotaxime and tobramycin), but Amikacin resistance was found in 100% of the isolates.

BIOFILM ASSAY

Thirty bacterial isolates were chosen for the biofilm assay because they were more resistant to antibiotics; biofilm production is thought to be a marker of virulence; numerous new methodologies have recently been developed for, or modified for, biofilm studies that have contributed to a deeper understanding of biofilm physiology, structure, and composition [30]. The potential of *P. aeruginosa* biofilm-producing isolates was assessed in this study using pre-sterilized 96-well polystyrene microtiter plates, which are used as a standard test for biofilm biomass exposure [30, 31, 32]. A microplate reader was used to measure absorbance at 630nm in order to estimate biofilm intensity. As a result, absorbance values expressed the strength of biofilm thickness formed at the surface of the microtiter well by the studied isolates. The outcomes were divided into three categories (weak, moderate, and strong). According to the findings, 5 (16.70%) of *P.aeruginosa* isolates were found to be strong biofilm producers, while 18 (60%) and 7 (23.30%) were found to be moderate and weak producers, respectively. Changes in biofilm thickness could be caused by a variety of factors, such as differences in isolates' ability to process biofilm, differences in the primary number of cells that thrived in adherence, and differences in the quality and quantity of quorum sensing, signaling molecules (autoinducer) produced by each isolate [33, 34].

MOLECULAR ANALYSIS

DNA EXTRACTION

Pseudomonas aeruginosa DNA was successfully extracted from cultures of seventeen isolates (5 strong and 12 moderate). The intact DNA bands were confirmed by gel electrophoresis, and the concentration was confirmed with biodrop, which was 105 ng/ µL.

DETECTION OF 16SRRNA GENE

One of the most attractive applications of 16srRNA gene sequence informatics is the provision of genus, species, or taxa recognition for isolates [35]. Although *16srRNA* gene sequencing is highly valuable in respects to bacterial classification [36], the result indicated that the tested isolates possess this gene (956 bp) Figure (1).

DETECTION OF ALGDGENE

The *AlgD* primer was used to amplify the constitutional genes *AlgD* in two isolates (P9 and P21), and bands were confirmed with gel electrophoresis. *AlgD*gene (313 bp)

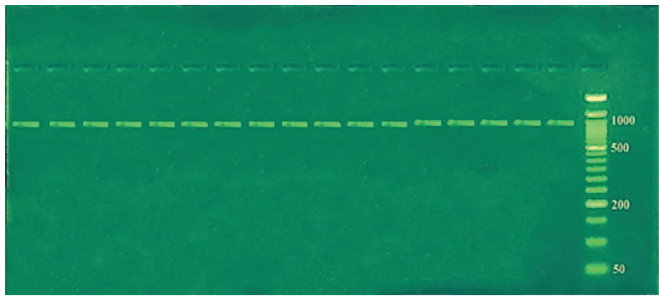

Fig. 1. Electrophoresis agarose gel of 16SrRNA gene of *P.aeruginosa*, L: 50bp DNA ladder, 16SrRNA amplicon (956bp). The electrophoresis was done in 2% agarose gel and TAE 1X, 100volt for 90 min stained with red stain.

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

was found in two *P. aeruginosa* isolates, according to the findings (P9 and P21). Figure (2)

P. aeruginosa produces alginate, an exopolysaccharide that protects bacteria from changing environmental conditions and allows bonding to solid surfaces [37].

Minimal Inhibitory Concentration Test of (Ciprofloxacin, Prodigiosin, Synergistic Between of Them) for *P. aeruginosa*

Two *P. aeruginosa* isolates (P9, P21) were examined to determine the minimum inhibitory concentration for ciprofloxacin, prodigiosin, and the synergistic effect of prodigiosin and ciprofloxacin against *P. aeruginosa* using the microdilution method specified by the clinical trial and laboratory results [29]. The results showed that the MICs of ciprofloxacin were (31.2 and 15.6µg/ml) and MIC of prodigiosin (87.5 and 175 µg/ml); MIC of synergistic between them was 2.7 and 1.36 µg/ml for prodigiosin and 15.6 and 7.8 µg/ml for ciprofloxacin against two isolates (P9, P21) respectively. The synergistic effect of prodigiosin reduced the MIC of ciprofloxacin against the two isolates, decreasing it from 31.2, 15.6 to 15.6, 7.8 against the two isolates (P9, P21) respectively [38]. The MIC of ciprofloxacin against *P.aeruginosa* was 6.3 µg /ml, [39] the MIC of ciprofloxacin against the pathogen where distributed between 0.008 and 4.0 µg/ml [40]. Synergistic validity was likely a key to prevent antimicrobial resistance. This factor is highly important during combination therapy, according to studies of combination therapies published empirically [41].

The anti-microbial and antioxidant properties of *S. marcescens* prodigiosin were examined. This pigment showed anti-bacterial activity against *B. cereus*, *S. aureus*, and *E. coli* within an inhibition zone of 12 and 7 and 6 mm, respectively.

Effect of sub-MIC of prodigiosin, Ciprofloxacin and synergism between of them 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 of them (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 showed that the biofilm formed by them became weak and therefore was a reduction in biofilm formation as presented in Table (V). 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 (V).

As a result, the findings showed that prodigiosin had a

Table V. shows the reduction biofilm formation by *P. aeruginosa*

Isolates (Biofilm formers)	Biofilm reduction% after treated with prodigiosin		Biofilm reduction% after treated with ciprofloxacin		Biofilm reduction% after treated with synergism 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%

Notes: Pro.: prodigiosin, Cipro.: ciprofloxacin

Table VI. Fold change of AlgD gene and 16srRNA gene in *P. aeruginosa* isolates (P9, P21) in presence of two sub-MICs for (prodigiosin, ciprofloxacin and synergism between of them)

Isolate/ treatment	AlgD gene		16sRNA gene		CTΔ		CTΔΔ		Fold	
P9C Sub-MIC 1	CT-B	22.6	CT-B	21.6	B	1				
	CT-A6	27.1	CT-A6	26.1	A6	1	A6	0	A6	1
	CT-A10	34.1	CT-A10	33.1	A10	1	A10	0	A10	1
P9C Sub-MIC 2	CT-B	22.6	CT-B	21.6	B	1				
	CT-A6	25.8	CT-A6	24.8	A6	1	A6	0	A6	1
	CT-A10	29.9	CT-A10	28.9	A10	1	A10	0	A10	1
P9T1 Sub-MIC 1 (43.7)	CT-B	23.7	CT-B	17.8	B	5.9				
	CT-A6	32.3	CT-A6	24.8	A6	7.5	A6	1.6	A6	0.32987697
	CT-A10	Undetermined	CT-A10	28.1	A10	-----	A10	-----	A10	No gene expression
P9T1 Sub-MIC 2 (21.9)	CT-B	23.7	CT-B	17.8	B	5.9				
	CT-A6	28.5	CT-A6	22	A6	6.5	A6	0.6	A6	0.65975395
	CT-A10	32	CT-A10	25.3	A10	6.7	A10	0.8	A10	0.57434917
P9T2 Sub-MIC 1 (15.6)	CT-B	24.5	CT-B	19.6	B	4.9				
	CT-A6	34.8	CT-A6	22.1	A6	12.7	A6	7.8	A6	0.00448710
	CT-A10	Undetermined	CT-A10	29.1	A10	-----	A10	-----	A10	No gene expression
P9T2 Sub-MIC 2 (7.8)	CT-B	24.5	CT-B	19.6	CT-B	4.9				
	CT-A6	32	CT-6	27.2	A6	4.8	A6	0.1	A6	0.93303299
	CT-A10	34	CT-A10	29.4	A10	4.6	A10	0.3	A10	0.81225239
P9T3 Sub-MIC 1 (pro:1.36; cipro: 7.8)	CT-B	31.5	CT-B	20.8	B	10.7				
	CT-A6	Undetermined	CT-A6	25.5	A6	-----	A6	----	A6	No gene expression
	CT-A10	Undetermined	CT-A10	28.6	A10	-----	A10	----	A10	No gene expression
P9T3 Sub-MIC2 (pro: 0.68 cipro:3.9)	CT-B	31.5	CT-B	20.8	B	10.7				
	CT-A6	35	CT-A6	34	A6	1	A6	9.7	A6	0.00120228
	CT-A10	37	CT-A10	36.4	A10	0.6	A10	10.1	A10	0.00091116
P21C Sub-MIC 1	CT-B	23.4	CT-B	22.4	B	1				
	CT-A6	31.7	CT-A6	30.7	A6	1	A6	0	A6	1
	CT-A10	36.1	CT-A10	35.1	A10	1	A10	0	A10	1
P21C Sub-MIC 2	CT-B	23.4	CT-B	22.4	CT-B	1				
	CT-A6	29.6	CT-A6	28.6	A6	1	A6	0	A6	1
	CT-A10	35	CT-A10	34	A10	1	A10	0	A10	1
P21T1 Sub-MIC 1 (87.5)	CT-B	22.1	CT-B	23.6	B	-1.5				
	CT-A6	Undetermined	CT-A6	33.1	A6	-----	A6	-----	A6	No gene expression
	CT-A10	Undetermined	CT-A10	Undetermined	A10	-----	A10	-----	A10	No gene expression
P21T1 Sub-MIC 2 (43.7)	CT-B	22.1	CT-B	23.6	B	-1.5				
	CT-A6	30	CT-A6	26.5	A6	3.5	A6	5	A6	0.03125
	CT-A10	Undetermined	CT-A10	30	A10	-----	A10	----	A10	No gene expression

P21T2 Sub-MIC 1 (7.8)	CT-B	25.1	CT-B	26.6	B	-1.5				
	CT-A6	29.7	CT-A6	Undetermined	A6	-----	A6	-----	A6	No gene expression
	CT-A10	34.1	CT-A10	Undetermined	A10	-----	A10	-----	A10	No gene expression
P21T2 Sub-MIC 2 (3.9)	CT-B	25.1	CT-B	26.6	B	-1.5				
	CT-A6	29	CT-A6	27	A6	2	A6	3.5	A6	0.08838834
	CT-A10	35	CT-A10	Undetermined	A10	-----	A10	----	A10	No gene expression
P21T3 Sub-MIC 1 (pro: 0.68 cipro: 3.9)	CT-B	33.6	CT-B	28.9	B	4.7				
	CT-A6	38.9	CT-A6	30.6	A6	8.3	A6	3.6	A6	0.08246924
	CT-A10	Undetermined	CT-A10	32.7	A10	-----	A10	-----	A10	No gene expression
P21T3 Sub-MIC 2 (pro: 0.34 cipro: 1.95)	CT-B	33.6	CT-B	28.9	B	4.7				
	CT-A6	38	CT-A6	30	A6	8	A6	3.3	A6	0.10153154
	CT-A10	Undetermined	CT-A10	36	A10	-----	A10	-----	A10	No gene expression

Notes: C: control; T1: prodigiosin; T2: Ciprofloxacin; T3: synergism between prodigiosin and ciprofloxacin for two isolates (P9, P21), B: before, A6: after 6hr, A10: after 10hr, pro: prodigiosin, cipro: ciprofloxacin.

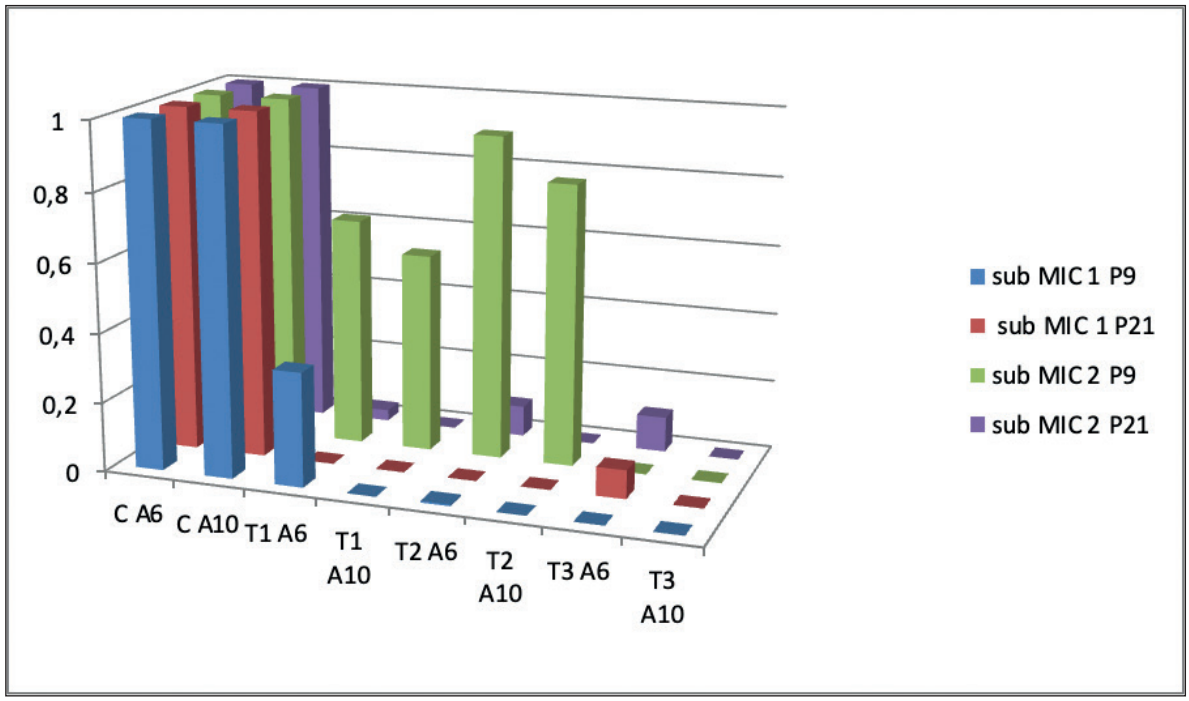

Fig. 3. Fold change of AlgD gene and 16srRNA gene in *P. aeruginosa* isolates (P9, P21).
C: control, T1: prodigiosin, T2: ciprofloxacin, T3: synergism between prodigiosin and ciprofloxacin, A6: after 6 hr, A10: after 10 hr.

greater impact on biofilm inhibition than ciprofloxacin on both isolates, and that synergism between them produced better results than either of them alone. Also, since P21 was stronger than P9 in biofilm formation, the biofilm formation reduction of P9 was greater than P21 under three treatments (i.e. the effect of treatments was greater on P9 than P21) [42]. *P. aeruginosa* biofilm formation was inhibited by ciprofloxacin at subinhibitory concentrations, reducing pathogenicity. Reduced virulence factor activity renders the organism defenseless, allowing for greater immune response and bactericidal agent action against *P. aeruginosa* [43]. Bacterial biofilm is known as bacterial community surrounded in a self-produced polymeric matrix that adherent into an inert or living surface. Also, [44] when compared to planktonic bacteria, proved that biofilm-forming bacteria are less responsive to antibiotics. The exopolysaccharide structure and decreased metabolic activity may be to blame for this sensitivity. [45] Antibiotics decreased biofilm formation; however, depending on the antibiotic class and the bacterial strain, some studies found that antibiotics may significantly stimulate biofilm formation. [46] The high rate of

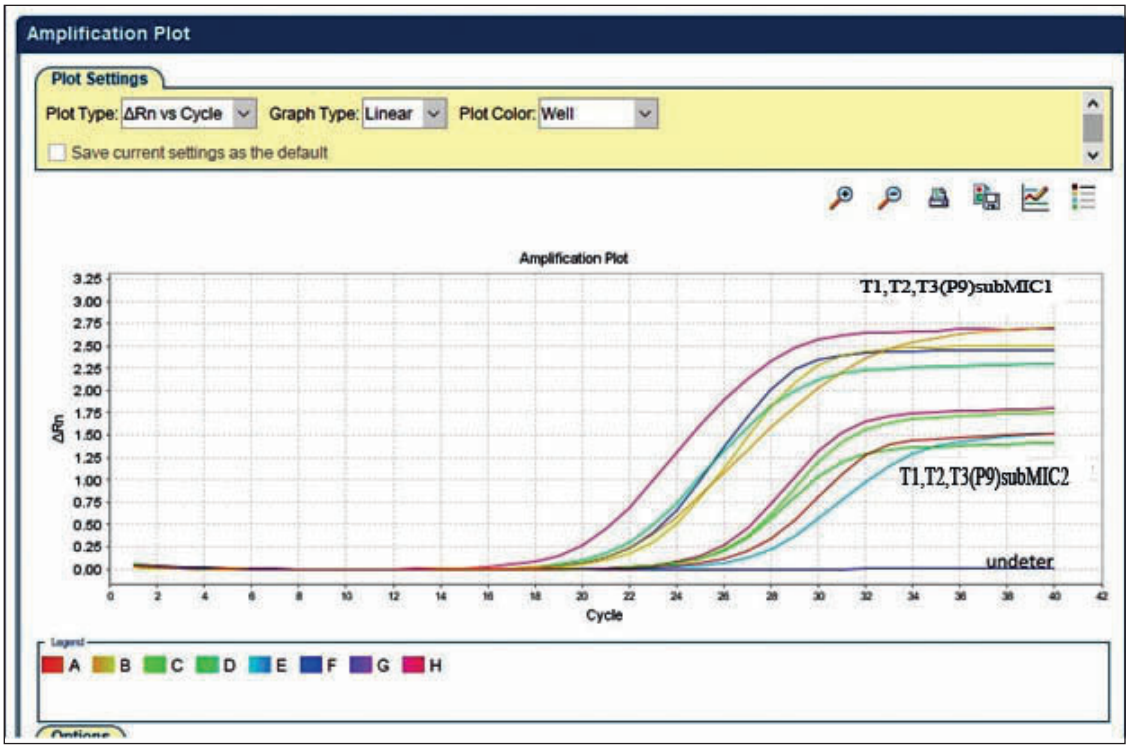

Fig. 4. The amplification curve of AlgD gene and 16srRNA gene in *P. aeruginosa* for isolates P9 treated with sub MICs of prodigiosin, ciprofloxacin, synergism between of them.
T1: prodigiosin, T2: ciprofloxacin, T3: synergism between prodigiosin and ciprofloxacin

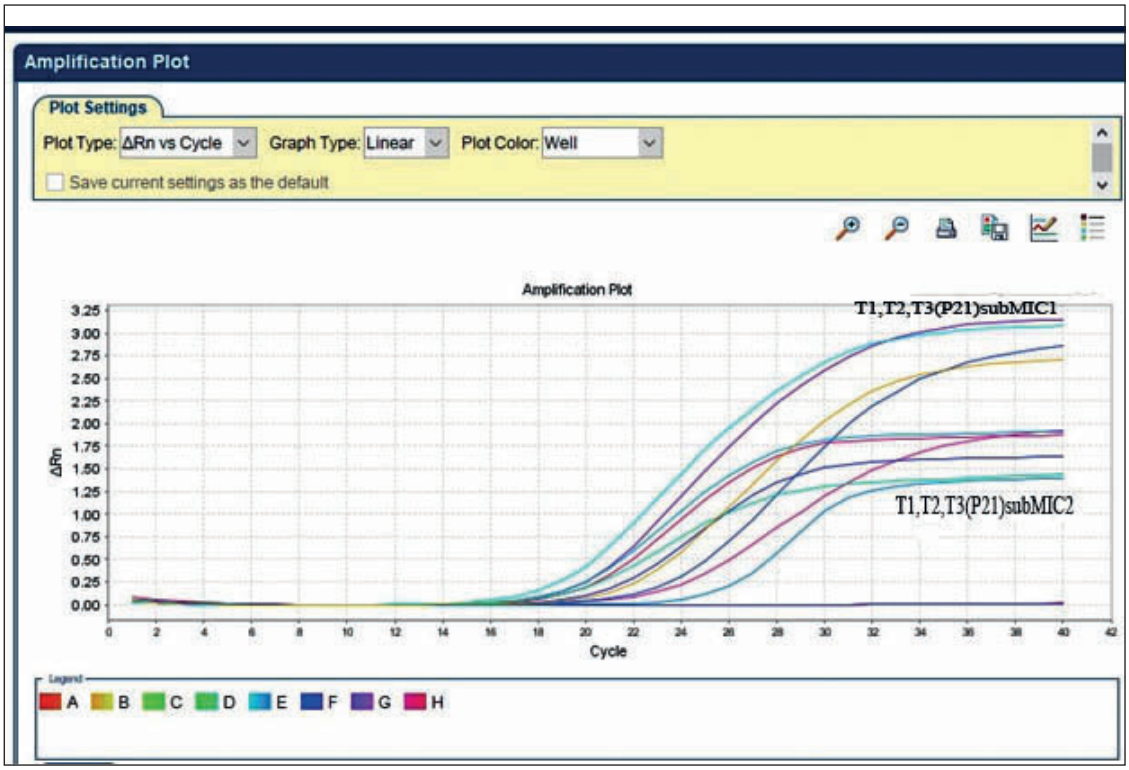

Fig. 5. The amplification curve of AlgD gene and 16srRNA gene in *P. aeruginosa* for isolates P21 treated with sub MICs of prodigiosin, ciprofloxacin, synergism between of them.
T1: prodigiosin, T2: ciprofloxacin, T3: synergism between prodigiosin and ciprofloxacin

biofilm formation may be linked to biofilm phenotypes and genes such as capsule, lipopolysaccharide, and fimbriae. [47] Biofilm formation is inhibited by natural compounds at low concentrations due to the inhibition of receptors and molecules involved in the quorum sensing pathway, which is needed for biofilm formation.

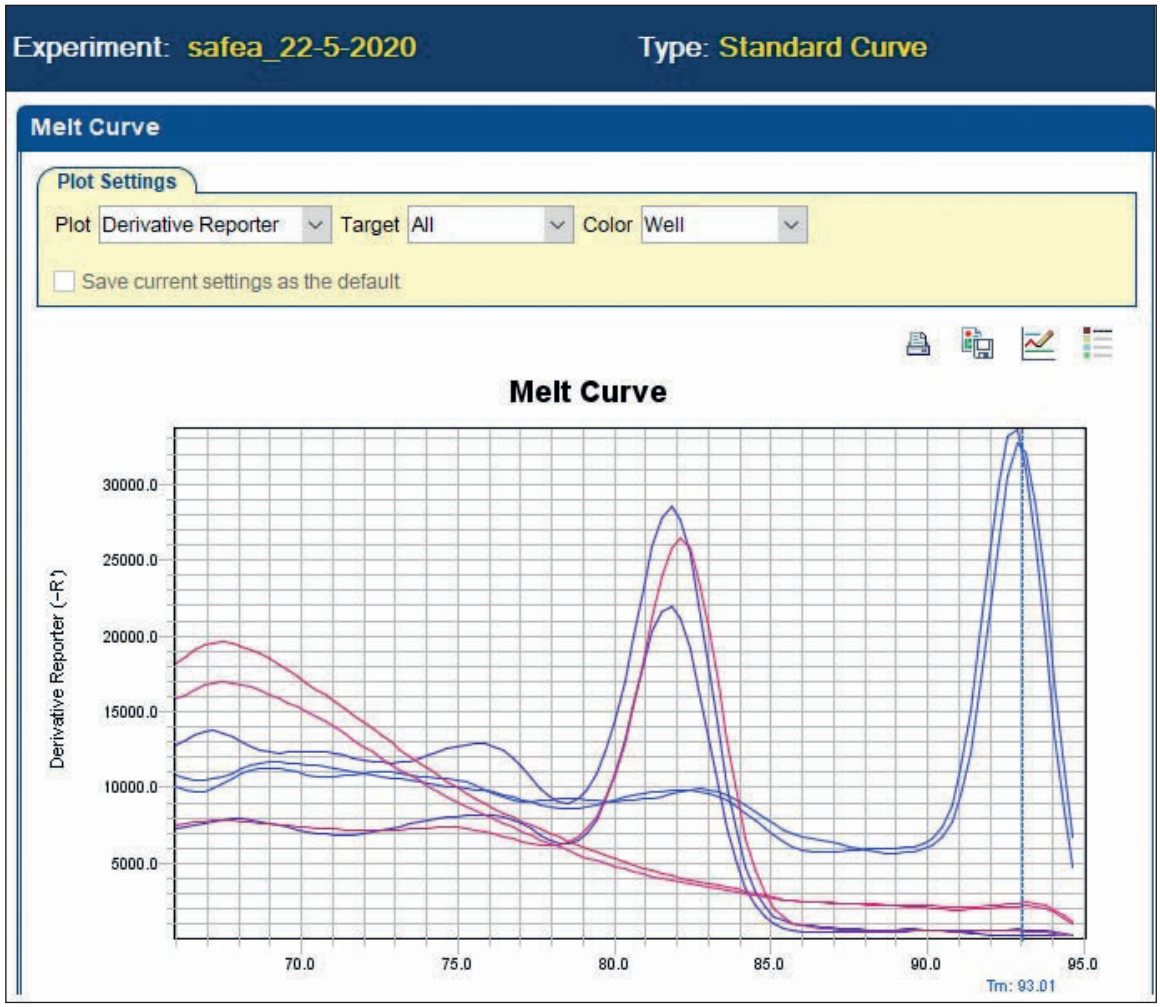

Fig. 6. Melt curve for isolates P21 for 16srRNA gene and AlgD gene after treatment.

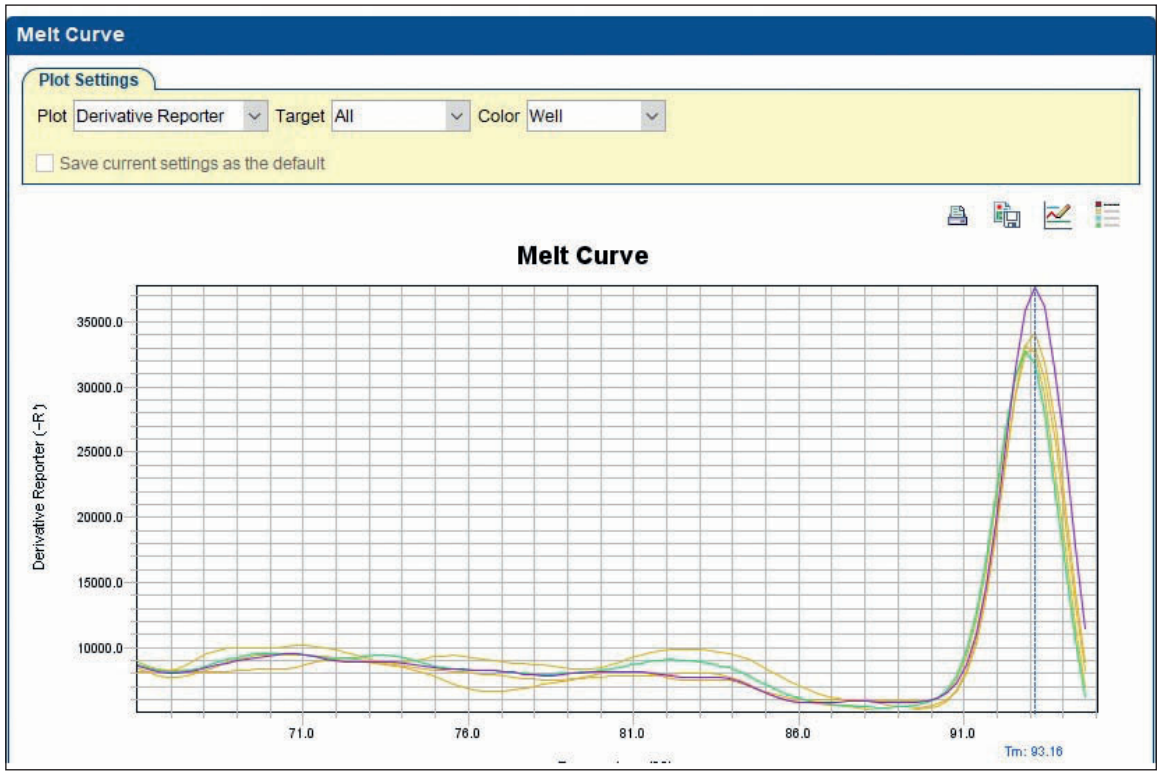

Fig. 7. Melt curve for isolates P9 for AlgD gene after treatment.

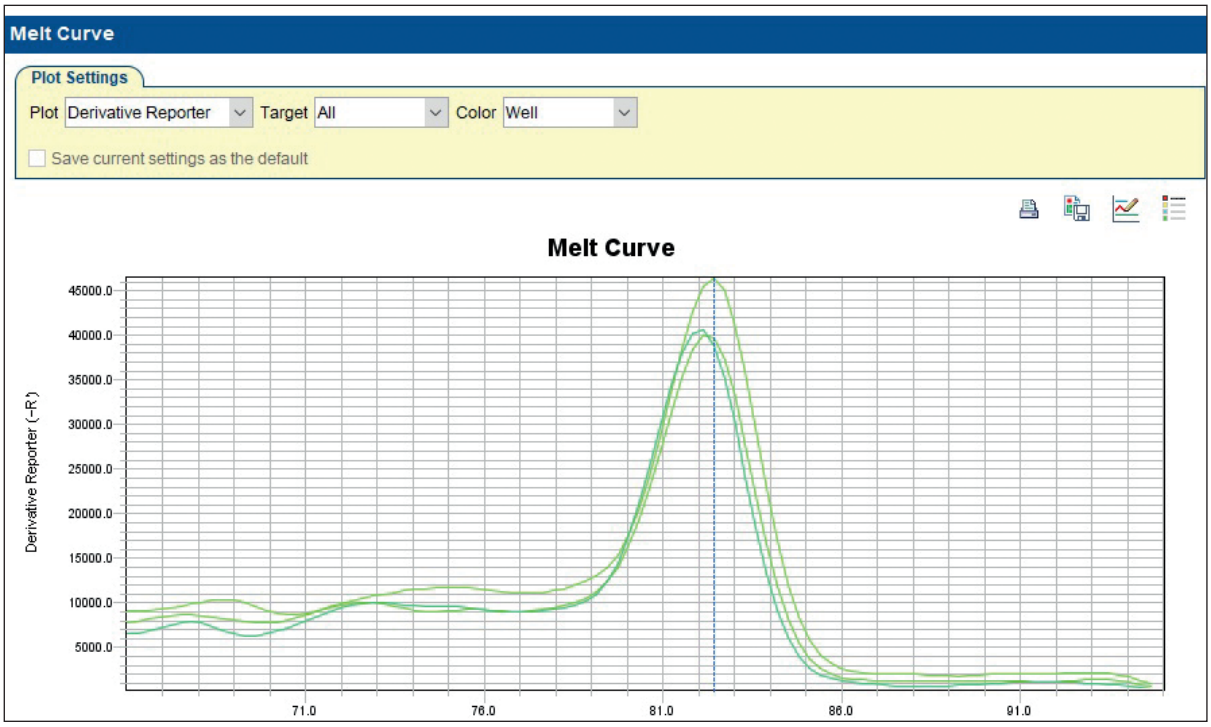

Fig. 8. Melt curve for isolates P9 for 16srRNA gene after treatment.

REAL TIME-QPCR

RNA EXTRACTION

RNA was extracted from the selected isolate *P. aeruginosa* (P9, P21), grown in cetrimide agar as control. Additionally, these samples were treated with sub-MICs of prodigiosin sub-MIC1 (43.7 and 87.5 µg/ml), sub MIC2 (21.9 and 43.7 µg/ml) and sub-MICs of ciprofloxacin sub-MIC1 (15.6 and 7.8 µg/ml), sub-MIC2 (7.8 and 3.9 µg/ml) and synergistic between prodigiosin and Ciprofloxacin sub-MIC1 (1.36 and 0.68 µg/ml for prodigiosin, 7.8 and 3.9 µg/ml for Ciprofloxacin), sub-MIC2 (0.68 and 0.34 µg/ml for prodigiosin, 3.9 and 1.95 µg/ml for ciprofloxacin) for two isolates *P. aeruginosa* (P9, P21). Total RNA of samples was extracted by using GENE$_{zol}$ ^{TM} TriRNA pure kit, the concentration ranging between 150-264 ng/µl and the purity fluctuated from 1.96 to 2.1.

Effect of (prodigiosin, ciprofloxacin, synergism between prodigiosin and Ciprofloxacin) on the expression of *AlgD* gene in *P. aeruginosa* Isolate

Quantitative real time PCR was carried out using SYBR green through a two-step RT-PCR method in order to determine the effect of sub-MICs of prodigiosin, ciprofloxacin and synergism between on the expression of *AlgD* gene in *P. aeruginosa* isolates (P9 and P21). The results are presented in Table (VI), Figure (3, 4, 5, 6, 7, 8). We used Livak equation $2^{-\Delta\Delta ct}$ which is suitable method to investigate the comparative variations in gene expression from real-time quantitative PCR experiments [48].

The results showed down regulation in the *AlgD* gene expression level under some treatments, while there was no gene expression in most treatments with both sub-MIC1 and sub-MIC2 of prodigiosin, ciprofloxacin and synergism between them, but there was slight differences in the results according to the isolate, treatments, concentrations, and duration of treatment effect. Both isolates (P9 and P21) were greatly affected by the synergism between prodigiosin and ciprofloxacin under the two sub-MICs of them. Sub-MIC1 (higher concentration) of all treatments was more efficient than sub-MIC2 (lower concentration) and in most treatments there was no gene expression either after 6 or 10 hours in addition to the presence of down regulation in *AlgD* expression by other treatments, while sub-MIC2 had lower effect. The antibiotics are capable of modifying cellular processes of bacteria when they are incorporated into culture media at sub-MIC [49]. In line with previous studies, [50] the pyocin/phage region is upregulated in response to subinhibitory ciprofloxacin 0.1 µg/ml for *P. aeruginosa.* Also, [51] ciprofloxacin is a bactericidal antibiotic of the fluoroquinolone drug class. It inhibits DNA replication by inhibiting bacterial DNA topoisomerase and DNA-gyrase. Of the fluoroquinolone class, ciprofloxacin is the most potent against gram-negative bacilli bacteria (namely, the *Enterobacteriaceae* suchas *Escherichiacoli*, *Salmonella spp*., *Shigella* spp., and *Neisseria*). [52] Ciprofloxacin also has effectiveness against some gram-positive bacteria. Ciprofloxacin is the most active against *Pseudomonas aeruginosa,* among the quinolones. [53] Sub-MIC of clindamycin modified the ability of *S. aureus* to form biofilms and shifted the nature and composition of the biofilm matrix toward rich eDNA content. [54] Detected the effect of antibiotics on *pvl, hla* and spa mRNA level and found the sub-MIC of clindamycin decrease *PVL*, *Hla* and *SpA* production in all tested strains.

CONCLUSIONS

So, in our study, both prodigiosin and ciprofloxacin greatly affected *AlgD* expression, although both of them approximately had same affect but prodigiosin can be considered better than ciprofloxacin since using antibiotics may lead to bacterial resistance. [55] Prodigiosin due to its hydrophobic properties is proposed to influence the function of biological membranes. Prodigiosin can enter into the cytoplasm of bacterial cells and, at higher concentrations, affects the membrane integrity by depletion of lipopolysaccharide layer of *E. coli* and *B. cereus*. [56] The antibacterial activity of prodigiosin (PG) is the result of ability to pass through the cellular membrane and damage it and capacity for inhibiting target enzymes involved in DNA replication, such as topoisomerase IV and DNA gyrase, which inhibit the cell growth.

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