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ORIGINAL ARTICLE

Inhibition of Quorum Sensing Character in *Pseudomonas aeruginosa* isolates and Its Effect on Biofilm Formation and Anti-Microbial Susceptibility Profile

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ABSTRACT

Quorum sensing (QS) plays a very important role in virulence and biofilm formation of Pseudomonas aeruginosa. We tested the effect of the aqueous garlic extract (AGE) on QS regulated biofilm formation and on antimicrobial susceptibility profile of P. aeruginosa isolates. One hundred P. aeruginosa isolates from patients with nosocomial infections from different departments of Sohag University Hospitals were collected during the period from April 2016 to April 2017. These isolates were tested for antibiotic sensitivity by the disc diffusion method and were screened phenotypically for biofilm formation by the Congo Red Agar (CRA) method and Tissue Culture Plate (TCP) technique. The presence of LasI, LasR, Rh1I and Rh1R genes in biofilm forming P. aeruginosa isolates was tested by using polymerase chain reaction (PCR). Biofilm producing strains were investigated by exposure to sub-inhibitory concentration of AGE as a quorum-sensing inhibitory agent. P. aeruginosa showed the highest antibiotic resistance rate to Piperacillin (85%), followed by Ticarcillin-Clavulanate (84%), while the highest sensitivity was to Colistin (73%), followed by Polymyxin B (64%), and lastly to Meropenem (56%). Eighty % of the isolated samples were biofilm producers, and most of these were from ICU patients. Strains were found to have different distribution of individual QS genes. LasI gene was present in 74% of isolates, LasR gene was present in 58% of isolates, Rh1I gene was present in 43% of isolates and Rh1R gene was present in 36% of isolates. On exposure of biofilm producing strains to the AGE there was significant improvement of the antibiotic sensitivity profile and significant decrease of biofilm formation. This work highlighted the bioactivity of garlic extract, as a Quorum Sensing inhibitory agent, in effectively inhibiting biofilm formation and increasing the sensitivity of the pathogens to antibiotics.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen that infects immunocompromised patients with cancer, AIDS, surgery, burn wounds, and patients with cystic fibrosis or nosocomial infections [1]. These infections are difficult to be eradicated because it is resistant to conventional antibiotic therapies; which are mainly due to its ability of biofilm formation [2] in which bacterial cells stick together and are embedded within a self-produced extracellular polysaccharide matrix [3]. For these reasons, *P. aeruginosa* infections are characterized by high morbidity and mortality rates[4]. *Pseudomonas aeruginosa* regulates its ability of biofilm formation and production of extracellular virulence factors as elastase, exotoxin A, exoenzyme S, pyoverdin, LasA proteases and haemolysins through cell-to-cell communication known as quorum sensing [5,6]. It is a process of bacterial communication used to collectively control group behaviors. This process depends on the production, release, and group-wide detection of signal molecules called autoinducers, which are homoserine lactones (HSLs) [7]. *P. aeruginosa* depends on two major quorum-sensing systems, the LasR-LasI and RhlR-RhlI, systems. In *P. aeruginosa*, LasI typically produces and LasR responds to autoinducer 3OC12–HSL. The LasR: 3OC12–HSL complex activates transcription of many genes as RhlR that encodes a second quorum sensing receptor[8]. RhlR binds to the autoinducer C4–HSL, the product of RhlI. RhlR: C4– HSL also directs a large regulon of genes, which are also members of the LasR regulon. This tandem regulatory arrangement helps LasI/R to control the first wave of quorum-sensing–controlled gene expression and helps RhlI/R to control the second [7]. Furthermore; a third signaling system uses another kind of signal molecule; 2-heptyl-3-hydroxy-4-quinolone, which has been named the Pseudomonas quinolone signal (PQS) and is able to affect the expression of Las- and Rhl-controlled genes was recorded[9]. With the widespread appearance of multi antibiotic resistant *P. aeruginosa*, it is becoming

increasingly more difficult to treat such infections with conventional known antibiotics [10]. Therefore, interfering with the QS activity of *P. aeruginosa* isolates using QS inhibitor compounds constitutes a very useful novel way for the management and the treatment of these infectious diseases. The presence of natural non-toxic QS inhibitor (QSI) agents in certain natural foods is interesting; offer a natural treatment of chronic *P. aeruginosa* infections; increase the sensitivity of the infecting organism to host defenses and its clearance from the host. Allium sativum (Garlic), a dietary medicinal herb has also been investigated for its QSI potential. Its constituents include oil soluble and water soluble organosulphur (thiosulphinates) compounds, vitamins, minerals and proteins [11]. The mechanism by which garlic can block QS is presently still unknown. However, the effect is at a posttranscriptional level, since the amounts of mRNA of neither LasI, lasR, rhlI nor rhlR were notably affected by the garlic treatment [12]. This suggests that the QSI molecules may interact directly with the QS receptors by either competitive or non-competitive N-acylhomoserine lactone mechanism. On this background; the main objectives of our study were to isolate and identify *P. aeruginosa* strains from hospital acquired infections supposed to be associated with biofilm formation; to study their antimicrobial sensitivity profile and to assess their ability of biofilm formation; and to evaluate the anti-QS and antibiofilm activity of garlic as a natural quorum sensing inhibitory agent against the isolates; and to assess the impact of this exposure on biofilm formation ability and antimicrobial sensitivity profile

METHODOLOGY

Study design and patients

This study was carried out in the Department of Medical Microbiology & Immunology, Faculty of Medicine, Sohag University during the period from April 2016 to April 2017. Two hundred and eighty five patients were in the study with health care associated infections (developed 48 to 72 hours after patient's admission) supposed to be caused by *P. aeruginosa* and associated with biofilm formation as; infected orthopedic implants, infected diabetic foot ulcer, ventilator-associated pneumonia (VAP), urinary tract infections in catheterized patients, chronic chest infections, infection in cystic fibrosis patients , surgical wound infection, blood stream infections , and infected burn wounds. Exclusion Criteria: infections caused by organisms other than *P. aeruginosa*. Oral informed consents were obtained from the patients. The study was approved by the ethical committee of Sohag Faculty of Medicine.

Microbiological analysis:

- Samples were collected under complete aseptic conditions using sterile cotton swabs and dry sterile well-closed plastic cups.
- Midstream urine samples were collected from the studied patients in sterile wide mouthed containers with cap tops after instructing them to clean the genital area with soap and water. In catheterized patients; the urine sample was collected through a sterile syringe after 10 min of clamping the catheter. Urine samples were inoculated on culture media using sterile disposable calibrated loops (1 µl &10 µl) to perform viable count and incubated aerobically at 37°c for 24-48 hours. After incubation, the colonies on plates were counted. Counts $\geq 10^5$ CFU/ ml of uncentrifuged urine were considered UTI.
- Collected samples were inoculated on Cetrimide agar *(Oxoid Ltd., Basingstoke UK)* and incubated aerobically at 37°c for 24-48 hours. Cetrimide agar is a type of agar used for the selective isolation of *Pseudomonas aeruginosa* as it contains cetrimide for inhibition of organisms other than *P. aeruginosa*.
- Colonies are smooth 2-3 mm in diameter, may be coalesed together with the detection of the yellow-green or blue-green fluorescent growth characteristic of *P. aeruginosa* as both pyocyanin and fluorescein are typically produced by strains of *P. aeruginosa*. Figure 1
- The isolated colonies were then further identified as *P. aeruginosa* by colony morphology, Gram staining appears as Gram negative cocco-bacilli and their biochemical characteristics were detected using API 20E test strips *(BioMerieux, France).*

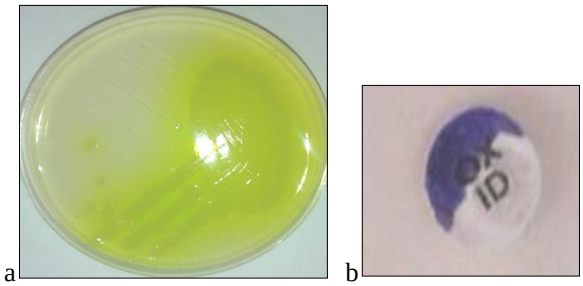

Fig. 1: a) Yellow green colonies of *Pseudomonas aeruginosa* on Cetrimide agar; b) oxidase positive.

Antibiotic Susceptibility Testing:

By modified Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines *(CLSI, 2016)*[13] using commercially available discs *(Oxoid Ltd., Basingstoke UK*). After incubation at 37˚C for 24 h; inhibition zone diameters were measured and the examined isolates were reported as susceptible, intermediate, or resistant to the antibiotics. (table 1)

Table 1: Performance standards for antimicrobial sensitivity testing according to CLSI guidelines *(CLSI, 2016)*[13]

Antimicrobial agent	Symbol	Disk content	Zone Diameter (mm)		
			Sensitive	Intermediate	Resistant
Piperacillin	PRL	100 ug	≥21	15-20	≤14
Piperacillin-tazobactam	TPZ	100/10ug	≥21	15-20	≤14
Ticarcillin-clavulanate	TIM	75/10 ug	≥24	16-23	≤15
Ceftazidime	CAZ	30 ug	≥18	15-17	≤14
Cefepime	FEP	30 ug	≥18	15-17	≤14
Aztreonam	ATM	30 ug	≥22	16-21	≤15
Imipenem	IPM	10 ug	≥19	16-18	≤15
Meropenem	MEM	10 ug	≥19	16-18	≤15
Colistin	CT	10 ug	≥11	-----	≤10
Polymyxin B	PB	300 units	≥12	-----	≤11
Gentamicin	CN	10 ug	≥15	13-14	≤12
Tobramycin	TOB	10 ug	≥15	13-14	≤12
Amikacin	AK	30 ug	≥17	15-16	≤14
Ciprofloxacin	CIP	5 ug	≥21	16-20	≤15
Ofloxacin	OFX	5 ug	≥16	13-15	≤12
Norfloxacin	NOR	10 ug	≥17	13-16	≤12

Phenotypic detection of biofilm formation by P. aeruginosa isolates:

a) Congo Red Agar method [14]*:*

A simple qualitative method for detection of biofilm formation by using Congo Red Agar (CRA) medium; CRA medium was prepared with brain heart infusion agar (37 g/L) *(Oxoid, UK)*, sucrose (50 g/L) *(Oxoid, UK)* and Congo Red indicator (0.8 g/L) *(Oxoid, UK)*. CRA plates were inoculated with the isolates and incubated at 37°C for 24 h aerobically [15]. Positive results were indicated by black colonies with a dry crystalline consistency. Weak biofilm producers were pink, with occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial picture indicated indeterminate results (Figure 2).

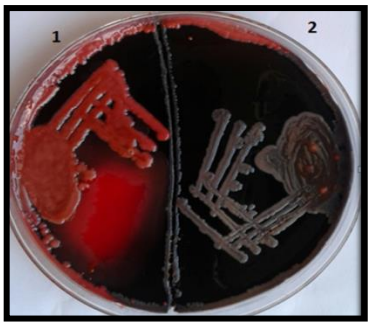

Fig. 2: CRA medium method; (1) Non biofilm forming isolate; (2) Biofilm forming isolate.

b) Tissue culture plate (TCP) method [16]:

This quantitative test is known as the gold-standard technique for biofilm detection. Organisms were isolated from fresh agar plates and were inoculated on 10 mL of trypticase soy broth with 1% glucose and were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well- flat bottom polystyrene tissue culture treated plates *(Oxoid, UK)* were filled with 200 µL of the diluted cultures. Negative control wells contained sterile broth. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed gently by tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times with ELISA washer *(Stat Fax 2600 washer, Awareness Technology Inc., Palm City,USA)*. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate for 20 minutes and were stained by crystal violet (0.1%) for 15 minutes. Extra stain was removed by using distilled water. Then the plates were air dried and then 95% ethanol was added for 30 minutes. Optical density (OD) of stained adherent bacteria was determined with ELISA auto reader *(Stat Fax 2600 autoreader, Awareness Technology Inc., Palm City,USA)* at wave length of 630 nm. The mean OD value obtained from negative control well was deducted from the average test OD values to obtain the OD value of each test strain. Biofilm formation by isolates was analyzed and grouped relying on the absorbance of the crystal violet-stained attached cells as weak, moderate or strong biofilm forming strain.[17] (Table 2, Figure 3&4)

Table 2: Interpretation of biofilm production by TCP method

Mean OD values	Adherence	Biofilm formation
< **0.062**	Non	Non
0.062 – 0.124	Weak	Weak
0.124 - 0.248	Moderate	Moderate
> **0.248**	Strong	Strong

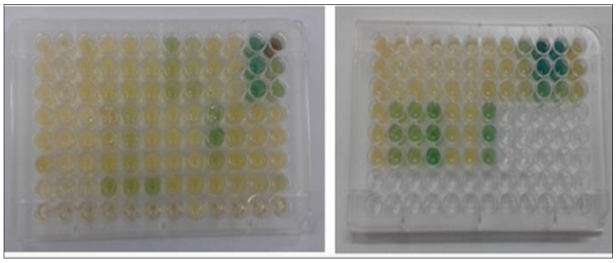

Fig. 3: TCP containing bacterial suspension and tryticase soya broth after incubation at 37 ^{0}C for 24 h.

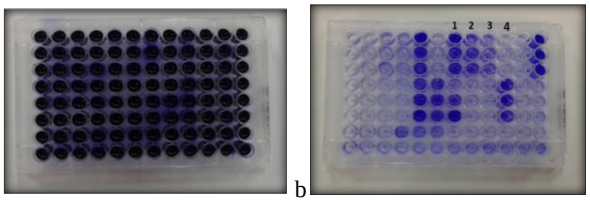

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Fig. 4: a) TCP after staining with crystal violet 0.1 %; b) TCP after addition of 95 % ethyl alcohol for 30 minutes: (1) Strong biofilm forming strain, (2) Moderate, (3) Weak, (4) Non biofilm forming strain.

Genotypic detection of genes coding for biofilm formation (LasI, LasR, Rh1I and Rh1R genes) by PCR:

DNA extraction was done according to the manufacturer's instructions by use of QIAamp DNA Kits ***(QIAGEN GmbH, Germany)***. The primers sequences ***(synthesized by Metabion International AG, Germany)***, sizes of the expected amplification products and thermal cycling conditions used for PCR amplification were listed in table 3.

Table 3: Primers sequences and thermal cycling conditions used for PCR amplification

Target genes	Primers used (sequence 5´-3´)	Thermal cycling condition	PCR product size
lasI Forward Reverse	CGTGCTCAAGTGTTCAAGG TACAGTCGGAAAAGCCCAG [18]	5 min of denaturation at 95°C (1 cycle), followed by 30 cycles of amplification; each of heat denaturation at 95 °C for 40 s, primer annealing at 52.5°C for 1 min, and DNA extension at 72 °C for 30 s then one cycle for final extension at 72°C for 7 minutes. The reaction was stopped by cooling at 4^{o}C.	295 bp
lasR Forward Reverse	AAGTGGAAAATTGGAGTGGAG GTAGTTGCCGACGACGATGAAG [18]		130 bp
Rh1I Forward Reverse	TTCATCCTCCTTTAGTCTTCCC TTCCAGCGATTCAGAGAGC [18]	5 min of denaturation at 95°C (1 cycle), followed by 35 cycles of amplification; each of heat denaturation at 95 °C for 40 s, primer annealing at 58.2 °C for 1 min, and DNA extension at 72 °C for 30 s then one cycle for final extension at 72°C for 7 minutes. The reaction was stopped by cooling at 4^{o}C.	155 bp
Rh1R Forward Reverse	TGCATTTTATCGATCAGGGC CACTTCCTTTTCCAGGACG [18]		133 bp

PCR and DNA amplification:

In a sterile 0.5 ml thermal cycler tube, 25µl PCR reaction mix containing 12.5µl PCR Master Mix (containing Mgcl, Taq, 10x PCR buffer and dNTPs) *(Jena Bioscience GmbH, Lobstedter, Germany)*, 8 µl PCR grade water, 1.25 µl of each primer and 2 µl of the extracted DNA sample was added. In each set of experiments, a negative control was included by replacing the DNA template with PCR grade water. Biometra thermal cycler *(T Gradient software PCR system version 4 - Biometra Whatman Company, Goettingen, Germany*) was used for amplification of DNA. Agarose gel electrophoresis was done as 10µl of each amplified DNA & 100bp ladder (molecular weight marker) *(GeneDireX)* were separated on 2% agarose gel stained with ethidium bromide using *Electrophoresis power supply (Biometra Whatman Company, Goettingen, Germany)*. The bands were visualized using UV transillumination and photographed using *InGenius3; gel documentation system (Syngene, Synoptics Ltd).*

Preparation of aqueous garlic extract (AGE) [19]:

100 grams garlic bulbs were peeled to obtain the edible portion, surface disinfected and then chopped and homogenized in 100 mL of autoclaved water. The extract was squeezed out and then filtered through sterile muslin cloth followed by filter sterilization (by the use of Millipore filters of pore size 0.25) to give a crude aqueous extract of 1000 mg of garlic/mL. This was collected in a sterile vial and stored at 4°C until used.

Determination of MIC (Minimum Inhibitory Concentration) and SIC (Sub-Inhibitory Concentration) of AGE of *P. aeruginosa* isolates by broth dilution method [20]:

Two fold serial dilutions of AGE solutions were done to prepare concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 mg/ml in sterile Trypticase Soya broth. The tubes were then inoculated with 0.1 mL of freshly grown cultures (18-20 hr old). Uninoculated tubes containing growth medium and extract were used as controls. The tubes were incubated at 37°c for 24 hours. The MIC defined as the lowest concentration (the highest fold dilution) of garlic extract that gave no increase in turbidity indicating no growth of the organisms. SIC defined as one fold dilution greater than the MIC. At this concentration of garlic extract some growth could be evident. Loop full of suspension from Trypticase Soya broth tubes containing SIC of garlic extract at the end of 24 hrs of incubation was streaked on sterile Cetrimide Agar plates. The plates were incubated at 37°C for a period of 24 hrs and checked for growth of culture at the end of the incubation period. Colonies that appeared on these plates were then considered as garlic exposed cultures. Antibiotic sensitivity profile of these cultures exposed to garlic extract, detection of biofilm formation by CRA and TCP methods and PCR for detection of quorum sensing genes were done.

Statistical Analysis

Quantitative data were expressed as means ± standard deviation. The data were tested for normality using Shapiro-Wilk test. The nonparametric Mann–Whitney test was used for data which wasn't normally distributed. Independent Samples t-test was used for normally distributed data. Chi-square ($\chi 2$) test was used for comparison as regarding qualitative variables, univariate and Multiple logistic regression tests was used to determine factors which associated with biofilm formation among studied population. A 5% level was chosen as a level of significance in all statistical tests used in the study.

RESULTS

- A total of 100 isolates (35.3%) were detected as *Pseudomonas aeruginosa* by conventional and biochemical methods. The patients' age ranged from 10 - 66 years (mean age ± SD was 49.36 ± 10.2 years). These patients were 67 (67%) males and 33 (33%) females.
- The majority of *P. aeruginosa* isolates were isolated from ICU patients (39%); followed by chest department (20%); then orthopedic department (15%); (10%) from department of general surgery; (10%) from department of vascular surgery and (6%) from plastic surgery department.
- The highest percentage of *P. aeruginosa* isolates were from patients with cystic fibrosis (20%); followed by equal percentage of isolates from patients with infected orthopedic implants (15%) and patients with ventilator-associated pneumonia (VAP) (15%); then from patients with UTI (14%); followed by Surgical site infection (10%); infected Diabetic foot (10%); from patients with COPD (10%) and lastly (6%) from patients with infected burn.
- **Antibiotic sensitivity profile of the isolated Pseudomonas aeruginosa strains:** The highest resistance rate was to piperacillin (85%), followed by ticarcillin-clavulanate (84%), while the highest sensitivity rate was to colistin (73%), followed by polymyxin B (64%), and lastly to meropenem (56%). (Table 4, Figure 5)
- ***Phenotypic detection of biofilm formation:***

By TCP method; 42% of isolates was negative and 58% were positive distributed as follows: 17% were strong biofilm forming isolates, 19% were moderate and 22%were weak biofilm forming isolates. However; by CRA test only 9% of isolates were capable of biofilm formation and 91% were negative. By comparing the use of CRA method in phenotypic detection of biofilm formation and the use TCP method; the TCP method was more sensitive (84.5%), and with a higher Specificity (100%) than CRA method, also it has higher positive (100%) and negative predictive (46.2%) values.

Re-assessment of antimicrobial sensitivity profile after exposure to AGE

Antibiotic sensitivity profile of ***P. aeruginosa*** isolates to all antimicrobial agents tested was increased with a high statistically significant difference indicating great effect of AGE on improvement of the antibiotic sensitivity profile. (Table 4, Figure 5)

Table 4: Comparison between antibiotics sensitivity before and after exposure to SIC of AGE

Antibiotic	Before	After	*P*-value
Piperacillin			
Sensitive (%)	1 (1%)	22 (27.4%)	0.000*
Intermediate (%)	14 (14%)	23 (28.8%)	
Resistant (%)	85 (85%)	35(43.8%)	
Piperacillin-tazobactam			
Sensitive (%)	19 (19%)	57 (71.3%)	0.000*
Intermediate (%)	25 (25%)	2 (2.5%)	
Resistant (%)	56 (56%)	21 (26.2%)	
Ticarcillin-clavulanate			
Sensitive (%)	2 (2%)	34 (42.5%)	0.000*
Intermediate (%)	14 (14%)	24 (30%)	
Resistant (%)	84 (84%)	22 (27.5%)	
Ceftazidime			
Sensitive (%)	10 (10%)	68 (85%)	0.000*
Intermediate (%)	11 (11%)	5 (6.3%)	
Resistant (%)	79 (85%)	7 (8.7%)	
Cefepime			
Sensitive (%)	18 (18%)	53 (65.4%)	0.000*
Intermediate (%)	9 (9%)	5 (6.2%)	
Resistant (%)	73 (73%)	23 (28.4%)	
Aztreonam			
Sensitive (%)	30 (30%)	68 (85%)	0.000*
Intermediate (%)	27 (27%)	1 (1.3%)	
Resistant (%)	43 (43%)	11(13.7%)	
Imipenem			
Sensitive (%)	42 (42%)	69 (86.2%)	0.000*
Intermediate (%)	13 (13%)	1 (1.3%)	
Resistant (%)	45 (45%)	10 (12.5%)	
Meropenem			
Sensitive (%)	56 (56%)	71 (88.8%)	0.000*
Intermediate (%)	8 (8%)	0 (0.0%)	
Resistant (%)	36 (36%)	9 (11.2%)	
Colistin			
Sensitive (%)	73 (73%)	71 (88.8%)	0.01*
Intermediate (%)	1 (1%)	2 (2.5%)	
Resistant (%)	26 (26%)	7(8.7%)	
Polymyxin			
Sensitive (%)	64 (64%)	72 (90%)	0.000*
Intermediate (%)	4 (4%)	0 (0.0%)	
Resistant (%)	32 (32%)	8(10%)	
Gentamicin			
Sensitive (%)	36 (36%)	68 (85%)	0.000*
Intermediate (%)	10 (10%)	3 (3.7%)	
Resistant (%)	54 (54%)	9(11.3%)	
Tobramycin			
Sensitive (%)	28 (28%)	62 (77.5%)	0.000*
Intermediate (%)	15 (15%)	5 (6.3%)	
Resistant (%)	57 (57%)	13(16.2%)	
Amikacin			
Sensitive (%)	44 (44%)	69 (86.3%)	0.000*
Intermediate (%)	15 (15%)	2 (2.5%)	
Resistant (%)	41 (41%)	9 (11.2%)	
Ciprofloxacin			
Sensitive (%)	37 (37%)	67 (83.7%)	0.000*
Intermediate (%)	13 (13%)	1 (1.3%)	
Resistant (%)	50 (50%)	12 (15%)	
Ofloxacin			
Sensitive (%)	41 (41%)	70 (87.5%)	0.000*
Intermediate (%)	9 (9%)	0 (0.0%)	
Resistant (%)	50 (50%)	10 (12.5%)	
Norfloxacin			
Sensitive (%)	44 (44%)	69 (86.3%)	0.000*
Intermediate (%)	9 (9 %)	0 (0.0%)	
Resistant (%)	47 (47%)	11 (13.7%)	

* Statistically significant

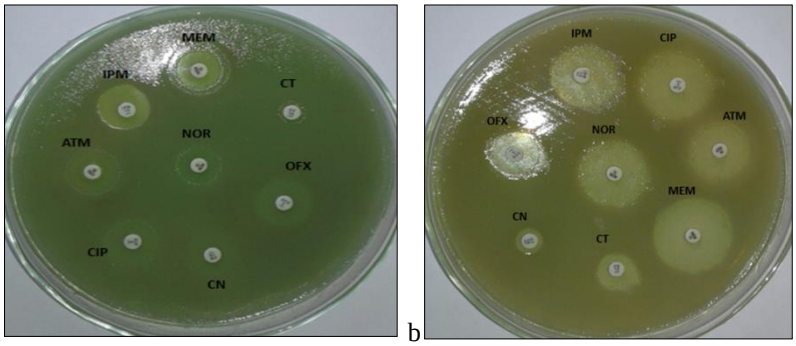

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Fig. 5 : a) Antibiotic sensitivity test by disc diffusion method for *Pseudomoas aeruginosa* strain 10 showing resistance to the following antibiotics: Meropenem (MEM), Imipenem (IPM),Colistin (CT), Aztreonam(ATM),Gentamicin (CN),Ofloxacin(OFX) ,Norfloxacin (NOR) and Ciprofloxacin(CIP) before exposure to the SIC of aqueous garlic extract; b) Antibiotic sensitivity test by disc diffusion method for the same strain after exposure to the SIC of aqueous garlic extract showing increased inhibition zone to the same antibiotic discs tested.

Re-assessment of biofilm forming ability after exposure to AGE

The number of biofilm forming isolates was statistically significantly reduced with a high statistically significant difference *P*-value < 0.05. (Table 5)

Table 5: Biofilm-forming isolates before and after exposure to aqueous garlic extract

Test	Before NO. (%)	After NO. (%)	*P*-value
Congo red test			
Positive	9 (9)	1 (1.3)	0.044*
Negative	91 (91)	79 (98.7)	
TCP test			
Positive	58 (58)	4 (5)	0.000*
Negative	42 (42)	76 (95)	

* Statistically significant

Genotypic detection of biofilm formation genes (LasI, LasR, Rh1I and Rh1R genes) by PCR:

Quorum sensing encoding genes were present in 80% of ***P. aeruginosa*** isolates. The distribution of quorum sensing genes among *P. aeruginosa* isolates was as follows: *LasI* gene was present in (74%) of isolates, *LasR* gene was present in (58%) of isolates, *Rh1I* gene was present in (43%) of isolates, and *Rh1R* gene was present in (36%) of isolates. (9%) of isolates were found to contain *LasI* gene only , (1%) of isolates contain *LasR* gene only , (2%) of isolates contain *Rh1I* gene only , (23%) of isolates contain *LasI*+ *LasR* genes , (4%) of isolates contain *LasI*+ *Rh1I* genes , (2%) of isolates contain *LasI*+*Rh1R* genes, (1%) of isolates contain *LasR*+ *Rh1I* genes, (5%) of isolates contain *LasI*+ *LasR*+ *Rh1I* genes, (2%) of isolates contain *LasI*+ *LasR* + *Rh1R* genes,(5%) of isolates contain *LasI*+ *Rh1I*+ *Rh1R* genes, (3%) of isolates contain *LasR*+ *Rh1I*+ *Rh1R* genes and (23%) of isolates contain *LasI*+ *LasR*+ *Rh1I*+ *Rh1R* genes . (Figures 6)

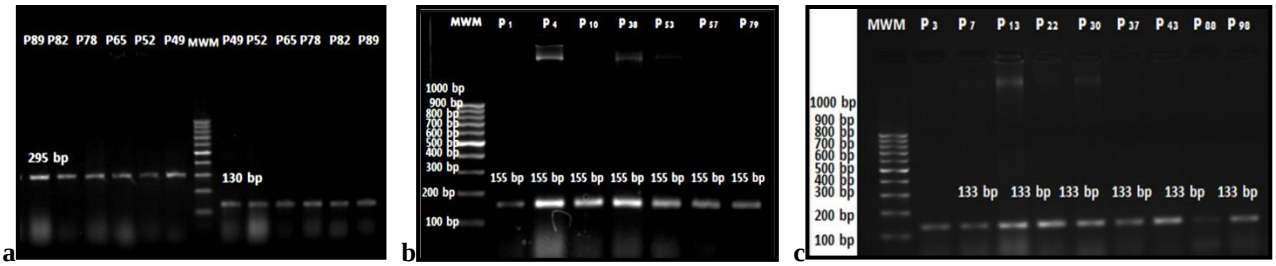

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Fig. 6: Agarose gel electrophoresis of PCR products. MWM-molecular weight marker (100 bp). a) P49 , P52 , P65 , P78 , P82 and P89 different strains of *P. aeruginosa* positive for *lasI* gene and *lasR* gene (*lasI* gene products at 295 bp) (*lasR* gene products at 130 bp). b) P1 , P4 , P10 , P38 , P53 , P57 and P79 different strains of *P. aeruginosa positive for rhlI* gene (*rhlI* gene products at 155 bp). c) P3 , P7 , P13 P22 , P30 , P37 , P43, P88 and P98 different strains of *P. aeruginosa positive for rhlR* gene (*rhlR* gene products at 133 bp).

DISCUSSION

Due to an increasing number of untreatable, persistent infections caused by *P. aeruginosa*; there is a rising need to develop novel strategies which deal with this phenomenon. Formation of biofilm is a survival strategy for *P. aeruginosa* to adapt to their living environment. Under the protection of biofilm, microbial cells become tolerant and resistant to antibiotics and the immune responses, which increase the difficulties for the clinical treatment of biofilm infections[21].

Therefore, there is a need for new treatment options to treat such biofilm-associated infections. Biofilm is one of the virulence factors controlled by the QS molecules of *P. aeruginosa* which adhere into the living surface of the host cells [22].

Therefore, targeting QS has emerged as an alternative strategy for controlling bacterial virulence, and studies have identified both synthetic and natural compounds capable of disrupting QS [23].

It has previously demonstrated that a crude extract of garlic specifically inhibits QS-regulated gene expression in *P. aeruginosa*, as judged from DNA microarray-based transcriptomic analysis [12]. The expression of 167 genes, 92 of which were regulated by QS, was repressed by the extract [12]. In this study, we tested the effects of aqueous garlic extract (AGE) on QS regulated biofilm formation by *P. aeruginosa* clinical isolates. In this study we detect biofilm formation before exposure to SIC of aqueous garlic extract by phenotypic methods: (CRA method & TCP); With the CRA method, 9 strains (9%) were found to be biofilm producers and 91 strains (91%) were non-biofilm producers. These results were near to that of the study of Afreenish et al[24]. With TCP method, 58 strains (58%) were found to be biofilm producers and 42 strains (42%) were non-biofilm producers.

These results were near to that of the study of Afreenish et al. [24] TCP method was more sensitive (84.5%), and had a higher Specificity (100%) than CRA method, also it had higher positive (100%) and negative predictive (46.2%) values. Knobloch et al.[25] did not recommend the CRA method for biofilm detection in their study. In this study we used PCR for detection of Quorum Sensing genes (LasI, LasR , Rh1I and Rh1R) as indicators for biofilm formation ability. In the present study, all the strains were found to have varied distribution of individual QS genes. Regarding frequency of quorum sensing genes among isolates; LasI gene was present in 74% of isolates, LasR gene was present in 58% of isolates, Rh1I gene was present in 43% of isolates, and Rh1R gene was present in 36% of isolates. Regarding distribution of genes among isolates; 9% of isolates were found to contain LasI gene only, 1% of isolates contain LasR gene only, 2% of isolates contain Rh1I gene only. These results were near that of Kadhim and Ali[26].

On comparison between TCP and PCR for detection of biofilm formation; Sensitivity of TCP method in comparison with PCR was 70%, specificity was 90%, positive predictive value was 96.6%, and negative predicative value was 42.9%. Twenty two strains of *Pseudomonas aeruginosa* were positive for biofilm formation by PCR but negative by TCP. Two isolates of *Pseudomonas aeruginosa* were negative for all QS genes but they were biofilm producers by TCP. This agrees with Dénervaud et al.[27] who confirmed that there may be other virulence factors which may not be stringently controlled by QS. In this study we used aqueous garlic extract (Allium Sativum) as quorum sensing inhibitor. After exposure of biofilm producing isolates to the sub-inhibitory concentration of AGE (SIC = 12.5 mg/ml) we reassessed antibiotic susceptibility and biofilm formation by phenotypic and genotypic methods. The antibiotic susceptibility profile showed significant improvement with *P*-value 0.000. These results were in accordance to those of Sneha et al.[20]. On reassessment of the ability of biofilm formation using CRA method and TCP method after exposure to the SIC of aqueous garlic extract, the number of biofilm forming isolates was significantly reduced with a high statistically significant difference *P*-value < 0.05.

CONCLUSION

This study highlighted the antibacterial effect of garlic as it has been exhibited a broad antibiotic spectrum against *Pseudomonas aeruginosa* isolates in addition to the reduction of biofilm formation ability. However; we did not find any loss of quorum sensing genes amongst the garlic exposed cultures probably indicating loss of drug resistance as a result of point mutations of quorum sensing genes and inhibition of biofilm formation. Such results explained the promising anti-QS-dependent therapeutic function of AGE against *P. aeruginosa.*

Conflict of Interests

The authors state that they have no conflict of interests.

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