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Pyocyanin and Biofilm Formation in *Pseudomonas aeruginosa* Isolated from Burn Infections in Baghdad, Iraq

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Abstract

Pseudomonas aeruginosa is emerging as important hospital pathogenic bacteria, which can persist in the environment for extended periods of time. Sixty-three isolates belonging to the *P. aeruginosa* were isolated from different clinical sources. The antimicrobial susceptibility test was performed by the disk diffusion method, and the biofilm formation was assayed by the micro titer plate. Pyocyanin was extracted from the culture supernatants, and the absorbance values were measured using a spectrophotometer. The percentage of resistance shown by the isolates were 87.3 % for cefotaxime, 52.38 % carbenicillin, 39.68 % imipenem, 11.11 % colistin, and 7.93 % Piperacillin-tazobactam. About 9.09 % exhibited positive results for the phenotypic production of extended spectrum β-lactamase, whereas 40 % of the imipenem-resistant isolates were Metallo-β lactamase (MBLs) producers. Among the burn isolates, 22.22 % formed moderate biofilms and 6.35 % formed strong biofilms. 34.92 % of the burn isolates were pyocyanin producers. All the pyocyanin-producer isolates carried phenazine biosynthetic operon *phzA-G* gene. The results of this study revealed a high prevalence of pyocyanin production and biofilm formation in the *P. aeruginosa* isolates from burn infections.

Keywords: *Pseudomonas aeruginosa*, Phenazine , Biofilm, Burn infections, Iraq.

1. Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen primarily causing nosocomial infections in immunocompromised patients and is responsible for high mortality rates in burn centers (Samira and Fereshteh, 2015; Klrissa and Mohammad, 2016). However, it is capable of causing a wide range of infections with damaged epithelial barriers (Engel and Balachandran, 2008). *P. aeruginosa* synthesizes a characteristic blue redox-active secondary metabolite, that is chloroform-soluble and a member of the tricyclic compounds "phenazine" called pyocyanin (1-hydroxy-5-methyl-phenazine (Ran *et al*., 2003). One of the most important symptoms of critical infections generated by these bacteria is the production of blue pus. Pyocyanin has been shown to inhibit respiration in mammalian cells (Rada and Leto, 2013), and the beating of human respiratory cilia *in vitro* (Wilson *et al.,* 1987). *P. aeruginosa* strains that do not produce pyocyanin have a low pathogenicity and a higher susceptibility to the immune response (Lau *et al*., 2004). *P. aeruginosa* PAO1 consist of two homologous core loci (operon *phzA1B1C1D1E1F1G1* and *phzA2B-2C2D2E2F2G2*) to be coded of phenazine-1-carboxylic acid and two phenazine genes (*phzM* and *phzS*) responsible for converting the enzymes of phenazine-1-carboxylic acid to pyocyanin (Mavrodi *et al*., 2001). Another important factor contributing to the *P. aeruginosa* pathogenicity in causing fatal infections is its potential to form biofilms on abiotic and biotic surfaces (Karatuna and Yagci, 2010). The populations of bacteria in biofilms are usually more resistant to antibacterial agents and host-mediated clearance strategies compared to their planktonic counterparts, giving rise to chronic infections that are notoriously difficult to eradicate (Mah *et al*., 2003). Bacterial cells which grow in biofilms produce extracellular polymeric matrices which hold the cells of the biofilm community together. Polysaccharides are important components of the biofilm matrix, as they contribute to the overall biofilm structure and to the resistance of grown bacteria in biofilm to certain antibacterial agents (Wozniak *et al*., 2003). This study is aimed at investigating the ability of different isolates of *P. aeruginosa* to produce pyocyanin and biofilm formation.

2. Materials and Methods

2.1. Bacterial Isolates

A total of sixty-three clinical isolates of *P. aeruginosa* were collected from several hospitals in Baghdad between December 2016 and April 2017 and identified using vitek 2 system and 16SrRNA gene by PCR.

2.2. Antibiotic Susceptibility Testing

The susceptibility of isolates to different antibiotics was tested using the Kirby-Bauer disk diffusion method following the Clinical and Laboratory Standards Institute

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guidelines (CLSI, 2011). Using antibacterial agents included: gentamicin (GM), tobramycin (TN), amikacin (AK), ciprofloxacin (CIP), levofloxacin (LEV), colistin sulphate (CO), piperacillin tazobactam (PTZ), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (ATM), carbenicillin (PY), and imipenem (IMP). On Mueller-Hinton agar plate (Himedia, India) using overnight culture at McFarland standard 0.5 followed by incubation at 35°C for eighteen hours.

2.3. Detection of Extended Spectrum β Lactamases Production

All isolates that showed resistance to the third generation of cephalosporin were tested to investigate the production of ESβLs by the disc approximation test, and the results were interpreted according to Collee *et al*. (1996) and Drieux *et al*. (2008). The tested isolates were inoculated according to Kirby-Bauer method onto the plate of Mueller-Hinton agar media. The Augmentin disc was placed in the center of the plate; cefotaxime and ceftazidime discs were placed at 3cm from center disc. The inhibition zones of the ceftazidime, cefotaxime and augmentin discs were compared after 16-18 hours of incubation at 35°C. The breadth of the zone of inhibition between ceftazidime, cefotaxime and augmentin disc was considered as isolates which produce extended spectrum β Lactamases.

2.4. Detection of Metallo-β Lactamase (MβLs)

The disc synergy test was used to detect the MβLs production according to Bashir *et al*., (2011) as follows: The tested *P. aeruginosa* isolates were inoculated according to the Kirby-Bauer method onto plates of Mueller-Hinton agar media. Two discs of imipenem antibiotic were placed on the plate; 5 µL of EDTA solution (final concentration is 0.5M) was added to one of them. The zones of inhibition of the imipenem disc and imipenem + EDTA disc were compared after incubation at 35°C for 16-18 hours. An increase in the inhibition zone size of at least 7 mm around the imipenem-EDTA disc more than the imipenem disc alone was considered as producers of MBLs.

2.5. Detection of Biofilm Formation

In the present study, *P. aeruginosa* isolates were screened for their ability to form biofilm by microtiter plate according to the method described by Mathur *et al*. (2006) and Lotfi *et al*. (2014). Twenty micro liter of bacterial suspension from an overnight culture was used to inoculate microtiter wells containing 180µL of Brain Heart Infusion (BHI) broth with 2 % sucrose. Control wells contained 200µL of BHI broth with 2 % sucrose. The covered microtiter plate was sealed with parafilm during incubation at 37°C for twenty-four hours. Unattached bacterial cells were removed by washing the wells three times with PBS (pH 7.2), they were dried at room temperature for fifteen minutes, and then 200µL of crystal violet (0.1%) was added to the wells for fifteen minutes. After removing the crystal violet solution, the wells were washed three times with PBS (pH 7.2) to remove the unbounded dye and, and were allowed to dry at room temperature, then they were extracted twice with 200µL of 95 % ethanol. The absorbance of each well was measured at 630nm using ELISA reader. The O.D value for control well was deducted from all the test O.D value.

2.6. Phenotypic Detection of Pyocyanin Production

Pyocyanin production was detected for all the sixty-three isolates on King's B medium (King *et al*., 1954) and Mueller-Hinton agar media by streaking the overnight culture followed by incubation at 30°C for twenty-four hours.

2.7. The Quantitative Pyocyanin Assay

The quantitative pyocyanin assay depends on the absorbance of pyocyanin at 520 nm in acidic solution. Five mL of culture grown in Tryptone broth (T-broth) for the production of pyocyanin was extracted with chloroform (3 mL) and then re-extracted into 1 mL of HCl (0.2-N) to give a pink to deep red solution. The absorbance of this solution was measured, and the concentrations expressed as µg/mL of pyocyanin produced by the culture supernatant were determined by multiplying the OD at 520 nm by 17.072 (Essar *et al*., 1990).

2.8. Molecular Detection of Pyocyanin Genes.

Detection of pyocyanin genes for producing isolates, using primers for phenazine biosynthetic operon *phzA-G*, and two phenazine modifying genes *phzM* and *phzS* genes as shown in Table 1 according to Jamileh *et al*.(2012).

Table 1. Primers for phenazine biosynthetic operon *phzA-G*, and two phenazine modifying genes *phzM*and *phzS* genes (AlphaDNA, Canada).

Genes	Primers	Primers Sequence(s)	PCR product
Phenazinebiosynthetic operon (*phzABCDEFG*)	PHZAF	5´-CCGTCGAGAAGTACATGAAT-3´	448 bp
	PHZAR	5´ -CATAGTTCACCCCTTCCAG-3´	
Phenazine-specific methyl- transferase (*phzM*)	PHZMF	5´-AACTCCTCGCCGTAGAAC-3´	313 bp
	PHZMR	5´-ATAATTCGAATCTTGCTGCT-3´	
Flavinecontaining Mono- oxygennase (***phzS***)	PHZSF	5´-TGCGCTACATCGACCAGAG-3´	664 bp
	PHZSR	5´-CGGGTACTGCAGGATCAACT-3´	

The typical colonies of *P. aeruginosa* were cultured on BHI agar (Himedia, India) for twenty-four hours at 37˚C. The total DNA was isolated from the colonies on BHI using microwave method according to Ahmed *et al*. (2014); cell pellets were washed with 1 mL of Tris- EDTA and were resuspended in 100 µL of TE. After the addition of 50 µL of 10 % Sodium dodecyl sulfate (SDS), the mixture was incubated for thirty minutes at 65°C. The lysates were centrifuged and the supernatants were removed. The microtubes were then placed in a microwave oven and heated. The pellets were dissolved in 200 µL of TE, and were extracted with an equal volume of

phenol/chloroform/ isoamyl alcohol (25:24:1) for fifteen minutes. The aqueous phase was recovered by centrifugation for twenty minutes and precipitated with ethanol. The total DNA was used as DNA template for PCR amplification. For PCR amplification, the reaction mixture (25 µL) contained: 1 µL (10 pmol/µL) of each forward and reverse oligo nucleotide primers as described in Table 1, 12.5µL of GoTaq®Green Master Mix2X (Promega, USA), 5µL of template, and 5.5µL of nuclease free water (Promega, USA). Amplifications were performed by a DNA Thermal Cycler (Gradient thermocycler Polymerase Chain reaction,TechNet-5000, USA). The cycling program included a five-minute initial denaturation step at 94˚C, followed by thirty cycles of denaturation for thirty seconds at 94˚C, annealing of primers for thirty seconds at 60 ˚C, and primer extension for thirty seconds at 72˚C with auto extension. After the last cycle, the PCR tubes were incubated for ten minutes at 72 ˚C. PCR products were detected by electrophoresis on 1 % agarose gel by staining with ethidium bromide.

3. Results and Discussion

3.1. Bacterial Isolates

P. aeruginosa infections are opportunistic bacteria in nature and ranged from those associated with catheter, ventilator, wound and burn to pulmonary infections in cystic fibrosis patients and keratitis in contact lens (Choy *et al*., 2008). In this study, 63 clinical isolates of *P. aeruginosa* obtained from patients with suspected infection were collected primarily from burn (n=37), wound swab (n=9), fluids (n=4), ear (n=4), keratitis (n=3), urine (n=3), catheters (n=2), and from blood (n=1).

3.2. Antimicrobial Susceptibility of P. aeruginosa isolates

The isolates showed high susceptibility for piperacillin + tazocin the resistance rate was 7.93% (n=5) in comparison to other antibiotics used in this study, and the high resistance was investigated to cefotaxime 87.3 % (n=55). The isolates were resistant to carbenicillin (52.38 %), and exhibited the same resistance for aztreonam and imipenem (39.68 %) (Table 2). Resistance to gentamicin, tobramycin, and amikacin was confirmed in 69.84 % (n=44), 74.6 % (n=47), and 61.9 % (n=39) of the isolates, respectively. Whereas 55.55 % (n=35) and 57.14 % (n=36) of the isolates exhibited resistance to ciprofloxacin and levofloxacin respectively.

Among the third generation cephalosporin resistant isolates, 9.09 % exhibited positive results for phenotypic production of extended spectrum β-lactamase (ESBLs), whereas 40 % of the imipenem resistant isolates were Metallo-β lactamase (MBLs) producers.

In the present study, a total of sixty-three MDR P. aeruginosa clinical isolates collected from several hospitals as a result of the emergence of MDR P. aeruginosa became difficult infections to treat. Clinically, antibiotics-resistant bacteria are responsible for the increased length of hospitalization, cost and mortality (Mansouri *et al*., 2013).

In the current study, isolates exhibited resistances for β-lactams in a varied levels especially for the third-generation cephalosporin, but at the same time several isolates were ESBLs producers.

Table 2. Antimicrobial susceptibility test results of *P. aeruginosa* isolates.

Antimicrobials Agents	Resistance %
Cefotaxime	87.3
Carbencillin	52.38
Aztreonam	39.68
Imipenem	39.68
Ceftazidime	30.15
Piperacillin-tazobactam	7.93
Tobramycin	74.6
Gentamicin	69.84
Amikacin	61.9
Levofloxacin	57.14
Ciprofloxacin	55.55
ColistinSulphate	11.11

Also, the isolates revealed resistance to gentamicin, tobramycin, ciprofloxacin, levofloxacin. These results are in agreement with those obtained by Al-Marjani and Khadam (2016). In fact, they isolated *P. aeruginosa* multi-drug resistant from different infection sites. This high multi resistance could be due to the production of hydrolytic enzymes and the acquisition of resistance mechanisms by *P. aeruginosa* strains. Nikokar *et al.* (2013) reported that 74.4 % of the isolates were resistant to carbenicillin, whereas Moazami-Goudarzi and Eftekhar (2013) showed that 94.7 % of isolates were imipenem resistant.

3.3. Biofilm Formation

All the isolates were divided into three categories according to biofilm analysis. 46.03 % (n=29) of isolates were categorized as weak biofilm producers, 39.68 % (n=25) produced moderate biofilm, and 14.28 % (n=9) formed strong biofilm. Isolates from burns and wounds had a greater ability to form strong and moderate biofilm compared with the isolates obtained from other sources (Table 3).

The results of the current study showed the ability of biofilm formation in clinical *P. aeruginosa* isolates. Biofilm formation is an important mechanism involved in resistance of bacteria.

Table 3. Type of specimen and biofilm formation in *P. aeruginosa* isolates

Biofilm Formation	Type of specimens								
	Burns No. (%)	Wounds No.(%)	Fluid No.(%)	Ear No. (%)	Keratitis No.(%)	Urine No.(%)	Catheters No.(%)	Blood No.(%)	Total No.(%)
Weak	19(30.16)	2(3.17)	2(3.17)	2(3.17)	2(3.17)	2(3.17)	0	0	29(46.03)
Moderate	14(22.22)	5(7.94)	1(1.59)	2(3.17)	0	0	2(3.17)	1(1.59)	25(39.68)
Strong	4(6.35)	2(3.17)	1(1.59)	0	1(1.59)	1(1.59)	0	0	9(14.2)
Total	37	9	4	4	3	3	2	1	63

In the current study 6.35% and 22.22% of burns isolates were strong and moderate biofilm producers respectively, and 3.17% and 7.94% of wounds isolates produced strongly and moderately biofilm respectively. Perez *et al*. (2011) showed the high potential of biofilm formation by clinical *P. aeruginos*a isolates regardless of the specimen source.

3.4. Phenotypic Detection of Pyocyanin Production

All fluids isolates 6.35 % (n=4) and keratitis 4.76 % (n=3) isolates produced pyocyanin on King's medium 12.7% (n=8) of wounds isolates and 34.92% (n=22) of burns isolates were pigment producers. Catheters isolates exhibited negative results (Table 4).

Table 4 . Type of specimen and pyocyanin production in *P.aeruginosa* isolates.

Pyocyanin production	Type of specimens								
	Burns No.(%)	Wounds No.(%)	Fluid No.(%)	Ear No.(%)	Keratitis No.(%)	Urine No.(%)	Catheters No.(%)	Blood No.(%)	Total No.(%)
Producer	22(34.92)	8(12.7)	4(6.35)	1(1.59)	3(4.76)	1(1.59)	0	1(1.59)	40(63.50)
Non	15(23.81)	1(1.59)	0	3(4.76)	0	2(3.17)	2(3.17)	0	23(36.50)
Total	37(58.73)	9(14.29)	4(6.35)	4(6.35)	3(4.76)	3(4.76)	2(3.17)	1(1.59)	63

3.5. The QuantitativePyocyanin Assay

The pyocyanin production was detected by quantitative assay for twenty-two isolates using spectrophotometer. *P. aeruginosa* isolated from burn infections were the highest pyocyanin producers. Pyocyanin production reached 10.85 µg/ml from the burn isolates, whereas the pyocyanin production in the rest of the *P. aeruginosa* isolates from wound infections ranged from 3.312 to 6.163 µg/ml, and from body fluids it ranged from 3.54 to 6.354 µg/ml. The variation in the production of pyocyanin among different *P. aeruginosa* isolates could be attributed to regulators. The overexpression of some genes in *P. aeruginosa* significantly reduced the homoserine lactone signals accumulation and affected the production of pyocyanin (El-Fouly *et al*., 2015).

3.6. Molecular Detection of Pyocyanin Genes.

All the pyocyanin-producer isolates carried phenazine biosynthetic operon *phzA-G* gene, and two phenazine modifying genes *phzS* and *phzM* genes.

phzM encodes a protein (36.4kDa) that most closely resembles O-methyl transferases of bacteria. *phzS* encodes a protein (43.6 kDa) similar to monooxygenases of bacteria. Among the pseudomonads, only *P. aeruginosa* has been found to contain two copies of the phenazine operon (Jamileh *et al*., 2012). *P. aeruginosa* isolates that do not produce pyocyanin have a low pathogenicity and a higher susceptibility to the immune response (Lau *et al*., 2004).

The EPS matrix of *P. aeruginosa* primarily consists of biomolecules, such as polysaccharides, proteins, extracellular DNA, metabolites (phenazines), siderophores and exotoxin. These molecules that are present in the matrix play an important role in the *P. aeruginosa* infections (Das *et al*., 2016).

Phenazines such as pyocyanin and the release of DNA from cells providing freely-available extracellular DNA by *P. aeruginosa*, are the major factors dictating the biofilm formation and the persistent infection within the host. Extracellular DNA is similarly a key factor in the biofilm formation of *P. aeruginosa* and in protecting the bacterial cells by inducing drug resistance. It is also a contributing factor to the high viscosity of cystic fibrosis sputum that blocks the respiratory airway passages (Das *et al*., 2016). Pyocyanin has been shown to intercalate with extracellular DNA to promote cell-to-cell interactions between the *P. aeruginosa* cells by influencing their physicochemical interactions and the cell surface properties. It has been suggested that pyocyanin may also contribute to the biofilm formation by the promotion of extracellular DNA (Das and Manefield, 2012).

4. Conclusion

In conclusion, this study determined the ability of MDR *P. aeruginosa* isolates for biofilm formation and pyocyanin production. Biofilm formation is associated with the increased resistance to the antibiotic and bacterial colonization within the burn infections. The resistant isolates achieve high levels of biofilm -specific resistance despite producing moderate or weak biofilms. Biofilm formation and pyocyanin can increase the toxicity and pathogenicity of this bacterium. All the *P. aeruginosa* even MDR and biofilm-forming isolates had low resistance to piperacillin-tazobactam.

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