

Colony Morphology and *pelA*, *pslD* and *algD* genes in *Pseudomonas aeruginosa* isolated from Baghdad Hospitals.

Maha M .Kadum, Mohammed F. AL- Marjani, Munim Radwan Ali

Department of Biology , College of Science , Mustansiriyah University, Baghdad, Iraq

** Corresponding Author: Mohammed F. Al- Marjani , Prof. of Microbiology in College of Science , Mustansiriyah University , Baghdad, Iraq*

E-mail: marjani20012001@gmail.com , dr.marjani@uomustansiriyah.edu.iq

Abstract:

Pseudomonas aeruginosa is emerging as an important pathogen in both hospital and community-acquired infections, as a result of its ability to produce biofilm formation and resistance for many antibiotics. In the current study, Sixty-three isolates belonging to the *P.aeruginosa* were isolated from different clinical sources and individually screened for antimicrobial susceptibility. The results showed high resistance of isolates for cefotaxime 87.3%, 52.38% of isolates were resistant to carbenicillin and 11.11% to colistin.

Colony morphology was determined on Congo red agar, *pelA*, *pslD* and *algD* genes were detected using PCR technique. All isolates were produced thin layer pellicle at air-liquid interface and mucoid, smooth, semi-wrinkled colonies on Congo red agar. Only 5 isolates revealed different fragments of *pelA1*, 2 and *pslD* genes. On the other hand, 72.41% of isolates were carrying the *algD* gene. The appearance of colonies is evidence of mutations especially in *pel* genes, but some of them appeared with orange colour. Depending *algD*, *pelA*, and *pslD* results, and phenotype detection, in this study most of the local isolates are mucoid as a result of incidence of *algD* gene among these isolates and high-frequency mutations of *pel*, and *psl* genes, suggesting that biofilm formation process depends on alginate as essential ESP apart from *Pel* and *Psl* matrices.

Keywords: *Pseudomonas aeruginosa*, wrinkled colony, exopolysaccharide, *pelA*, *pslD*.

I. INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen in nosocomial infections and responsible for high mortality rates in burn centers (Lipovyt et al., 2010). *P.aeruginosa* can develop antimicrobial resistance either through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms or by the acquisition of resistance genes on the plasmid. Both strategies for developing drug resistance can severely limit the therapeutic options for serious infections treatment (Streeter and Katouli, 2016).

Infections caused by MDR *P.aeruginosa* are associated with significant increases in morbidity, mortality, need for surgical intervention double the length of hospitalization and overall cost of patient care (Akingbade et al., 2012). At least, three polysaccharides were produced by *P.aeruginosa* (alginic acid synthesis, pellicle formation locus (*Pel*), polysaccharide synthesis locus (*Psl*)) and that are determinant for the stability of the biofilm structure. Mucoid and non-mucoid *P.aeruginosa* strains differ by the qualitative composition of their polysaccharides in the biofilm matrix, predominantly alginate or *Psl* (mannose-rich polymer)/*Pel*(glucose-rich matrix material), respectively (Maet et al., 2012; Lavery et al., 2014). Alginates, of mucoid *Pseudomonas* sp. are anionic linear unbranched polymers composed of D-mannuronic (M) acid and L-guluronic acid (G) with a high molecular weight, contributes to the structural stability and biofilms protection and to the water and nutrients retention as well (Powell et al., 2018). The autoaggregation ability leads to several macroscopic phenotypes; among them are the pellicles production at the air-liquid interface of standing liquid cultures and the highly structured colonies production on agar plates, *pel* mutants do not form pellicles or mature solid-surface-associated (SSA) biofilms (Friedman and Kolter, 2004). Mannose rich polymer *Psl* plays an essential role in the initial steps of biofilm formation by non-mucoid *P. aeruginosa* as well as in its maintenance (Heydari and Eftekhari, 2015). This study aims to determine antibiotic susceptibility and investigate the colony morphology and *pelA*, *pslD* and *algD* genes in *P.aeruginosa* isolated from Baghdad Hospitals.

II. MATERIALS AND METHODS

Bacterial Isolates: A total of 63 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 16S rRNA using PCR according to Spilker et al. (2004).

Antibiotic Susceptibility Testing: The isolates were subjected to antimicrobial susceptibility testing using Kirby-Bauer disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,2016), using commercially available 6mm discs (MAST Group,UK). The susceptibility of the isolates was determined against 12 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), Imipenem (IMP). On Mueller- Hinton agar Plate (Himedia, India) using overnight culture at a 0.5 McFarland standard followed by incubation at 35°C for 18 hrs.

Pellicle formation assay: Pellicle forming was detected according to Friedman and Kolter (2004). T-broth prepared by dissolving 10g of tryptone and 5g of NaCl in 1L of distilled water, and dispensed into glass tubes to autoclaved, then inoculated with 10 µl of bacterial suspensions and incubated at 27°C for 24hrs., tubes contained T-broth without inoculum considered as a negative control. The existence of an opaque layer at the air-liquid interface in these tubes considered as a positive result.

Wrinkled colony assay: This assay was carried out as described by Friedman and Kolter (2004) as follows: Congo red agar: Prepared by dissolving 10g of tryptone, Congo red 40µg/ml and Coomassie brilliant blue 15µg/ml, in addition to 0.5-1.5% agar and then autoclaved. The overnight culture at a 0.5 McFarland standard for selected isolates were cultured by spotting 10µl on Congo red agar and incubated at 27°C for 6 days to assess the colony morphology, each plate inoculated with 5 isolates.

Detection of *pelA* and *pslD* : PCR amplification for *pelA1*, *pelA2*, and *pslD* genes was performed using specific primers designed in this study by (Alpha DNA/Canada), *pelA1* (forward: 5'-ACCGAGGTGAGGAACGGATA-3', reverse: 5'-GGTGACCTGGATGACCACTG-3') with amplicon size (612bp), *pelA2* (forward: 5'-CCACTGGTCATCCAGGTCAC-3', reverse: 5'-GCCTGGAACAGCCAGGTAAT-3') amplicon size (722 bp) and *pslD* (forward: 5'-CCGCATCAGCATGTTTCCAG-3', reverse: 5'-CTTGAAGCTCGCGCTGGTAGA-3') with amplicon size (914bp). Optimization of PCR conditions were accomplished by gradient PCR (TechNet-5000 /USA) to determine the accurate annealing and reaction conditions for these primers which were as following: Initial denaturation 95°C/5min, followed by 30 cycles each cycle included: denaturation 95°C/1min; annealing 61°C/45sec and extension 72°C/1min, and then final extension 72°C/10min. The Multiplex reaction mixture was prepared in a total volume of 25 µl, that composed of 12.5µl of GoTaq® Green Master Mix (Promega/ USA) 1µl of each forward and reverse primer and 1.5µl of nuclease-free water. Then 5µl of prepared template DNA by boiling was added. PCR mixture without template DNA was considered as the negative control. PCR amplicons were detected by gel electrophoresis.

Detection of the *algD* gene: Existence of *algD* gene was detected by PCR amplification using specific primers and reaction conditions as described by Stover et al. (2000), (forward: 5'-ATGCGAATCAGCATCTTTGGT-3', reverse: 5'-CTACCAGC AGATGCCCTCGGC-3'), with amplicon size (1310 bp). PCR mixture consisted of 12.5 µl of GoTaq® Green Master Mix, 1µl of each forward and reverse primer, and 5.5µl of nuclease-free water, then 5µl of prepared template DNA was added to reach total volume 25 µl. Negative control was represented by PCR mixture without template DNA. Gel electrophoresis analysis for PCR amplicons. PCR conditions were Initial denaturation 96°C/5min, followed by 35 cycles each cycle included: denaturation 95°C/1min; annealing 63.3°C/30sec and extension 72°C/1min, and final extension 72°C/7min.

III. RESULTS

Bacterial Isolates

The 63 clinical isolates of *P. aeruginosa* obtained during routine diagnostic assessment of 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).

Antimicrobial Resistance

All isolates showed high susceptibility for Piperacillin+Tazocin 7.93% (n=5) in comparison to other antibiotics used in this study, and high resistance 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%) (Figure.1). 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. Colistin 11.11 % (n=7). Whereas 55.55 % (n=35) and 57.14% (n=36) of the isolates exhibited resistance to Ciprofloxacin and Levofloxacin respectively.

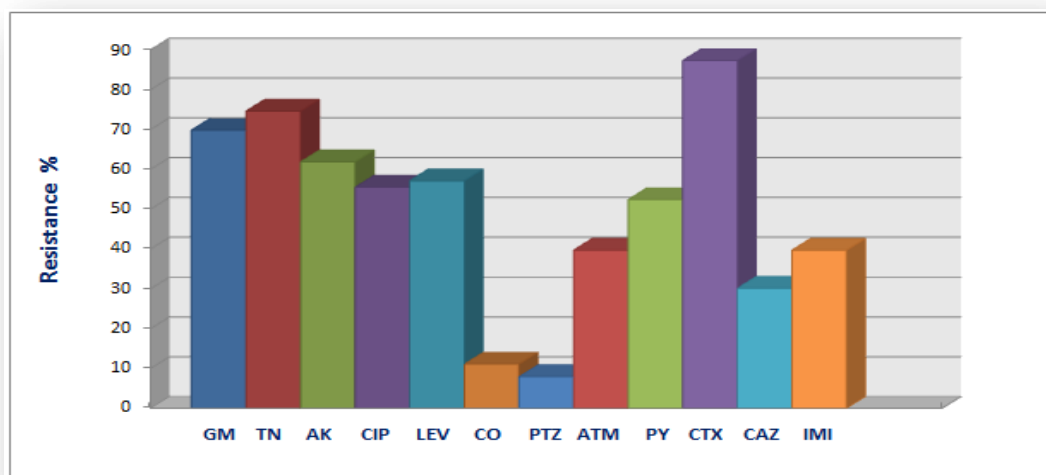


Figure (3-3): Antibiotic resistance of *P. aeruginosa* isolates: GM = Gentamicin, TN = Tobramycin, AK= Amikacin, CIP = Ciprofloxacin, LEV=Levofloxacin, CO = Colistin, PTZ= Piperacillin/Tazobactam, ATM= Aztreonam, PY = Carbencillin, CTX = Cefotaxime, CAZ = Ceftazidime, IMI = Imipenem.

Pellicle Formation assay: The results showed the ability of *P.aeruginosa* isolates to form a thin layer pellicle at the air-liquid interface of standing cultures.(Figure.2 A).

Wrinkled colony assay: Wrinkled colony assay for isolates on Congo red agar revealed that all isolates phenotypically displayed partially wrinkled, mucoid and smooth colonies on Congo red agar. Some of them appeared with orange colour. (Figure.2 B).

Detection of *pelA* and *pslD*: Three burns isolates showed different fragments represented by B25 which showed expected amplicons for *pelA1*, *pelA2* and *pslD*, when electrophoresed. Furthermore, isolate B22 revealed PCR products for *pelA1*, and *pslD*, whereas B35 isolate had *pelA1* only. Whereas 2 wounds isolates W7 and W9, were carrying *pelA1*, *pelA2*. All the 22 isolates were negative for both genes.(Figure.3).

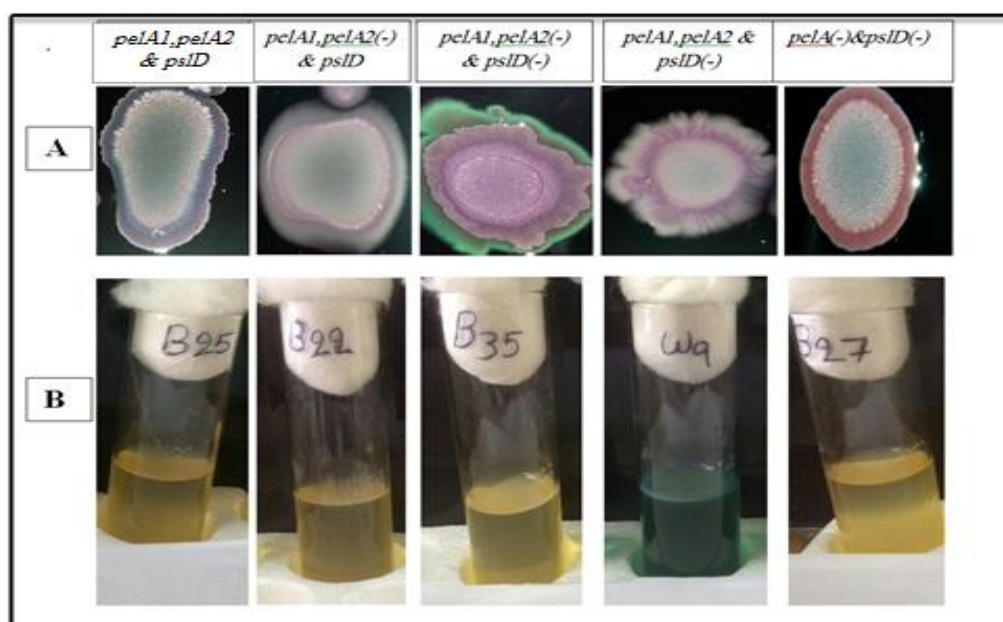


Figure (2).A): Genotype and colony morphology for *P. aeruginosa* isolates B25, B22, B35, W9, and B27. B): Thin layer pellicle for the same isolates.

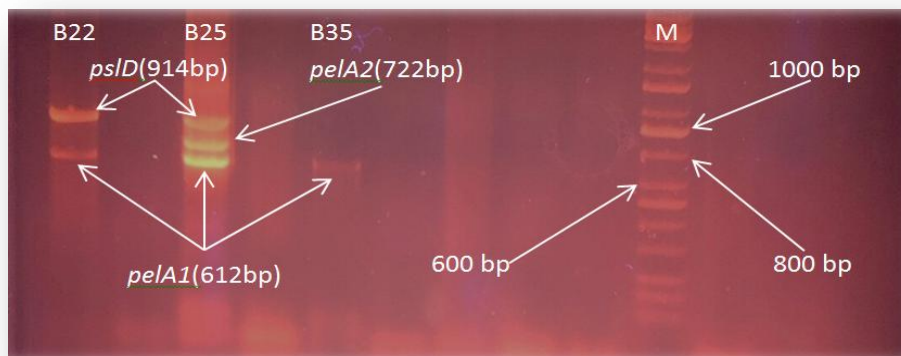


Figure (3)Gel electrophoresis of *P.aeruginosa* burn isolates: B36:pelA1, pelA2 and pslD genes. B22: pelA1(612bp) and pslD(914bp), B25: Multiplex for 3fragments, and B35: pelA1 (612 bp). M.100bp ladder. Agarose gel electrophoresis was performed using 1% agarose gel, and the run lasted for 50 min /100V.

Detection of algD gene:

Alginate production was investigated by algD gene detection in isolates by PCR, which showed that all burns isolates carrying algD except 5 isolates gave negative results, as well as 1 wound, 1 catheter and 1urine isolates. (Figure 4).

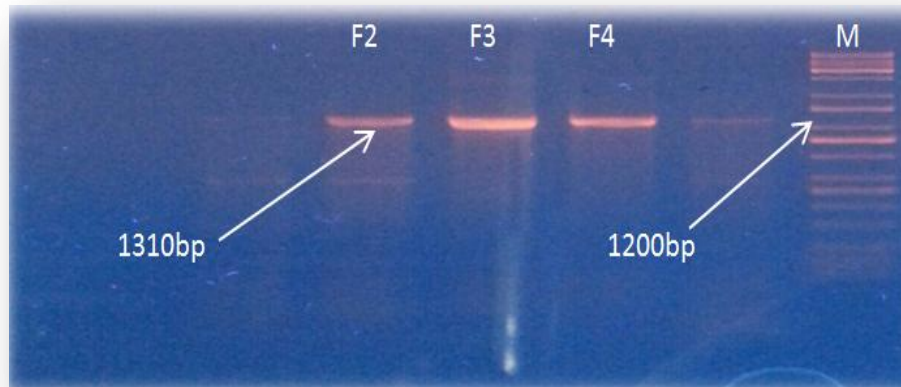


Figure (4): Gel electrophoresis of algD gene (1310bp). Lanes:1-3, isolates F2, F3, F4. M: 100bp ladder. Agarose gel electrophoresis was performed using 1% agarose gel, and the run lasted for 50 min /100V.

IV. DISCUSSION

P.aeruginosa infections usually occur and/or with hospitalized immune-compromised patients. Many infections such as burn, wound , blood and pneumonia can lead to severe illness and death in these people. However, healthy people can also develop mild illnesses like generalized skin rashes and ear infections especially in children with *P.aeruginosa* (CDC, 2018).In the present study total 63 MDR *P.aeruginosa* clinical isolates collected from several hospitals as a result in the emergence of multiple drug-resistant *P.aeruginosa* which became a major challenge in the treatment of corresponding infections.Clinically, drug resistance bacteria are responsible for increased cost,length of hospital stay and mortality (Mansouriet al.,2013).In this study, isolates exhibited resistance for β -lactams in a varied levels especially for third-generation cephalosprin's, and for imipenem, isolates revealed resistance to gentamicin,

tobramycin, ciprofloxacin, levofloxacin, and fewer resistance levels to colistin. The local study done by AL Saray (2016) showed that 43.48% and 91.30% of isolates were resistant to ceftazidime and to both of cefotaxime and imipenem respectively, carbencillin 76.19%, for gentamycin 71.43% and tobramycin 47.62%, ciprofloxacin 74.62%, while all isolates were sensitive to colistin. Nikokar et al. (2013) reported that 74.4% of isolates were resistant to carbencillin. Whereas Goudarzi and Eftekhari (2013) study showed that 94.7% of isolates were imipenem resistant. Present results showed that all selected isolates produced smooth and mucoid colonies on Congo red agar which agreed with Amina & Ahmed (2017) who showed mucoid *P.aeruginosa* colonies seemed to be most adherent with smooth appearance due to the alginate slime production. These colonies adsorb Congo red by the production of Congo-red-binding component of the biofilm matrix (Colvin et al., 2013). In PA14, the major exopolysaccharides produced is the glucose-rich and cellulase-sensitive Pel. The Pel role in biofilm formation was first identified in a screen for pellicle formation deficient mutants (i.e., biofilms formation at the air-liquid interface of standing cultures). Later, Pel was shown to be important for initiating and maintaining cell-cell interactions in biofilms (Jennings et al., 2015). Among *P.aeruginosa* isolates, only 5 isolates revealed different fragments of *pelA*1,2 and *pslD* genes. The Pel polysaccharide serves as an intercellular adhesin for the formation and maintenance of biofilms in *P.aeruginosa* (Colvin et al., 2013). Otherwise, Cohen et al. (2015) observed that deletions in *pslD* abolished biofilm formation also referred to the c-di-GMP regulatory role of expression of the genes for synthesis of the biofilm matrix, including extracellular polysaccharides (*pel* and *psl*) and an adhesion. Either Pel or Psl can be used by non-mucoid strains as the primary matrix structural polysaccharide, surface attachment for most isolates required Psl. Significantly, there was strain-to-strain variability in the contribution of Pel and Psl to mature biofilm structure (Colvin et al., 2013). In the current study, 72% of *P.aeruginosa* isolates were carrying the *algD* gene. These results supported previous results obtained from phenotype detection, which showed mucoid, smooth and slime phenotypes of local isolates as a result of *algD* gene prevalence among these isolates suggesting that biofilm formation process of these isolates depends on alginate or slime production, which represents a significant virulence factor for some species as well as play an important role in the pathogenesis of infections. Slimes are generally polysaccharide materials, also other polymers may also be present and are probably involved in the microbial cells protection. So local isolates improve their pathogenicity by increasing their ability to transition to chronicity by conversion to mucoidity as well as their antibiotic resistance mechanisms to evade host immunity and antibiotic therapy. Alginate is the ESP that often and mainly produced by *P.aeruginosa* clinical isolates from the CF patients lungs. This ESP overproduction is responsible for typical mucoid phenotype, which protects *P.aeruginosa* from harsh environments in CF lungs (Dobrindt et al., 2013; Wei and Ma, 2013), and causes lung lesions by facilitating bacterium adhesion to the respiratory epithelium providing an extracellular matrix in biofilms. Therefore, biofilm protects the bacterium from the host's immune response and from antibiotics. Longitudinal studies in CF have demonstrated that initial pseudomonal colonization occurs by non-mucoid (wild-type) *P.aeruginosa*. *mucA* mutations of *P.aeruginosa* are resulted from disease progression and adaptation to the lung environment, which confer mucoid phenotype acquisition with overproduction of the ESP alginate (Marvig et al., 2015; Powell et al., 2018).

V. CONCLUSION.

Depending *algD*, *pelA*, and *pslD* results, and phenotype detection, in this study most of the local isolates are mucoid as a result of incidence of *algD* gene among these isolates and high-frequency mutations of *pel*, and *psl* genes, suggesting that biofilm formation process depends on alginate as essential ESP apart from Pel and Psl matrices. So local isolates improve their pathogenicity by increasing their ability to transition to chronicity by conversion to mucoidity in addition to their antibiotic resistance mechanisms to evade host immunity and antibiotic therapy.

References

- [1] Akingbade, O.A.; Balogun, S.A.; Ojo, D.A.; Afolabi, R.O.; Motayo, B.O.; Okerentugba, P.O. & Okonko, I.O. (2012). Plasmid Profile Analysis of Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Wound Infections in South West, Nigeria. *J. World Appl. Sci.* 20(6):766-775.
- [2] Alikhani, M.Y.; Tabar, Z.K.; Mihani, F.; Kalantar, E.; Karami, P.; Sadeghi, M.; Khosroshahi, S.A. & Farajnia, S. (2014). Antimicrobial Resistance Patterns and Prevalence of *bla*PER-1 and *bla*VEB-1 Genes Among ESBL-producing *Pseudomonas aeruginosa* Isolates in West of Iran. *Jundishapur J. Microbiol.* 7(1).
- [3] Al-Saray, Z.A.K. (2016). Effect of Gamma Rays on Some Virulence Factors From Burn and Wound Isolated Bacteria. M.Sc. Thesis. College of Science, Mustansiriyah University, Iraq.
- [4] Amina, M. & Ahmed, B. (2017). *Pseudomonas* motility and antibiotics resistance. *J. Bioengin. Biomed. Sci.* 7: 220.
- [5] CDC. (2018) Centers for Disease Control and Prevention. *Pseudomonas aeruginosa* in healthcare settings <https://www.cdc.gov/hogranisms/pseudomonas.html>.

- [6]CLSI.(2016).Performance standards for antimicrobial susceptibility testing.Twenty-six informational supplement. 36(1):M100-S26.
- [7]Cohen, D.; Mechold, U.; Nevenzal, H.; Yarmiyhu, Y.; Randall, T.E.; Bay, D.C.; Rich, J.D.; Parsek, M.R.; Kaefer, V.; Harrison, J.J. & Banin, E. (2015). Oligoribonuclease is a central feature of cyclic diguanylate signaling in *Pseudomonas aeruginosa*. PNAS.112 (36):11359–11364.
- [8]Colvin, K.M.; Alnabelseya, N.; Baker, P.; Whitney, J.C.; Howell, P.L.; Parsek, M.R. (2013). *pelA* deacetylase activity is required for Pel polysaccharide synthesis in *Pseudomonas aeruginosa*. J.Bacteriol.195(10) :2329 –2339.
- [9]Dobrindt, U.; Hacker, J.H. & Svanborg, C. (2013).Current Topics in Microbiology and Immunology. Between Pathogenicity and Commensalism.e-book. Springer-Verlag- Berlin.Pp:104.
- [10]Friedman, L. & Kolter, R. (2004). Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. J. Bacteriol. 186(14):4457-4465.
- [11]Goudarzi, S.M. & Eftekhari, F. (2013). Assessment of Carbapenem Susceptibility and Multidrug-Resistance in *Pseudomonas aeruginosa* Burn Isolates in Tehran. Jundishapur J. Microbiol. 6(2):162-165.
- [12]Heydari, S. & Eftekhari, F. (2015). Biofilm formation and B-lactamase production in burn isolates of *Pseudomonas aeruginosa*. J. Jundi. Microbiol.8(3): e15514(1-5).
- [13]Jennings, L.K.; Storek, K.M.; Ledvina, H.E.; Coulon, C.; Marmont, L.S.; Sadovskaya, I.; Secor, P.R.; Tsenga, B.S.; Sciane, M.; Filloux, A.; Wozniak, D.J.; Howell, P.L. & Parsek, M.R. (2015). Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. Proc. Natl. Acad. Sci. U.S.A. 112:11353–11358.
- [14]Laverty, G.; Sean P. Gorman, S.P. & Gilmore, B.F. (2014). Biomolecular Mechanisms of *Pseudomonas aeruginosa* and *Escherichia coli* Biofilm Formation . J. Pathogens. 3:596-632.
- [15]Lipovsky, B.; Rihova, H.; Hanslianova, M.; Gregorova, N.; Suchanek, I. & Brychata, P. (2010). Prevalence and resistance of *Pseudomonas aeruginosa* in severely burned patients: a 10-year retrospective study. J. Acta. Chir.Plast.52(2-4):39-43.
- [16]Ma, L.; Wang, J.; Wang, S. et al. (2012).“Synthesis of multiple *Pseudomonas aeruginosa* biofilm matrix exopolysaccharides is post transcriptionally regulated,” J. Environ.Microbiol.14(8):1995–2005.
- [17]Mansouri, S.; Safa, A.; Najari, S.G. & Najari, A.G. (2013). Inhibitory activity of Iranian plant extracts on growth and biofilm formation by *Pseudomonas aeruginosa*. Mal. J. Microbiol. 9(2):176-183.
- [18]Marvig, R.L.; Sommer, L.M.; Molin, S. & Johansen, H.K. (2015). Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. J. Nat. Genet. 47:57–64.
- [19]Nikokar, I.; Tishayar, A.; Flakiyan, Z.; Alijani, K.; Rehana-Banisaeed, S.; Hossainpour, M.; Alvaei, S.A. & Araghian, A. (2013). Antibiotic resistance and frequency of class 1 integrons among *Pseudomonas aeruginosa*, isolated from burn patients in Guilan, Iran. Iranian J. Microbiol. 5(1): 36-41.
- [20]Powell, L.C.; Pritchard, M.F.; Ferguson, E.L.; Powell, K.A.; Pate, S.U.; Rye, P.D.; Sakellakou, S-M.; Buurma, N.J.; Brilliant, C.D.; Copping, J.M.; Menzies, G.E.; Lewis, P.D.; Hill, K.E. & Thomas, D.W. (2018) Targeted disruption of the extracellular polymeric network of *Pseudomonas aeruginosa* biofilms by alginate oligosaccharides. npj Biofilms and Microbiomes. 4:13.
- [21]Spilker, T.; Coenye, T.; Vandamme, P. & LiPuma, J.J. (2004). PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* Species recovered from cystic fibrosis patients. J. Clin. Microbiol.42: 2074–2079.
- [21]Stover, C.; Pham, X.; Erwin, A.; Mizoguchi, S.; Garber, R.; Yuan, Y.; Brody, L.; Kas, A.; Larblg, K.; Lim, R.; Wong, G.; Wu, Z.; Saler, M.; Lory, S. & Olson, M. (2000). Complete genome sequence of *P.aeruginosa* PAO1, an opportunistic pathogen. J. Nature. 406: 959-964.

[22]Streeter, K. &Katouli, M. (2016).Pseudomonas aeruginosa: A review of their Pathogenesis and Prevalence in Clinical Settings and the Environment.J. Infect. Epidemiol. Med.2(1): 25-32.

[23]Wei, O. & Ma, L.Z.(2013).Biofilm Matrix and Its Regulation in Pseudomonas aeruginosa.Int. J. Mol. Sci. 14:20983-21005.