Molecular study for the virulence strain of Pseudomonas aeruginosa isolated from burn patients

By: Dr.Lubna Abdul jabbar Al-Zubaidi
College of Engineering, Department of environmental engineering, Al Mustansiria University.
lubna_alzoubaidy@yahoo.com post box: 46172 Baghdad-Iraq
Keywords: Pseudomonas aeruginosa, virulence factor, PCR, amino glycoside group.
(Received 16 / 1 /2014 , Accepted 10 / 3 / 2014)

الخلاصة:
أصبحت الزوائف الزنجارية من المسببات المهمة لاصابات المهمة للجروح والحروق وتسبب تزايد حالات الوفاة للمصابين بالحروق. تتضمن عوامل الضراوة لهذة البكتربا هي قدرتها على انتاج البايوفلم ، القابلية على سرعة الحركة داخل وخارج الخلايا ، مقاومتها لاغلب المضات الحيوية المستعملة لذلك اجريت هذة الدراسة لغرض تسليط الضوء على اهم عوامل الضراوة في بعض سلالات الزوائف الزنجارية والمعزولة من مرضى مصابين بالحروق. اظهرت كل السلالات وعددها 10 سلالات قابلية انتاج البايوفلم وكانت السلالة رقم 3 من اكثر السلالات قابلية على الانتاج بمعدل امتصاص طيفي 2.5 وكانت هذة السلالة من اكثر السلالات مقاومة للمضادات الحيوية لذلك انتخبت هذة السلالة لغرض اجراء الفحوصات الجزيئة، وفاعلية الحركة وقد ظهرت حزم واضحة بحجم 1.2 زوج قاعدي استجابة للجين المقاوم لمجموعة الامينوكلايكوسايد. ان فهم ميكانيكية عوامل الضراوة للزوائف الزنجارية يعتبر من اهم العوامل التي يمكن ان تساهم في تقليل حالات الوفاة لدى مرضى الحروق.

Abstract:

Pseudomonas aeruginosa became an important cause of wound and burn infections and serious mortality in burn patients. The virulence factors of these bacteria including their capacity to form biofilm, their ability to hold antibiotics resistance genes as well as rapid motility in vivo and in vitro. Thus the present study was designed to highlight some the virulence factors among different strains of Pseudomonas aeruginosa. All isolated strains (10 isolates) had the capacity to produce biofilm ,one of them (No.3) has shown high level of biofilm (OD.2.5) and it was resisted to most antimicrobial agents; for this reason further investigation was carried out including gene expression by PCR technique and motility. Results showed that all strains were able to resist most common antibiotics; the molecular study for Strain 3 gave clear band of gene size 1.2pb which is responsible for resistance to aminoglycoside group. Understanding the virulence factors mechanisms of Pseudomonas aeruginosa is of a big importance in order to reduce the mortality in burn patients.

Introduction:

Pseudomonas aeruginosa elaborates a large number of virulence factors inluding biofilm formation, production of exotoxin and proteolytic enzymes and multidrug resistance ability (1). Almost all the clinical cases of P.aeruginosa infection can be associated with the compromise of the host defense mechanism especially in burn patients, in this scenario P.aeruginosa utilize their own virulence factors to invade body and increase the mortality rate among burn patients.(2) Pili and flagella of P.aeruginosa play an important role in bacterial virulence while the mutant strains will lose their virulence capacity.(3,4).P.aeruginosa has a large and complex genome, genomic sequencing of this bacterium isolated from wound indicated that this strain has a genome size of 6.3Mbp and contains 5,570 predicted outer reading frame (ORF). (5) Multidrut g resistance ability of P.aeruginosa is one of the most important challenges for physion during

through therapeutic regime; the genome sequence of this bacterium confirmed the presence of gene cassettes encoding antibiotic resistance elements. Transposable elements that affect on gene expression play an important role with other gram negative bacterial genes by spreading through environment and causing gene diversity (6). Strains of P. aeruginosa secrete a variety of redox-active phenazine compounds, the most well studied being pyocyanin. Pyocyanin is responsible for the blue-green color characteristic of Pseudomonas spp. It is considered both as a virulence factor and a quorum sensing signaling molecule for P. aeruginosa. Pyocyanin is an electrochemically active metabolite, involved in a variety of significant biological activities including gene expression, maintaining fitness of bacterial cells and biofilm formation. It is also recognized as an electron shuttle for bacterial respiration and as an antibacterial and antifungal agent.(7).

Materials and methods:

This work was carried out on ten strains of Pseudomonas aeruginosa obtained from burned patients. All strains were pyocianin produce and they were subjected for further investigations to compare their virulence factors.

Biofilm formation test: the biofilm formation protocol was adapted from that of O'Toole and Kolter. 11 Polystyrene 12- by 75-mm tubes containing 0.5 ml of LB medium (LB Consist of salts, yeast extract tryptone) were inoculated from overnight LB cultures and incubated at 37°C without agitation. triplicate tubes were rinsed thoroughly with phosphate buffer saline (PBS), and a 1% solution of crystal violet was added to stain the attached cells. After 10 to 15 min of incubation at room temperature, the tubes were rinsed with PBS, and the biomass of attached cells (biofilm) was quantified by solubilization of the dye in 2 ml of 95% ethanol. The absorbance was measured using ELISA plate reader at 454nm (7).

Motility assay: Swimming. Tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) were inoculated with a sterile toothpick and incubated for 16 h at 25°C. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the

point of inoculation.

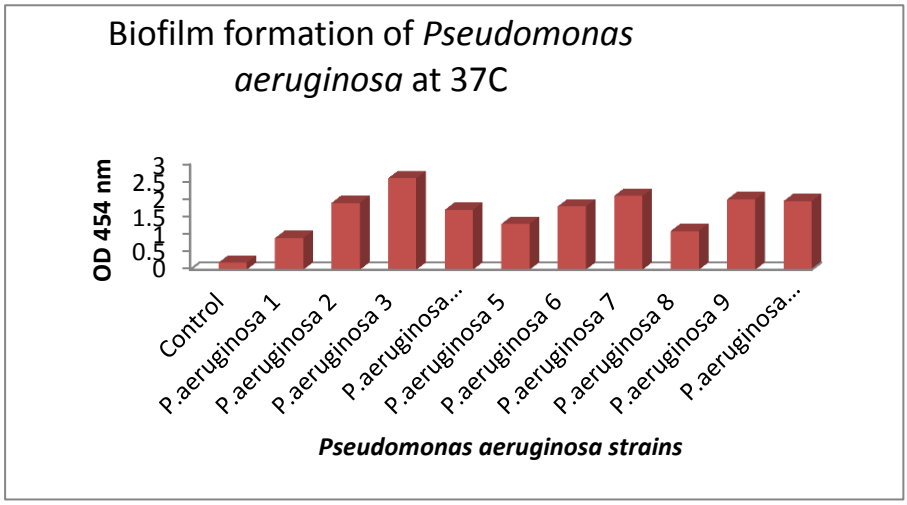
Sensitivity to antimicrobial agents: Disc diffusion method was carried out according to the method of Bauer et al 196. 8. Ten strains of pseudomonas aeuginosa were tested invitro, bacteria were inoculated on Muller Hinton Agar. Antibiotics discs were applied by using aseptic technique including (Ampicillin, Aguamantin, Colistin , Ciprofloxacin, Cotrimazol , Cephalexin , Gentamicin, Nalidexic Acid, Tetracyclin, Trimethoprim sulfamethoxazol) . Plates were incubated at 37C for 18 hours and the zone of inhibition was measured to the nearest whole millimeter .

Molecular study for Pseudomonas aeruginosa 9 reference

Bacterial stain: Pseudomonas aeruginosa strain no 3 was chosen for further molecular test, since it showed high level of biofilm formation and multidrug resistance criteria, Gantamicin was the target gene for molecular study, therefore forward and reverse primers were designed using advance available soft ware, for molecular study DNA was extracted using DNA extraction kit, Bam III restriction enzyme was used, (20µl was prepared including 18 µl Master mix and 2 µl genomic DNA, Master mix including Taq polymerase enzyme, buffer , forward primer TCGTCAAGCAGGAAGTGC and reverse primer TTTCGTTCAGCACCTGCG, nanopurified water, PCR thermal cycler was set with annealing temperature 58C. gel documentation was done to observe the presence of bands in comparing with DNA ladder(control).9

Results and discussion:

Biofilm formation was detected in all strains of *pseudomonas aeuginosa* , strain No 3 showed higher OD level in biofilm production Fig (1) all stains had the ability to produce biofilm with different OD level in comparism with contol (LB medium only).



Fig(1) Biofilm formation of *Pseudomonas aeruginosa* at 37C

Variation in biofilm formation could be due to the differences among strains motility or acquiring more genomic cassett that enhance bacterial activity although environmental factors found to be an important factors effecting bacterial growth and virulence including biofilm formation (Data not seen). (Stoodly *et al* 2002)[10] demonstrated that biofilm formation among *P.aeruginosa* strains may affected by the presence of carbon source, carbon may support the growth, also the presence of glucose presence in environment of bacteria , certain mechanism of biofilm formation like mushroom shaped multicelluar may produced.[11]

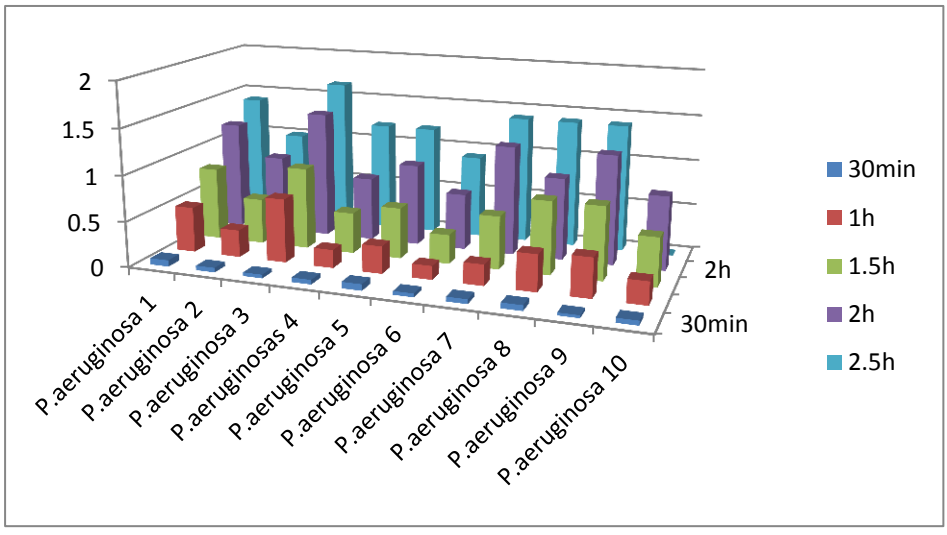


Fig (2) Growth curve of *Pseudomonas aeruginosa* strains

Fig (2) demonstrate the growth curved of different *P.aerumonas* strain, strain no 3 showed high rate of growth within 2.5 hours, that means the log phase occurs with 30-45min. increasing in bacterial growth may depend on the availability of nutrients or chemotactic factors that enhance the speed of bacterial growth. Bacterial motility plays an important virulence factor since bacteria may utilize the availability of nutrients in cells and tissue of human body and that allows bacteria to be more virulent. Recent studies showed new genes of pseudomonas strains like motB which is resemble to vibrio cholera gene. [11]. Gene expression of P.aeruginosa PAO1 biofilm cell and planktonic cells has been completed (Whitely *et al*,2000)[17] reported that the gene responsible for synthesis of Flagella and type IV pili were found to repressed in biofilm cell, mutant of pili does not produce biofilm which explain that the flagella play an important role in virulence. Other study carried out by Doyle *et al* [(12)] demonstrated that the twitching motility depends on the presence or production of lactoferrin through iron chelton mechanism, the presence of iron normally activates growth mechanism of bacteria, the presence of many genes may also plays an important role for bacterial motility

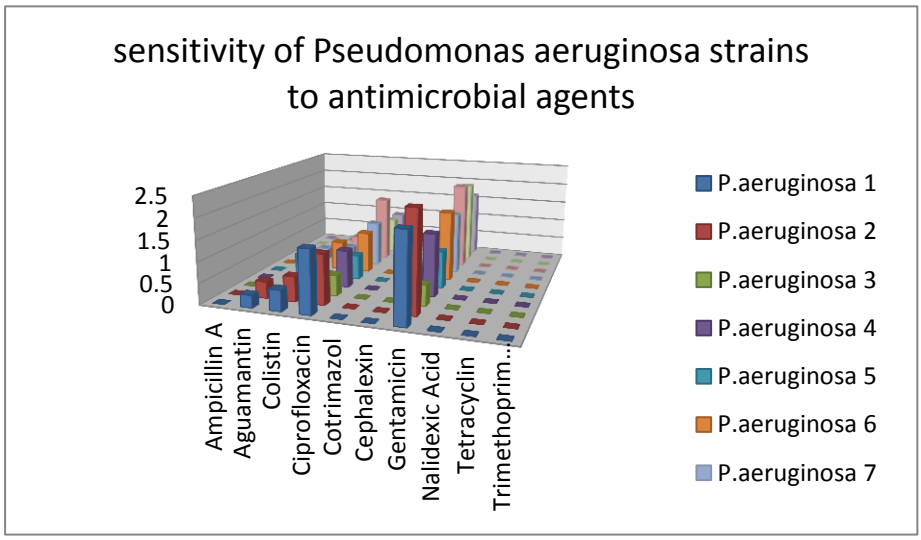
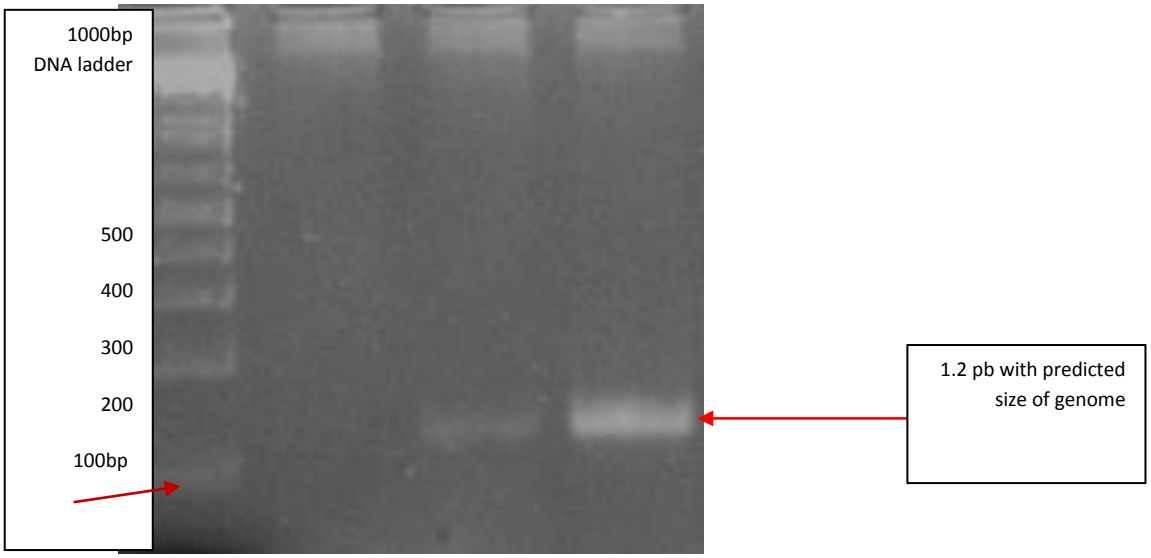


Fig (3) Sensitivity of *Pseudomonas aeruginosa* strains to antimicrobial agents

Antibiotic therapy has considerably improved the management of infectious diseases in general; many *P. aeruginosa* infections are not fully treated or eradicated by the application of anti-pseudomonal drugs leading to chronic infections. Burn patients that survive the initial burn trauma can become colonized with antibiotic-resistant, hospital-derived *P. aeruginosa* strains that are not easily eradicated with antibiotic therapy. [13]

Fig (3) demonstrated the sensitivity of different strains of *P.aeruginosa* to different sets of antibiotics, it's clear form this figure that all strains showed multidrug resistant pattern although moderate to high rate of sensitivity was detected for Gentamicin and Ciprofloxacin antibiotics. Recent reports have demonstrated the presence of widespread MDR *P. aeruginosa* clones in hospitals. These clones are responsible

for an important proportion taking resistance to lactams as an example, these MDR clones were repeatedly reported to carry extended-spectrum lactamase-encoding genes located in diverse horizontally acquired elements (integrons, transposons, and plasmids) . ([14,15])



Fig(4) Illusterate PCR assay for gentamicin resistance gene for *P.aeruginosa* starin 3

Aminoglycosides are a vital component of antipseudomonal chemotherapy implicated in the treatment of a variety of infections. In current study aminoglycoside resistance gene was observed in some strains including strain no 3, modification in genomic expression or modification of enzymes or activity of bacteriophage that may carry new genes from other related MDR bacteria (Farzam et al, 2011) ([16, 18]) indicated that in Europe, *aac(6')II* was the most prevalent resistance gene (32.5%) in *P.aeruginosa* strains , followed by *ant(2")-I* (16.9%).

In conclusion: Biofim formation of *P aeruginosa* may varies among strain, genetic variation and genomic exchange may also responsible for increasing the virulence factor, therefore this study focused what is believed to be the first points to be in consideration for biofilm formation and further gene sequencing for local strains for successful treatment and reduce mortality rate among burn patients.

References:

1.Holder I; Neely N. Pseudomonas elastase act as virulence factor in burned host by Hageman factor dependant activation of host kinin cascade.1989;57(11):3345-3348.

2. Japoni A; Farshad S; Alborzi A. *Pseudomonas aeruginosa*: Burn infection, treatment and antibacterial resistance. Iranian red crescent Medical journal.2009;11(3):244-253.

3. Katie B; Ashley M. At Loyola University, establishment of *Pseudomonas aeruginos*a infection : Lesson from versatile opportunistic. Microbes and infection. 2000;2(9):1051-1060.

4.Klausen M; Hydrom A; Ragas P and *et al*. Biofilm formation by *Pseudomonas aeruginosa* wild type, Flagella and type IV pili mutants. Molecular Microbiology.2003; 48(6):1511-1524.

5. Kung V; Ozer E ; Huser A. The accessory genome of *Pseudomonas aeruginosa*. Microbiology and Molecular biology reviews. 2010;74(4):621.

6. Holmes A; Gillings B; Neild B and etal.The gene cassette metagenome is basic resources for bacterial genome evaluation. Environ.Microbiol.2003; 5: 383-394.

7. Deziel C; Comeau Y; Villemur R. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlated with emergence of Hyperpiliated and highly swarming and twitching motilities. Journal of Bactriology. 2001; 183(4):1195-1204.

8.Bauer A; Kirby M; Sherris M. Antibiotic susceptibility by standardized single disc method.Amer J clini.Pathol.1966;45:493-396.

9. Parales, R.E. and Harwood, C.S. Construction and use of anew broad-host-range lacZ transcriptional fusion vector, pHRP309,for Gramn3bacteria. Gene. 1993. 133, 23-30.

10. Stoodley P; Sauer K; David D and et al. Biofilm as complex differentiated communities'. Annu Rev Microbiol. 2002.56:187-209.

11. OToole G; Kaplan H; Kolter R. Biofilm formation as microbial development. Ann Rev Microbiolo.2000.54:49-79.

12. Doyle T; Hawkins A, McCarter L. The complex flagellar Torque generator of *Pseudomonas aeruginosa*. Journal of bacteriology. 2004; 186(19):6341-6350.

13. Valerie Aloush, Shiri Navon-Venezia, Yardena Seigman-Igra, Shaltiel Cabili and Yehuda Carmeli. Multidrug-Resistant *Pseudomonas aeruginosa*: Risk Factors and Clinical Impact. 2006. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. 2006. Vol 50, No.1: 43–48.

14.Gingh K ;Parsek M, Greenberg E. A component of innate immunity prevent bacterial biofilm development. Nature.2002; 417:552-555.

15. Juan, C., et al. Metallo-beta-lactamase-producing Pseudomonas putida as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones. J. Antimicrob. Chemother. 2010. 65:474–478.

16. Farzam V; Shahin P; Qorban N; Abbas F. The prevalence of aminoglycoside-modifying enzyme genes *(aac (6')-I, aac (6')-II, ant (2")-I, aph (3')-VI*) in *Pseudomonas aeruginosa*.2011; Clinics vol.66 no.9:1519-1522.

17. Whiteley, M., Parsek, M. R. & Greenberg, E. P. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa* J. Bacteriol. (2000);182, 4356-4360.

18. Rashid M; Kornberg A. In organic polysphosphate is needed for swimming and twitching motilities of *Pseudomonas aeruginosa*. Pro.Natl.Acad.sci.USA.2000; 97:4885-4890.