The Virulence of Pigmented and Non-Pigmented *Pseudomonas Aeruginosa* in Mice with Antibiotics Susceptibility

**Abstract**

*Pseudomonas aeruginosa* employ a large virulence armamentarium to overcome host defenses, including the production and dispersal of Pyocyanin exotoxin and other phinazine molecules that are toxic to their hosts. The aim of the present study is to evaluate the mice killing capacity of different clinical isolates of pigmented and non-pigmented *Pseudomonas aeruginosa*. Three reference isolates isolated previously from otitis media and otitis externa (pyocyanin highly producer, fluorescein highly producer, non-pigmented strain) where chosen to be inoculated intraperitoneally in mice. The results of the present study showed that the Mortality occurred within 24 h in group one (pyocyanin producer) by 100% of mortality rate and within 48h in group two (fluorescin producer strains) by 100% of mortality rate whereas mortality occurred in group three (non-pigmented strains) at the end of 96 h post infection by 66.6% of mice death when all compared with control group (Intraperitoneally saline injection). Our study concludes the highly significant mice killing capacity of highly pyocyanin *P. aeruginosa* producer when compared to other pigmented and non-pigmented and these different isolates retain the capability to develop otitis media.

**Introduction**

*Pseudomonas aeruginosa*, is an opportunistic pathogen that causes extensive morbidity and mortality in individuals who are immunocompromised or have underlying medical conditions such as, urinary tract, respiratory tract and skin infections and primarily causes of nosocomial infections [1,2]. Its non sporulating, gram negative, oxidase positive motile bacterium with a polar flagellum [3], *P. aeruginosa* is a common nosocomial pathogen because it is capable of thriving in a wide variety of environmental niches [4]. It is a leading cause of hospital associated infections in the seriously ill, and the primary agent of chronic lung infections in cystic fibrosis patients [5]. They exist in very large numbers in the human environment and animal gut, they are capable of inhabiting/contaminating water, moist surface and sewage ,hospital environment usually have resident *P. aeruginosa* [6]. Despite the apparent ubiquity of *P. aeruginosa* in the natural environment and the vast array of potential virulence factors, the incidence of community-acquired infections in healthy subjects is relatively low. However, in the hospital environment, particularly in immunosuppressed, debilitated and burns patients, the incidence of *P. aeruginosa* infection is high [7]. It produces many numbers of extracellular toxins, which include phytotox factor, pigments, hydrocyanic acid, proteolytic enzymes, phospholipase enterotoxin, exotoxin and slime [1]. *P. aeruginosa* grows well on media and most strains elaborate the blue phenazine pigment pyocyanin and fluorescein (yellow), which together impart the characteristic blue –green coloration to agar cultures [6]. Pyocyanin is a blue redox-active secondary metabolite [8], which induces rapid apoptosis of human neutrophils, with a10 fold acceleration of constitutive neutrophil apoptosis *in vitro* but no apoptosis of epithelial cell or macrophages [9]. The redoxactive exotoxin pyocyanin is produced in the concentration up to 100 mol/l during the infection of CF patients and other bronchiectasis airways .The contribution of pyocyanin during infection of bronchiectasis airways are not appreciated [10]. Notably pyocyanin mediated ROS inhibit catalase activity, deplete cellular antioxidant reduced glutathione and increased the oxidized reduced glutathione in the bronchiolar epithelial cell [11,12]. Excessive and continuous producing of ROS and inhibit of antioxidant mechanisms overwhelm the antioxidant capacity, leading to tissue damage, also pyocyanin inhibit ciliary beating of the airway epithelial cell [13] pyocyanin. Also increases apoptosis and inactivates 1-protease inhibitor [14] reducing agents such as GSH and NADPH can reduce pyocyanin to pyocyanin radical, which then mono-or divalently reduce O2 to form superoxide anion O2- or H2O2 [15]. Pyoverdine per contra is the main siderophore in iron gathering capacity its function as a powerful iron chelator, solubilizing and transporting iron through the bacterial membrane via specific receptor proteins at the level of outer membranes [Herinrichet al,1991]. Pyoverdine is important because it has a high affinity for iron, with an affinity constant of 10[32] [16]. And has been shown to remove iron from transferrin in serum, probably assisting growth within, and ultimate colonization of the human host by *P. aeruginosa* (Cox and Adams,1985). Moreover experiments studying the burned models of *P. aeruginosa* infections have shown that ferric-pyoverdine is required infection and /or colonization, underlining the importance of ferric-pyoverdine to virulence of *P. aeruginosa* [16]. *P. aeruginosa* it is highly resist to antibiotics this resistance can be conferred by the outer membrane which provides an effective intrinsic barrier in the cell wall (or) cytoplasmic membrane (or)
within the cytoplasm and modifications in outer membrane permeability via alternations in porin protein channel represent a component of many resistance mechanisms. In addition to activating enzymes released from the inner membrane, bacteria can function more efficiently within the confines of the periplasmic space, the mechanisms by which intracellular concentrations of drugs are limited include decreased permeability through the outer membrane and active efflux back out across the cytoplasmic membrane. The production of B-lactamase is the most prevalent mechanism of resistance to B-lactam antibiotics, the B-lactamase have been reported to hydrolyze all antipseudomonal agents. Moreover, P. aeruginosa can develop a biofilm, in which bacterial cells are emmeshed into amicodex polysaccharide becoming more resistant to beta-lactams as well as decrease the outer membrane permeability that enable bacteria to gain resistance development.

Material and Methods

Bacterial isolates

Three reference isolate from otitis media uptake from Science College (highly producer pyocyanin, highly producer fluorescein, non-pigmented strain). All strains passages in mice to retain their virulence. Stock cultures were maintained at 70°C in brain heart infusion broth containing 5% glycerol.

Laboratory animals

Swiss albino male mice were purchase from (institute of biological and pharmaceutical research laboratory, Baghdad) aged 4-8 week and weigh 22-30gm were bred at animal breeding house at the College of Science, Tikrit University, all mice were kept at 22-25°C in plastic cage and fed pellet and water every day.

Experimental infection

Swiss albino mice trated with multiple strains of P. aeruginosa (highly pyocyanin producer strains + fluorescein producer strains + non-pigmented strains) bacterial culture adjusted to 0.5 McFarland and each mice (3 in each group) challenge intraperitoneally with 1ml of bacterial suspension and mortality rate calculated for 5 days and in compared with control (injected only with normal saline).

Result and Discussion

Effect of P. aeruginosa on the laboratory animals

Mice were treated with (pyocyanin producer + non-pigmented strains + fluorescein producer strains). Mortality occurred within 24h in group one in a percent 100% and within 48h in group two in a percent 100% whereas mortality occurred in group three at the end of 96h post infection in a percent 66.6%. our results are compatible with Al-shamaa et al. [20] that elucidate pyocyanin is the important virulence factor among many virulence factors of P. aeruginosa which caused the death of injured rat within 24h. Whereas pyoverdine treated rat death within 4h, pyocyanin also alter specific immune defenses and potentiates and per perpetuates harmful inflammatory reactions in the infected cystic fibrosis [21]. O'Malley et al. [22] also recorded that pyocyanin exhibits paradoxical pro-oxidant property. A. zwiter ion that can easily penetrate biological membranes, pyocyanin can directly accept electrons from reducing agent such as NADPH and reduced glutathione. Thereafter, the electrons reoxidized to oxygen to generate ROS, such as peroxide and single oxygen, also in harmony with Finlayson et al. [23] who elucidated pigmented strains of P. aeruginosa were highly virulence than non pigmented strains. Furthermore, virulence factor produced in large ratio than non pigmented strain in which pigmented strains produce significant more (P<0.05) DNase, elastase, protease and siderophore. Pyocyanin is the highest virulence factor which altered the host immune response in several ways to aid evasion of immune system and establish chronic infection, evidence suggest that pyocyanin could prevent the development of an-effective T-cell response against Pseudomonas and prevent activation of monocyte and macrophage [24], also pyocyanin in neutrophils induce a sustained increase in ROS and subsequent decrease in intracellular Camp which triggers the time and concentration dependent acceleration of apoptosis [9]. As confirm in studies using wild type and isogenic pyocyanin deficient mutant P. aeruginosa pigment dependent acceleration of neutrophil apoptosis and adenin one release of chemokine might represent an immune suppression mechanism of the pathogen [25]. The fundamental ability of pyocyanin to alter the redox cycle and increase oxidative stress central to its divers detrimental effect on host cell, for example pyocyanin disrupt Ca+2 homeostasis in human airway epithelial cells by oxidant-dependent increases in inosited triphosphate and abnormal releases of Ca+2 from intracellular stores, because Ca+2 is important for regulating ion transport, mucus secretion and ciliary beat. These alterations probably have important ramifications for P. aeruginosa lung infection [26]. Also pyocyanin function as inhibitor of ATPase and this explains the pyocyanin toxicity including ciliary dysmotility, disruption of calcium homeostasis and diminished apical membrane localization of the cystic fibrosis trans membrane conductance regulator (CFTR) [27] (Figure 1). Other potential toxic effects of pyocyanin include preturbance of cellular respiration, epidermal growth inhibition, prostacyclin release from lung endothelial cell and alter balance of protease-antiprotease activity in the cystic fibrosis lung [11]. The prooxidant effect of pyocyanin can thus augment such innate immune response circuits, for example, pyocyanin increases the release of the neutrophil chemokine (IL-8) from lung epithelial cells and up regulates the expression of the neutrophil receptor intracellular adhesion molecule (ICAM-1) [26,27] in spite of all above toxic effects of pyocyanin, pyocyanin producer strains show highly virulence because pyocyanin act as a signaling molecule for quorum sensing regulation, which is regulated virulence faceto expression [11,24], in spite of also pyowerdine (PVD) importance virulence factor which is function as a powerful iron chelators solubilizing and transporting iron through the bacterial membrane via specific receptor process before it reaches its targets [29-31] elucidate that PVD is essential element in vivo iron gathering and virulence expression in P. aeruginosa who found that PVD deficient mutants demonstrated no virulence when injected into burned mice [32].
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Figure 1: Mortality rate by pyocyanin and fluorescein and non-pigmented strains treating mice.

References

1. Pollack M (2000) Pseudomonas aeruginosa. In principle and practice of Infection Diseases. Mandell GL, et al. (Eds.). Philadelphia: Churchill Livingstone, USA, pp.2310-2325.
2. Stover CK, Pham QX, Erwin AL, Mizogushi SD, Warrenre Pet al. (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen Nature 406(6799): 959-964.
3. Akanji BO, Ajide JO, Onasanya A, Oyelakin O (2011) Genetic Fingerprinting of Pseudomonas aeruginosa Involved in Nosocomial Infection as Revealed by RAPD-PCR Markers. Biotechnology 10(1): 70-77.
4. Romling U, Wingender H, Muller H, Tummler B (1994) A major Pseudomonas aeruginosa clone common to patients and aquatic habitats. Appl Environ Microbiol 60(6): 1734-1738.
5. Brooks GF, Butel JS, Morse SA (2001) Medical Microbiology. Jawetz, Medical Clinics of North America 85(1).
6. Anderlini P, Przepiorka D, Champlin R, Korbling M (1996) Biologic and clinical effects of granulocyte colony-stimulating factor in patients with persistent infection. J Immunol 168(4): 1861-1868.
7. Holder IA (1977) Epidemiology of Pseudomonas aeruginosa in a burn hospital. Young VM (Eds.). In Pseudomonas aeruginosa: Ecological Aspects and Patient Colonization, Raven Press, New York, USA, p. 77-95.
8. Xu H, Lin W, Xia H, Xu S, Li Y, et al. (2005) Influence of ptsP gene on pyocyanin production in Pseudomonas aeruginosa. FEMS Microbiol 253(1): 103-109.
9. Usher LR (2002) Induction of neutrophil apoptosis by the Pseudomonas aeruginosa exotoxin pyocin: a potential mechanism of persistent infection. J Immunol 168(4): 1861-1868.
10. Zychlinsky A, Sansonetti P (1997) Perspectives series: host-pathogen interactions: apoptosis in bacterial pathogenesis. J Clin Invest 100(3): 493-495.
11. Lau GW (2004) The role of pyocyanin in Pseudomonas aeruginosa infection. Trends Mol Med 10(12): 599-560.
12. Lau GW, Hassett DJ, Britigan BE (2005) Modulation of lung epithelial functions by Pseudomonas aeruginosa. Trends in Microbiology 13(8): 389-397.
13. Wilson RD, Sykes A, Watson D, Rutman A, Taylor GW, et al. (1988) Measurement of Pseudomonas aeruginosa phenazine pigments in sputum and their contribution to sputum sol toxicity for respiratory epithelium. Infect Immun 56(9): 2515-2517.
14. Shellito J, Nelson S, Sorensen RU (1992) Effect of pyocyanine, a pigment of Pseudomonas aeruginosa, on production of reactive nitrogen intermediates by murine alveolar macrophages. Infect Immun 60(9): 3913-3915.
15. Cheluvappa R, Jamieson HA, Hilmer SN, Muller M, Le Couteur DG (2007) The effect of Pseudomonas aeruginosa virulence factor, pyocyanin, on the liver sinusoidal endothelial cell. J Gastroenterol Hepatol 22(8): 1350-1351.
16. Meyer JM, Neely A, Stintzi A, Georges C, Holder IA (1990) Pyoverdin is essential for virulence of Pseudomonas aeruginosa. Infect Immun 64(2): 518-523.
17. Henwood CJ, Livermore DM, James D, Warner M (2001) Antimicrobial susceptibility of Pseudomonas aeruginosa: results of a UK survey and evaluation of the British Society for Antimicrobial Chemotherapy disc susceptibility test. Journal of Antimicrobial chemotherapy 47(6): 789-799.
18. Bonfiglio G, Cardiotto V, Russo G, Stefani S, Schito G, et al. (1998) Antibiotic Resistance in P. aeruginosa. An Italian Survey. J Antimicrob Chemother 41(2): 307-310.
19. Gamarrellou H, Antoniadou A (2001) Antipseudomonal Antibiotics. Medical Clinics of North America 85(1).
20. Al Shamaa SD, Bahjat SA, Nasir NS (2011) Production of extracellular pigments as a virulence factor of Pseudomonas aeruginosa. colle of edureaf J 11: 689-697.
21. Votgu L (2006) Microevolution of cytochrome bd oxidase in Staphylococci and its application in resistance to respiratory toxins released by Pseudomonas. J Bactirol 188(3): 8079-8086.
22. O’Malley YQ, Reszka KJ, Spitz DR, Denning GM, Britigan BE (2004) Pseudomonas aeruginosa pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 287(1): 94-103.
23. Finlayson EA, Brown PD (2011) Comparison of Antibiotic Resistance and Virulence Factors in Pigmented and Non-pigmented Pseudomonas aeruginosa. West Indian Med J 60(1): 24-31.
24. Winstanley C, Fothergill JL (2008) The role of quorum sensing in chronic cystic fibrosis infections. FEMS Microbiol Lett 290(1): 1-9.
25. Allen L (2005) Pyocyanin production by Pseudomonas aeruginosa induces neutrophil apoptosis and impairs neutrophilmediated host defenses in vivo. J Immunol 174(6): 3643-3649.
26. Denning GM (1998) Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells. Infect Immun 66(12): 5775-5784.
27. Kong F (2006) Pseudomonas aeruginosa pyocyanin inactivates lung epithelial vacuolar ATPase-dependent cystic fibrosis transmembrane conductance regulator expression and localization. Cell Microbiol 8(7): 1121-1133.
28. Look DC (2005) Pyocyanin and its precursor phenazine-1-carboxylic acid increase IL-8 and intercellular adhesion molecule-1 expression in human airway epithelial cells by oxidant-dependent mechanisms. J Immunol 175(6):4017-4023.

29. Xie L, Bourne PE (2008) Detecting evolutionary relationships across existing fold space, using sequence order-independent profile-profile alignments. Proc Natl Acad Sci USA 105(14):5441-5446.

30. Oberhardt MA, Puchalka J, Fryer KE, Martins SVA, Papin JA (2008) Genome scale metabolic network analysis of the opportunistic pathogen Pseudomonas aeruginosa PA01. J Bacteriol 190(8):2790-803.

31. Merriman TR, Merriman ME, Lamont IL (1995) Nucleotide sequence of pvdD, a pyoverdine biosynthetic gene from Pseudomonas aeruginosa: PvdD has similarity to peptide synthetases. Journal of Bacteriology 177(1):252-258.

32. Cox CD (1985) Iron transport and serum resistance in Pseudomonas aeruginosa. Antimicrob Chemother 36:1-12.