Production of Extracellular Pigments as a Virulence Factor of Pseudomonas aeruginosa

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Abstract:

Pigment production is considered an important virulence factor among many virulence factors that are produced by *pseudomonas aeruginosa*. A strong relationship between pigment production and both virulence and pathogenicity of *Pseudomonas* isolates has been revealed in this study.

Among the important results considered in the present study, 80% of *Pseudomonas* isolates produce pigments. Culture media and broth that contain cetrimide were the most inducible media for pigment production within 24 hrs compared with modified MaConkey agar and broth in which pigmentation occurred within 4- 5 days .

To determine the virulence of pigmented and non- pigmented *Pseudomonas aeruginosa* isolates, 0.1 ml of each broth cultured bacteria and filtrate were injected in an intentionally inscised wound (1 cm) on dorsal side of mice. Pyocyanin (blue- green) pigment producer strains were highly virulent, since they caused death of mice within 24 hrs. On the other hand, pyoveridin (yellowish - green) producer strains caused death of animals after 48hrs. On the contrary, injection of mice with extracts of non pigmented strains caused local inflammatory reactions rather than death. However, culture filtrates of isolates also showed inflammations upon injection of mice. It is evident from the results of this study that the pigment production is considered an important virulence factor in *Pseudomonas aeruginosa*.

إنتاج الصبغات خارج الخلوية كعامل ضراوة لجراثيم الزوائف الزنجارية

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ملخص البحث:

يعد إنتاج الصبغات عاملاً مهماً من بين عوامل الضراوة المتعددة المنتجة من قبل الزوائف الزنجارية، فإنتاج الصبغات له علاقة وثيقة بضراوة العزلات المختلفة لهذه الجراثيم وامراضيتها كما بينتها الدراسة الحالية .

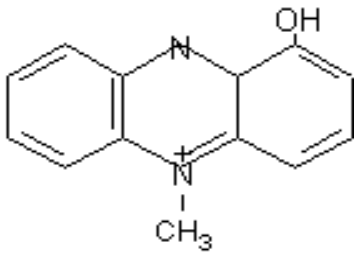
ومن النتائج المهمة التي تم الحصول عليها أن 80% من عزلات الزوائف الزنجارية *Pseudomonas aeruginosa* كانت منتجة للصبغات وكانت الأوساط الزرعية الحاوية على Cetrimide من أكثر الأوساط الزرعية المحفزة لإنتاج هذه الصبغات وخلال 24 ساعة مقارنة بأوساط الماكونكي المحورة MMA والتي بينت إنتاج الصبغات بعد 4- 5 أيام .

تمت مقارنة ضراوة العزلات المنتجة وغير المنتجة للصبغات وامراضيتها وذلك بحقن 0,1 سم^3 من مستخلصات المزارع ورواشحها الجرثومية في جرح مستحدث حوالي 1سم في المنطقة الظهرية للجرذان . وأظهرت النتائج أن هناك علاقة وثيقة بين إنتاج الصبغات المختلفة وامراضية هذه الجراثيم. فالجراثيم المنتجة للصبغات الخضراء المزرقة (البايوسيانين) كانت أكثرها ضراوة .

فقد أدى حقن 0,1 سم^3 من المستخلص الجرثومي في الجرح المستحدث إلى موت الجرذان خلال 24 ساعة. أما الجراثيم المنتجة للصبغات الصفراء – المخضرة (Pyoviridin) فقد أدى الحقن إلى موت الجرذان خلال 48 ساعة. في حين أن حقن المستخلصات الجرثومية غير المنتجة للصبغات أدت إلى حدوث التهابات موضعية فقط ولم يؤدِ إلى موت الحيوان. أما حقن رواشح المزارع الجرثومية المنتجة للصبغات فقد أدى إلى حدوث التهابات موضعية في موضع الحقن مما يدل على أن إنتاج الصبغات يعد عاملاً مهماً من عوامل ضراوة هذه الجراثيم.

Introduction

The oppurtinistic pathogen *Pseudomonas aeruginosa* is a gram negative bacterium that infects immunodeficient or otherwise compromised patients and causes a wide range of infections. Among those infected with this bacterium are patients with cystic fibrosis, sever burns, wounds, and especially those with nosocomial infection (Mavrodi *et al.*, 2001). This bacterium produces two types of soluble pigments; the fluorescent pigment pyoveridin and the blue pigment pyocyanin (Todar 2004). Pyocyanin (N- methyl 1- hydroxyl phenazin) is a blue phenazin pigment of low molecular weight, chloroform soluble and exists as a redox cyclic compound (Cox, 1986) .



Chemical structure of pyocyanin
(O'Malley *et al.*, 2003)

O'Malley *et al.*, 2003 concluded that pyocyanin may decrease cellular catalase activity via both transcriptional egulation and direct inactivation of the enzyme. Mammalian catalase binds four Nicotinamide Adenine Dinucleotide Phosphate (NADPH) molecules help in maintaining enzyme activity and pyocyanin directly oxidizes this NADPH producing superoxides. Inhibition of cellular respiration is one of the important mechanisms of pyocyanin toxicity to bacterial or eukaryotic cells (Ran *et al.*, 2003) Pyoveridin per contra is the main siderophore in iron- gathering capacity produced by *Pseudomonas aeruginosa* growth *in vivo* .

Pseudomonas aeruginosa has a strong iron requirement and it is able to excrete large amount of two chemically unrelated siderophores; mainly pyoveridin (PVD) (Cox and Adams, 1985) and to lesser extent pyochellin (Cox ,1986) into its environment. These siderophores function as a powerful iron chelator; solubilizing and transporting iron through the bacterial membrane via specific receptor proteins at the level of outer membranes (Heinrichs *et al.*, 1991). The results of Meyer *et al.*, 1990 strongly suggest that pyoveridin compets directly with transferrin for iron which is an essential element for *in vivo* iron gathering and virulence expression in *ps. aeruginosa*.

The aim of the present study is to demonstrate the best media which enhances pigment production of *pseudomonas aeruginosa*, and to elucidate the role of these pigment in pathogeneity of this bacterium .

Materials and Methods

Bacterial strains:

Twenty strains of *pseudomonas aeruginosa* obtained from the Department of Biology/ College of Science Mosul University isolated from different inflammatory conditions were used in this research.

Enhancing pigment media:

A broth medium which consists of an aqueous solution of w/v; ammonium acetate 0.2%; NaCl 0.3%; $MgSo_4$-$7H_2o$ 0.01% and $KHPO_4$ 0.07% was used . The approximate pH is 7.0 . This medium was autoclaved at 121°C for 15 min and distributed in test tubes (5 ml / tube).

All tubes were inoculated with a loopful of culture scraped from an overnight growth on cetrimide agar .

Solid media: Modified MacConkey agar (MMA); pepton 20 gm; potassium sulphate 9 gm; magnesium chloride 2 gm; agar 15 gm; sodium citrate 10 gm; lactose 10 gm; neutral red 30 µg; bile salts 1.5 gm, 1000 ml distilled water . Pseudomonas selective agar base (cetrimide agar). Composition (gm/ liter): pepton 20; potassium sulphate 10; magnesium chloride 1.4; cetyl-trimethyl ammonium bromide (cetrimide) 0.3; agar-agar 13.6; glycerol 10 ml. sterilization was by autoclave and then poured in sterile petri dishes. pH of the medium is 7.2 (Merck 1985).

Culture filtrate:

Pseudomonas aeruginosa was grown in broth media (mentioned previously) until pigment was produced. The broth culture was then centrifuged at 5000 rpm for 15 min. and finally, sterilization of supernatant by filtration was performed using 22 µm milipore filter paper. This filtrate was used for injection .

Experimental design

Twelve white male rats (*Rattus novregicus*) weighing 250 gm were divided into 6 groups (2 rats/ group), anesthetized with ketamin (50 mg/kg) and xylazin (5 mg/ kg) then injected intraperitionially. One centimeter wound was made at the endorse of rats after disinfecting the area with 70% ethanol. These rats were then injected with 0.2ml of broth culture and filtrate into the wounded area as follows:

Group 1 (Control) rats injected with distilled water inside rats the wounded area . Group 2 rats injected with cultured non pigmented strain of *pseudomonas auroginosa*. Group 3 rats were injected with cultured pigmented strains that exhibited blue - green color on the solid media .Group 4 rats injected with culture filtrate of blue – green pigmented strains. Group 5 rats injected with yellow - green pigmented strains of *pseudomonas auroginosa*. Group 6 rats injected with culture filtrate of yellow- green pigmented strains.

Results and Discussion

The present study revealed that (80%) strains of *Pseudomonas aeruginosa* elaborated detectable pigments on cetrimide broth and agar within 24h (Figure 1). While Modified MacConkey agar (MMA) detected 50% pigmented strains within 2-3 days. These results come in agreement with published results that have shown that cetrimide enhanced pigment production of *pseudomonas aeruginosa* in liquid and solid media (Totter and Moseley 1953). Daley *et al.* 1984 elucidated that systemic adjustment in concentration of MMA compounds had either a stimulatory or an inhibitory effect on pigment production in an attempt to compare the

pathogenicity of pigmented and non - pigmented strains of *pseudomonas aeruginosa*. Our results showed a considerable correlation between pigment production and bacterial pathogenicity *in vivo.* Injection with 0.1 ml of non pigmented strains culture in the wounded area of rats did not show any inflammatory reaction and the rats were active and healthy (Figure 3) . Same control results were seen in group 1 (control) after injection with distilled water (Figure 2) . Group (3) injected with 0.1 ml of cultured suspension of blue – green (Pyocyanin) pigmented strains were dead within 24 hrs (Figure 4). On the other hand rats in group (4) injected with 0.1 ml. cultured- filtrate of blue – green pigmented strains survived with positive inflammatory reaction in the wounded area (Figure 5). Group 5 died after 48hrs. of injection with 0.1ml of suspension of yellow- green- pigmented strains (Pyoveridin) (Figure 6) . Severe inflammatory reactions in wounded skin – area elevated after injection of the rats with 0.1 ml cultured filtrate of yellow – green pigmented strains of *pseudomonas arruginosa* (Figure 7).

These results indicate that the injection of culture filtrate (crude pigment extract) of pyocyanin and pyoveridin cause inflammatory reaction in wounded skin area while the injection of a Cultured suspension pigmented strain caused death within 24h. in the case of blue-green pigmented strains and within 48h. for green - yellow pigmented producer strains. These results clarify the relation between pigment production and strain virulence which comes in agreement with many studies such as (Votgu, *L.* 2006) that elucidate one of the important mechanism of pyocyanin toxicity in eukaryotic cells which inhibit Cellular respiration. Pyocyanin also alters specific immune defenses and potentiates and per petuates harmful inflammatory reactions in the infected cystic fibrosis lung. Yet the *in vitro* metabolic fate of pyocyanin has not been elucidated till now. (Muller, 2002) .

O' Malley *et al.* 2004 also recorded that *P. aeruginosa* pyocyanin exhibits a paradoxical pro- oxidant property. A zwitterion that can easily penetrate biological membranes pyocyanin can directly accept electrons from reducing agents such as NADPH and reduced glutathione, then transfers the electrons to oxygen to generate ROS such as hydrogen peroxide and singlet oxygen .

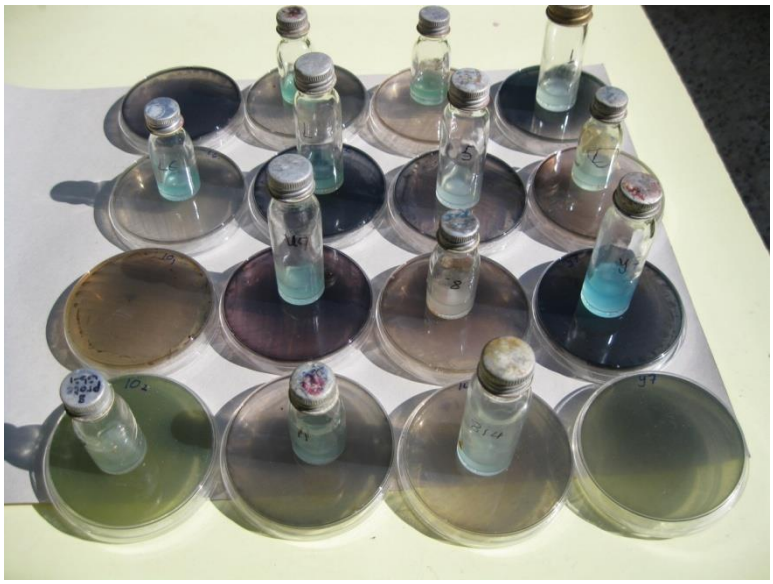
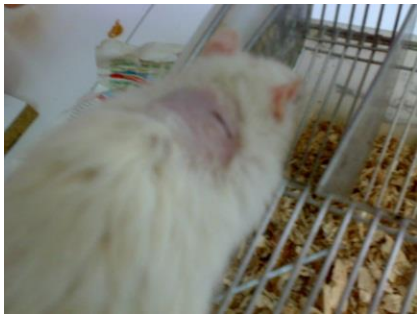
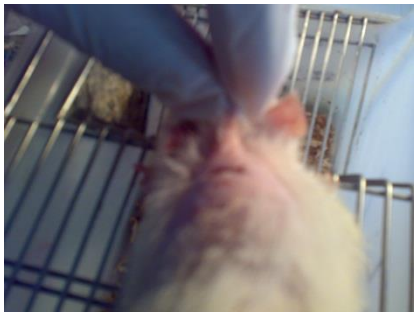


Fig (1) pigment production on solid and broth media containing cetrimide



After 24 hrs.

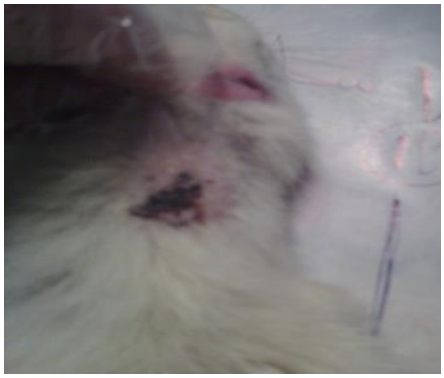


After 48 hrs.

After 72 hrs.

After 96 hrs.

Fig (2): Control group injected with distilled water .



After 72 hrs. After 96 hrs.

Fig (3): Rats injected with cultured nonpigmented strains of *ps.auroginosa* .

Fig (4): Rats injected with cultured blue-green pigmented strains (death occurred after 24 hrs.).

After 48 hrs. After 72 hrs. After 96 hrs.

Fig (5) : Rats injected with culture filterate of blue-green pigmented strains.

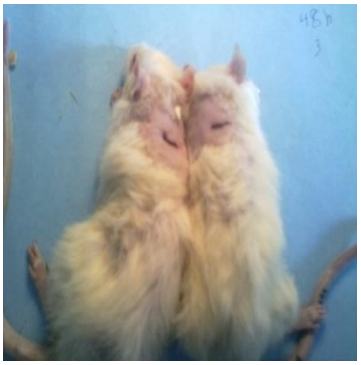
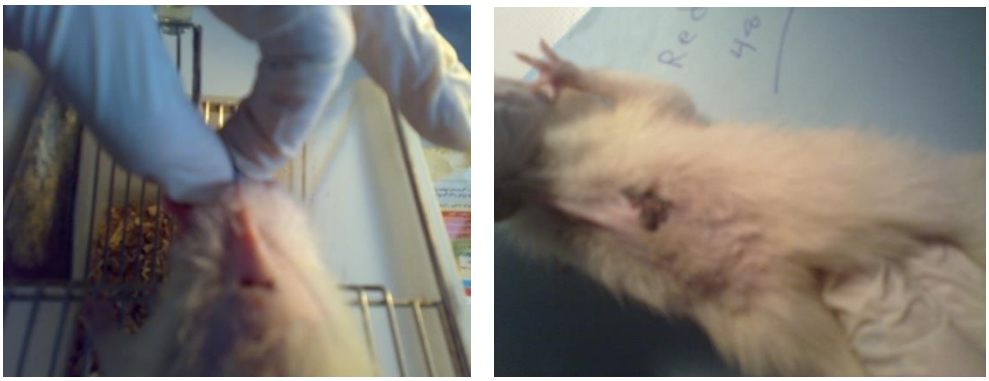


Fig (6): Rats injected with yellow green pigmented strains (death occurred after 48 hr.



After 24 hrs. After 48 hrs.

Fig (7): Rats injected with yellow – green culture filtrate .

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