Effect of pyocyanine extracted from *P. aeroginosa* on other pathogenic bacteria in vitro

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Abstract:

Clinical isolates of *Pseudomonas aeroginosa* was tested for their inhibitory effect on growth of other pathogenic bacteria ,Pyocyanine(PCN) is the major metabolites of this bacteria ,the pigmented strain were found to have stronger antimicrobial activity on Gram's +ve and Gram's–ve bacteria than unpigmented one .Our results revealed the PCN have strongly active against *Staph*. aureus & *E.coli* ,but weakly effect of *Klebs*. & *Proteus* , other strains of *P.aeroginosa* are not affected by PCN .

تأثير البايوسيانين المستخلص من *P. aeroginosa* على بعض الجراثيم المرضيه الاخرى بالوسط الزرعي

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الخلاصة:

تمتلك بكتيريا *Pseudomonas aeroginosa* خاصية تثبيط نمو بعض أنواع البكتيريا المرضية الأخرى، هذه الخاصية تأتي من امتلاكها بعض المواد المثبطة لنمو البكتريا مثل البايوسيانين . أعطت الدراسة نتائج مفادها أن البايوسيانين المستخرج من هذه البكتيريا له فعالية تثبيطية قوية على بكتيريا *Staph. aureus* و *E.coli* و بتراكيز قليلة ، بينما تقل الفعالية على بكتيريا *Klebs.* و*Proteus* وبتراكيز اكبر من البايوسيانين .وجدت الدراسة أيضا أن البايوسيانين لا يمتلك تأثير تثبيطي على سلالات *Pseudomonas* الأخرى.

Introduction:

Pseudomonas aeroginosa is an opportunistic human pathogen that causes an acute lung diseases with high mortality in seriously ill and immune compromised patients[1]. Beside many diseases as urinary tract infection , osteomylitis , otitis media and peritoneal infection in patients undergoing dialysis [2][3].This species produced colorful redox – active antibiotic called pyocyanine (PCN) which is responsible for the bluish tint of sputum and pus associated with P. aeroginosa infection in human [4] . Also its produced an arsenal of virulence factor that may act against the commensal flora as well as host cells [3] . These virulence factors include a number of secreted and surface –

associated molecules such as pilus adhesion[5],proteases [6] ,haemolysin and rhammnolipid [7] .Also among the secreted molecules are enzymes such as a staphylolytic endopeptidase [8] ,as well as copious respiratory inhibitors like PCN , hydrogen cyanide and a mixture of quinolone N-oxides [9] .

The mechanism(s) by which *P. aeroginosa* establishes infection in the patients as well as the mechanisms the underlie the pathophysiology of resulting disease remain poorly understood . However , numerous studies provide that factors secreted by the bacterium could contribute to disease pathogenesis [10] .

Among these factors is PCN ,a redox –active phenazine derivative that increase intracellular oxidant stress [11] .

PCN is been detected at concentration as high as 100mmol in pulmonary secretion from patients with *P. aeroginosa* –associated airways diseases[12] , and its production is increased when the organism is in the biofilm [13],PCN consider the first phenazine compound was discovered in nature from *P. aeroginosa* at (1890).Its characterized by thermostable ,pepsin stable and soluble in organic solvents as Acetone , Phenol and its rapid soluble in chloroform for this reason used in extraction and purification of PCN [14]. A number of bacterial infections result from colonization of more than one microorganisms [3][15] .PCN known as antagonistic against Gram positive and negative pathogenic bacteria except *Pseudomonas genus* which resist PCN activity [14]. Among bacteria specieses which sensitive to PCN are *E. coli , salmonella sp. ,proteus sp. Staphylococcus aureus and Klebs*.[16].

Materials and Methods:

A-Isolation and Identification of bacteria :

Isolation of *P. aeroginosa* from different samples of patients by using many bacteriological tests such as Gram's stain . catalase , oxidase , urease and gelatenase test , the tested bacteria characterized by growing at 42Cº and give positive results in all above tests .

Other pathogenic bacteria : isolated pure colonies of other pathogenic bacteria like *Staphylococcus aureus* . *E.coli, klebsiella sp*., and *Proteus sp*. For exposure to pyocyanine [17].

B- Extraction of pyocyanine :

For extraction of crude PCN from *P. aeroginosa* used method of Al-Shibib and Kandela (1993) by inoculate a high pyocyanine production *P.aeroginosa* isolate in King-A broth[17] at 37Cº for 11 days (media was mix will daily) .After incubation , centrifuge the media and put the supernatant in large conical flask to add chloroform in 1:2 ratio ,mix well and leave it even form two layer according to different in specify gravity .Remove the supernatant and take the lower layer due to contain the pigment which dissolved in solvent , to drying the pigment which used evaporator at 50Cº,then make different

concentration of dye (50,100,150,200 µgm/ml) by using PBS[18].

Agar diffusion assay :

To observed the antagonist effect of tested dye upon other bacteria , culture the bacteria on nutrient agar and make four well in agar , then few drops of different concentration of dye was added in each well and ,incubated for 24hr after that the distance of inhibition zone was measured by help of ocular lens .

Results:

Isolation and diagnosis of bacteria : *P.aeroginosa* was given from samples of patients that examined in consultory clinics in Al sadr educational hospital in Al najaf and to ensure the bacteria *P.aeroginosa* ,culture the samples in blood agar and MacConky agar , when the colonies revealed the characters of P.aeroginosa and when culture on Pseudomonas special media the colonies given opaque, circle , and regular edges .The microscopical examination revealed Gram' Negative bacilli , and the other biochemical tests given results as shown in table-1.

Table-1 . The characteristic of *Pseudomonas aeroginosa*

TESTS	P.aeroginosa
Pyocyaninogenic strain	88.5%
Gram' stain	+ve
Growth in 42 Cº	+ve
Catalase	+ve
Oxidase	+ve
Gelatenase	+ve
Urease	+ve
Glucose oxidation	+ve

Pyocyanine extraction : The PCN produced from *P.aeroginosa* was variable from one isolate to other, for this reason n our study , the sample that given the higher productivity of PCN was choosen .In table-2 observed the higher concentration of pigment in sample number (6) according to the optical density which measured by spectrophotometer .

Table-2. The optical density of pyocyanine and the site of samples .

Site of samples	Optical density
1- urine sample	0.12
2-wound swab	0.22
3-wound swab	0.31
4-Burn swab	0.30
5-wound swab	0.51
6-wound swab	0.61
7-wound swab	0.45
8-Ear swab	0.32
9-ear swab	0.22
10- Burn swab	0.21

inhibited in conc. (150µgm /ml) PCN , but *Klebs.sp*. and *proteus sp*. are inhibited in(200µgm/ml) as showing in table -3 .Also table -3 showed the *P.aeroginosa sp.* Resist all concentration of PCN and are growing in various types of inhibitors released from P.aeroginosa.

Antagonist action of PCN : While the studying the antagonistic activity of *P.aeroginosa* against other pathogenic bacteria species we found that crude PCN extracted from *P. aeroginosa* was inhibited the growth of *Staph. aureus* in conc. (100 µgm/ml)of PCN ,while the *E.coli*

Table-3. The effect of pyocyanine concentration on growth of other pathogenic bacteria

The isolates	PCN concentration(µgm/ml)							
	50		100		150		200	
Staphylococcus Aureus	G	IZ	G	IZ	G	IZ	G	IZ
	+	0mm	-	2mm	-	3mm	-	4mm
Klebsiella sp.	+	0mm	+	0mm	+	0mm	-	2mm
E .coli	+	0mm	+	0mm	-	2mm	-	3mm
Proteus Sp.	+	0mm	+	0mm	+	0mm	-	3mm
P.aeroginosa	+	0mm	+	0mm	+	0mm	+	0mm

G: Growth of bacteria , IZ: Inhibition zone in mm

Discussion:

In this study, found that *P.aeroginosa* exhibits antagonistic relationship with *Staph. aureus* and other pathogenic bacteria via secretion of respiratory inhibitors as pyocyanine .Our results indicated that *Staph. aureus* and *E.coli* may be resist PCN in low concentration ,but were in over concentration the corresponding colonies did not become PCN resistant [1]. The study assumed that resistant non pathogenic *Staph .Sp*. Share their natural habitat with *P.aeroginosa* and have therefore evolved a resistant phenotype ,and assumed that these *Staph. sp*. Live in an environment that is also occupied by *P.aeroginosa* and that they have learned to resist PCN to be able to coexist with Pseudomonas [19].

However, the resistance mechanism for PCN is unclear .It has been described that in vitro PVN oxidizes NADH+H+, forming O2 and H2O2 as by-products, and it was concluded that the production of these reactive oxygen species is responsible for antibiotic activity of pyocyanine [20]. The pathogenic G-ve bacteria species representatives have evolved a higher tolerance ,suggest that they share their biotype more frequently with P.aeroginosa than with G+ ve bacteria .It is intriguing that Staph. aureus and other pathogenic species have not evolved a pyocyanine resistant respiration .

Our hypothesis is that in their evolution, they have been so much adapted to their host that either have not gained or have lost the ability to resist respiratory toxins released by *P.aeroginosa*. Probably, there was no real selective pressure, as pathogenic Staph. and Pseudomonas species do not interact frequently during infection .for example, in the course of lung infection in cystic fibrosis (CF) patients ,*Staph. aureus* infection frequently comes first and is later followed by P.aeroginosa infection .But even when they infect the same organ , they must not necessarily reside at the same site .the persistence of Staph. aureus in CF has previously been associated with the isolation of subpopulation of *S.aureus* with small colony variant (SCV) phenotype [21].

Our results indicated when present of mix culture from site of infection in same time seem to be monoculture but it has two type of causative agents and when treated the bacteria lead to no response to treatment due to treat one species of bacteria for this reason comes the important of this study.

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