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Antimicrobial Activity of Pyocyanin Produced by *Pseudomonas aeruginosa* Isolated from Surgical Wound-Infections

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Abstract: Five different isolates of *Pseudomonas aeruginosa* were obtained from surgical specimens and minced meat. The isolates were tested for the production of the blue pigment; pyocyanin. Considerable amounts of blue pigment were produced by *P. aeruginosa* isolates when grown on the four tested media. Müller-Hinton agar was further used for growth, pigment production and sensitivity tests. Pigment production began during the first 24 hrs of growth and maximal pigment production was achieved after 48 hrs by isolates No. 1, 2 and 3. At this time, isolate No. 5 produced only moderate level of pyocyanin. While isolate No. 4 reached the highest yield after 72 hrs. Pyocyanin pigments obtained from culture supernatants of the tested *P.aeruginosa* isolates "grown in peptone water liquid medium" by serial chloroform extractions reached about 62.8µg/ml. The growth of all Gram-positive bacteria and *Candida* species was completely inhibited when cultivated on the agar plates containing the blue pigment. Whereas, Gram negative bacteria; *Klebsiella pneumoniae* and *P. aeruginosa* were resistant to pyocyanin. *Salmonella typhi* and *Proteus mirabilis* were intermediately affected. Thus, the five tested *P. aeruginosa* isolates were resistant to their own produced pyocyanin pigments. In a Conclusion, the Gram-positive bacteria were more susceptible as a group to the antibiotic action of pyocyanin than were the Gram negative bacteria. The antibacterial and antifungal nature of the pigment is attractive for the topical treatment of wound infections.

Key words: *Pseudomonas aeruginosa* · Antibiotic resistance · Pyocyanin · Chloroform

INTRODUCTION

Phenazines are heterocyclic compounds that are produced naturally and substituted at different points around their rings by different bacterial species [1]. Pyocyanin is a water-soluble blue-green phenazine pigment produced in large quantities by active cultures of *Pseudomonas aeruginosa.* Pyocyanin (*N*-methyl-1-hydroxyphenazine) has antibiotic activity against a wide variety of microorganisms [2-6].

The nature of bacterial inhibition by the phenazine is neither well understood nor well documented and no information is available concerning the effect of pyocyanin on the producer organism *P. aeruginosa.* The inhibitory action of pyocyanin is the result of its unique redox potential. It was also proposed that, during respiration, pyocyanin becomes reduced and univalently reduces oxygen to the toxic superoxide radical. The resistance of various bacteria to pyocyanin would therefore be dependent upon the levels of superoxide dismutase and catalase possessed by the organism and on the presence of oxygen [7]. Pyocyanin is a redox-active phenazine compound that kills mammalian and bacterial cells through the generation of reactive oxygen intermediates. *P. aeruginosa* resists pyocyanin because of the limited redox cycling of this compound and that under conditions favoring pyocyanin production; catalase and superoxide dismutase activities are increased [8].

The antagonistic effects of almost all of phenazine derivatives are usually attributed to one general characteristic redox activity. The 2-hydroxy- phenazine-1-carboxylic acid (2-OHPCA) produced by *Pseudomonas aureofaciens* is thought to kill off competing fungi through the production of reactive oxygen species [9]. Many effects of pyocyanin and phenazine-1-carboxylic acid (PCA) on a diversity of eukaryotic hosts as well as bacteria are thought to result from oxidative stress

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response [6, 10, 11]. A derivative of phenazine PCN zag2 (pyocyanin) produced by *P. aeruginosa* zag2 possess antagonistic activities against certain fungi. The antifungal activity of this compound was remarkable at 100°C for 20 minutes [12].

The ability of opportunistic human pathogen; *P. aeruginosa* to acquire resistance to a broad range of antibiotics has made effective therapy more difficult. Several recent investigations have dealt with the problem of antibiotic resistance in *P. aeruginosa* [13, 14]. A multidrug resistant *P. aeruginosa* strain has caused an outbreak in a neurosurgery ward [15]. Furthermore, *P. aeruginosa* and *Acinetobacter baumannil* infections were recorded in healthcare settings [16]. *Staphylococcus aureus* and *P. aeruginosa* strains showed different resistance patterns to various antibacterial agents. Strains of *Klebsiella pneumoniae, Acinetobacter baumannil, Citrobacter freundii*, *Enterococcus fecalis, Proteus mirabilis, P. vulgaris, Streptococcus agalactiae* and *Vibrio cholerae* were resistant to 4-9 antibiotics [17].

The increase in bacterial strains resistance, both to antibiotics and other disinfectants and germicides, led researchers to investigate other options, in treating both antibiotic-resistant and susceptible infections. In this concern, phenazines have been of great interest to pharmaceutical and clinical research groups for the last 50 years [6, 18, 19].

This work was undertaken to explore the growth conditions required for the production of a phenazine antibiotic pigment (pyocyanin) by *P. aeruginosa* isolated from clinical specimens; especially after surgical operations of some patients attending El-Olafey Hospital, Hodeidah Governorate, Republic of Yemen. The antibiotic action of pyocyanin pigment against different bacteria and yeast was investigated.

MATERIALS AND METHODS

Microorganisms: The selected four *P. aeruginosa* were isolated using swabs from surgical wounds of patients attending El-Olafey Hospital, Hodeidah Governorate, Republic of Yemen. One isolate of *P. aeruginosa* was obtained from minced meat. Thus, the five multi-drug resistant isolates; previously tested for antibiotic susceptibility test [20] were subjected to standard microbiological and biochemical techniques for identification in the Diagnostic Microbiology Laboratory, Faculty of Medical Sciences, Hodeidah University, Yemen.

Test microorganisms shown in table (3) were isolated from clinical specimens and identified at the Diagnostic Microbiol. Lab., Fac. Med. Sci., Hodeidah Uni., Yemen. The tested Gram positive and Gram negative bacteria were *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus mutans, Micrococcus* sp. and *Clostridium botiulinum* and *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhi, Salmonella paratyphi and Vibrio parahaemolyticus*. *P. aeruginosa* isolates were tested for production of pyocyanin [20]. The yeasts, *Candida albicans* and *Candida tropicalis* were also used for antimicrobial testing. All stock cultures were maintained on nutrient agar slants at 4°C with monthly transfer.

Media: Nutrient agar, blood agar, Mac Conkey agar and Müller Hinton agar were used for the growth of *P. aeruginosa* and detection of pyocyanin. In addition, peptone water liquid medium was used for the production of pyocyanin pigment.

Production of Pyocyanin: *P. aeruginosa* was inoculated into peptone water liquid medium and grown for 72 hrs at 37°C. Bacteria were then removed by centrifugation (10,000 *g* x 30 min) and filtration of the supernatant through 0.45-µm filters [8].

Purification of Pyocyanin: Pyocyanin was extracted from the culture supernatants of *P.aeruginosa* isolates by serial chloroform extractions followed by sequential extractions with acid and neutral water [21]. Concentrations expressed as micrograms of pyocyanin produced per ml of culture supernatant were calculated using an extinction coefficient at 520 nm of 17.072 [22, 23]. *Pseudomonas* proteins were removed during purification by chloroform extraction of the pyocyanin. After the completion of five separation sequences, the pH of the isolated acidified water layer was adjusted to 7.5 with a minimum volume of 0.1 M NaOH. Needlelike crystals were formed in the chilled solution over the following 2 hrs. These were trapped on a 0.45 µm (pore size) filter and washed with water. Finally, the pyocyanin was crystallized, dried under vacuum and weighed. It was re-suspended in water and stored at 4°C until used.

Antimicrobial Activity of Pyocyanin: Five isolates of *P. aeruginosa* were grown on Müller-Hinton agar for pigment production. After 48 hrs of incubation, bacterial cells were scraped and the plates were exposed

to chloroform to kill the remaining cells. Thereafter, some test bacterial and yeast strains were inoculated on the surface of chloroform-treated agar plates. The inhibition of the microbial growth indicates the antimicrobial activity of pyocyanin [24].

The paper-disc agar diffusion method, with circular discs of filter paper (5 mm in diameter), was also used to demonstrate the antimicrobial activity of the extracted pigment. The discs were impregnated with 10 to 20 µg of the purified pigment dissolved in chloroform. The solvent was evaporated and the discs were placed on Müller Hinton agar plates seeded with 100 µl of the test culture corresponding to 10^6 of cells. Plates were incubated for 24 hrs at 37°C and pigment sensitivity was interpreted by diameter of inhibition zones around the discs [25].

RESULTS AND DISCUSSION

Phenazine compounds have been shown to be important virulence factors in *P.aeruginosa*. In addition, they act as cell-cell signaling molecules and have inhibitory activity against other bacteria [19]. Phenazine compounds, such as pyocyanin produced by *P. aeruginosa*, are antibiotics in their own right that can function as competitive agents in microbial communities. The antibiotics secreted by other microorganisms in microbial communities could serve as a signal to alert *P. aeruginosa* to the existence or aggression of other bacteria and the subsequent increased pyocyanin production would help *P. aeruginosa* to compete with the other microbes [6].

Production of Pyocyanin on Different Media: Cultures of *P. aeruginosa* strains elicited a blue green pigment, reminiscent of pyocyanin, during growth on the four tested media; nutrient agar, blood agar, Mac Conkey agar and Müller Hinton agar (Table 1). Several nutritional media can be utilized by *P. aeruginosa* for the biosynthesis of pyocyanin. In previous investigations, *P. aeruginosa* was grown in low- and high-phosphate succinate media. Under culture conditions of limited phosphate, both pyocyanin production and catalase activity were enhanced [8]. Five clinical isolates (CIs) of *P. aeruginosa* were examined for the production of pyocyanin. Glycerol alanine minimal (GA) medium was utilized to determine the production of pyocyanin by the quorum sensing-deficient clinical isolates. Analysis of the QS-deficient CIs revealed that similar to *P. aeruginosa* PDO100, CI-4 produced no pyocyanin, whereas CI-1, CI-2 and CI-3 produced negligible levels of pyocyanin in comparison with *P. aeruginosa* PAO1. In contrast, the level of pyocyanin produced by CI-5 was comparable to that produced by PAO1 [26]. Glycerol-Luria broth was also used for pyocyanin production [27].

Effect of Incubation Period: Considerable amounts of the blue pigment were produced by *P. aeruginosa* isolates when grown on Müller-Hinton agar (Table 2). Pigment production began during the first 24 hrs of growth and maximal pigment production was achieved after 48 hrs by isolates No. 1, 2 and 3. At this time, isolate No. 5 produced only moderate level of pyocyanin. While, the isolate No. 4 reached the highest yield after 72 hrs. In previous studies, ninety-seven percent of a total of 135 clinical *P. aeruginosa* isolates elaborated detectable pigments on the modified Mac Conkey agar (MMA) within 24 hrs. Several strains of *P. aeruginosa* (83%) produced pigment on MacConkey agar and sheep blood agar as well, but most required 48 hrs of incubation [28]. An unusual phenotype of P. aeruginosa, characterized by early-and over-production of quorum sensing-regulated virulence factors such as the exoproducts pyocyanin and LasA protease, is widespread amongst cystic fibrosis isolates. In a simple glycerol-Luria broth test for pyocyanin production, whereby cultures were incubated overnight in 5 ml cultures, the levels of pyocyanin were lower than those measured during exponential growth in 50 ml cultures.

Table 1: Production of pyocyanin pigment by the selected *Pseudomonas aeruginosa* isolates grown on different media

	Pyocyanin production			
Bacterial isolates	Nutrient agar	Blood agar	Mac-Conkey agar	Müller Hinton agar
P. aeruginosa 1	++	++	+	+++
P. aeruginosa 2	++	++	+	++
P. aeruginosa 3	++	++	+	++
P. aeruginosa 4	++	++	+	++
P. aeruginosa 5	+	+	-	+

Visual assessment of pyocyanin amount (blue pigment): + low, ++ moderate, +++ high.

Table 2: Production of pyocyanin pigment by *Pseudomonas aeruginosa* isolates grown on Müller-Hinton agar for different incubation periods

	Pyocyanin production		
Bacterial isolates	24 hrs	48 hrs	72 hrs
P. aeruginosa 1	++	+++	+++
P. aeruginosa 2	++	+++	+++
P. aeruginosa 3	++	+++	+++
P. aeruginosa 4	++	++	+++
P. aeruginosa 5	+	++	++

Visual assessment of pyocyanin amount (blue pigment): + low, ++ moderate, +++ high

It is likely that this was either due to some breakdown of pyocyanin or because of greater aeration during growth of the 50 ml cultures in 250 ml flasks [27]. Pyocyanin was produced by *P. aeruginosa* in King's B medium in pH 7±0.2 after 96 hours at 37°C [12].

Sensitivity of the Test Microorganisms to Pyocyanin: The data presented in table 3 revealed that the growth of all Gram-positive bacteria and *Candida* species was completely inhibited when cultivated on the agar plates containing the blue pigment. Whereas, Gram negative bacteria; *Klebsiella pneumoniae* and *P. aeruginosa* were resistant to pyocyanin and *Salmonella typhi* and *Proteus mirabilis* were intermediately affected.

Gram positive bacteria; *Micrococcus luteus, Staphylococcus aureus, Bacillus licheniformis, Bacillus subtilis, Paracoccus denitrificans* were more susceptible to the antibiotic action of pyocyanin than were Gram negative bacteria; *Escherichia coli, Proteus vulgaris, Enterobacter aerogenes* and *P. aeruginosa* [2]. Pyocyanin has been detected in an oil-degrading culture containing *P. aeruginosa* and is a redox-active compound capable of inhibiting the growth of pyocyanin-sensitive members of the microbial community [5]. The antagonistic effects of almost all of phenazine derivatives are usually attributed to one general characteristic redox activity [1]. Pyocyanin, a phenazine produced by *P. aeruginosa*, suppresses wheat blotch caused by *Septoria tritici* [3].

Table 3: Sensitivity of the test microorganisms to pyocyanin produced by the selected *Pseudomonas aeruginosa* isolates grown on Müller-Hinton agar

	Pyocyanin of *P. aeruginosa* isolates (1-5)				
Test microorganisms	Pa 1	Pa 2	Pa 3	Pa 4	Pa 5
Gram positive bacteria					
Staphylococcus aureus 1	S	S	S	S	S
Staphylococcus aureus 2	S	S	S	S	S
Staphylococcus epidermidis	S	S	S	S	S
Staphylococcus saprophyticus	S	S	S	S	S
Streptococcus mutans	S	S	S	S	S
Micrococcus sp.	S	S	S	S	S
Clostridium botiulinum	S	S	S	S	S
Gram negative bacteria					
Escherichia coli	S	S	S	S	S
Klebsiella pneumoniae	R	R	R	R	R
Proteus mirabilis	I	I	I	I	I
Pseudomonas aeruginosa 1	R	R	R	R	R
Pseudomonas aeruginosa 2	R	R	R	R	R
Pseudomonas aeruginosa 3	R	R	R	R	R
Pseudomonas aeruginosa 4	R	R	R	R	R
Pseudomonas aeruginosa 5	R	R	R	R	R
Salmonella typhi	I	I	I	I	I
Salmonella paratyphi	S	S	S	S	S
Vibrio parahaemolyticus	S	S	S	S	S
Yeasts					
Candida albicans	S	S	S	S	S
Candida tropicalis	S	S	S	S	S

Pa: *P. aeruginosa,* S: sensitive (Inhibition zone: 15-32 mm),
I: intermediate (Inhibition zone: 7-14 mm), R: resistant (no inhibition of growth).

Production of phenazine antibiotics, mainly phenazine-1-carboxylic acid (PCA) and minor amounts of oxychloraphine (OCP), contributed to the capacity of *P. aeruginosa* PNA1 to suppress Fusarium wilt of chickpea, caused by *Fusarium oxysporum* f. sp. *ciceris* and Pythium damping-off of bean, caused by *Pythium splendens*. When grown in cultures, PNA1 also inhibited the mycelial growth of certain other phytopathogenic fungi [4]. The major antifungal agent of *P. aeruginosa* was found to be pyocyanin; 1-hydroxy-phenazine also possesses activity. Pyocyanin MICs for *Candida albicans* and *Aspergillus fumigatus* were > 64 µg/ml. These phenazines were active against nine other yeast species pathogenic for man. Preliminary experiments also suggested possible inhibition of yeast mycelial transformation in *C. albicans* by pyocyanin. There may be a role for pyocyanin and 1-hydroxyphenazine in the prevention of pulmonary candidiasis in patients colonized by *P. aeruginosa* [29]. Some metabolites produced by *P. aeruginosa* zag2 had antagonistic activities against certain fungi. The minimum inhibitory concentration of pyocyanin against *C. albicans* was 40.69ìg/ml. The antifungal activity of this compound was remarkable at 100°C for 20 mins [12].

Action of Pyocyanin on the Producer Organisms: Five isolates of *P. aeruginosa* were inoculated on the surface of chloroform-treated agar plates containing the blue pigment. Table 3 showed the sensitivity of the *P. aeruginosa* isolates to the tested pigments. The five tested isolates were resistant to their own produced pigments. Previous studies revealed that if *P. aeruginosa* uses pyocyanin production to its advantage in competing with other bacteria in the same ecological habitat, it must therefore have a mechanism to insure its own protection or immunity against the bactericidal agent it produces. This immunity could be via higher concentrations of superoxide dismutase (SOD) and catalase or by lack of permeability [7]. Similarly, all apyocyanogenic pseudomonads tested (reddish-brown strains of *P. aeruginosa, P. denitrificans, P. fluorescens* and *P. perfectomarinus*) were totally resistant to the pyocyanin pigment, suggesting that resistance may be a characteristic of the genus. *P. aeruginosa*, the producer organism was also essentially unaffected by high concentrations of the pigment [2]. Pyocyanin did not affect the intracellular killing of *P. aeruginosa* in human neutrophils [30]. Our results were coincided with those above mentioned studies because of the recorded pyocyanin resistance of the producing *P. aeruginosa* isolates.

Various levels of susceptibility to pyocyanin have been reported for individual microorganisms and susceptibility to pyocyanin is thought to depend on the rate of pyocyanin uptake and the level of antioxidant enzyme (SOD and catalase) activity [8]. It is currently assumed that differential expression of catalase and SOD activities is the principal mean of pyocyanin resistance [5]. It is also possible that other general antibiotic resistance mechanisms play a role in pyocyanin resistance [2].

Antimicrobial Activity of the Purified Pyocyanin: The purified pyocyanin extracted was 62.8µg/ml. The pyocyanin-resistant bacteria and fungi were completely resistant. It was also noticed that the Gram positive bacteria were more susceptible to the antibiotic action of pyocyanin (inhibition zones = 15 mm in diameter) than the Gram negative bacteria (inhibition zones = 14 mm in diameter) as shown in table 3.

P.aeruginosa antibacterial substance inhibited the growth of 177 out of 189 strains including nine staphylococcal species, all of 16 methicillin-resistant *S. aureus* and 27 of 39 strains of six other Gram positive genera. The antibacterial activity was heat stable and could be extracted into chloroform [31]. The sensitivity of Gram positive bacteria and resistance of some Gram negative bacteria as well as the heat stability of the produced pyocyanin preparations recorded in our study support its agreement with the above mentioned finding.

It can be concluded that pyocyanin possesses antimicrobial activity against several multidrug resistant pathogens and may be used topically in susceptible cases. Future studies are required to advocate its systemic use in infectious diseases.

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