

Purified Pyocyanin from Clinical Isolates of *Pseudomonas aeruginosa* Enhances Antibiotic Sensitivity Against Some Pathogenic Bacteria

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Abstract

Pseudomonas aeruginosa is one of the most life-threatening pathogen. It is considered nosocomial opportunistic microbe that cause wide range of infections including wound and burn infections, respiratory infections, and Otitis media. Despite the efficiency of antibiotics against infectious diseases, *P. aeruginosa* still causes complicated infections with antibiotic resistance in many clinical strains. The pigments produced by *P. aeruginosa* exhibits antibacterial properties. Thus, we have examined its ability to enhance antibiotics effect against some pathogenic microbes. 286 samples were collected from patients with different infections who visited Mosul hospitals. 76 samples were positive to *P.aeruginosa*. Among them, 38 (13.28%) of isolates were isolated from surgical infection, whereas, 12 (4.19%), 11(3.84%), and 7(2.447%) were isolated from Otitis media, Urinary tract infection, and pus, respectively. The pyocyanin in low concentrations showed synergistic effect with some antibiotics against *Staphylococcus aureus* and *Escherichia coli*. *E. coli* became sensitive to ciprofloxacin and nalidixic acid when mixed with 100 mg/ml pyocyanin. However, cloxacillin did not show any activity against *Staph.aureus* when mixed with 1 mg/ml and 6.25 mg/ml pyocyanin. *Staph aureus* became sensitive to nalidixic acid when mixed with 1 mg/ml pyocyanin.

Key word: *Pseudomonas aeruginosa*, pyocyanin, Antibiotics, Synergistic effect.

Introduction

Pseudomonas aeruginosa is widely spread in the environment. It is a gram negative, aerobic microbe in rod shape belongs to Pseudomonadaceae family. In this family, there are 12 other genus as well as *P. aeruginosa*. Although *P. aeruginosa* can be found in soil and water, it is considered the most opportunistic pathogen in hospitals¹⁹. It is also one of the nosocomial pathogens with antibiotic resistance strains¹³ *P. aeruginosa* composed of virulence factors including polysaccharide layer, exotoxins, pilli and biofilm formation ability. The key factor of pseudomonal infections is bacterial attachment to the host epithelial layer that keeps the microbe away from the host immune defense²¹.

P. aeruginosa produces many pigments, including pyocyanin (bluish green), pyoverdin (fluorescent yellow-green) and pyorubin (brownish red)²⁸. These pigments contribute to pseudomonal infections. Many studies suggest that pseudomonal pigments can be a virulence factor in the lungs of patients with cystic fibrosis. They also may interfere host cell respiration. The mechanism of the pigment pathology is still unknown²⁴.

The produced pyocyanin has antibacterial properties. It has been proved that pyocyanin prevents other bacterial growth is the site of infection¹. This characteristic provides a persistence growth for *P. aeruginosa*. Many studies examined the possible activity of pyocyanin against other pathogens.

Multi-resistant strains of *P. aeruginosa* as nosocomial pathogen has spread in hospitals leading scientists to dig out other efficient drugs. In this study, we hypothesized that pyocyanin is a drug nominate and

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whether can be used to enhance antibiotic efficiency by examining the synergetic effect of pyocyanin and antibiotics with less effect. We isolated and diagnosed clinical isolations from patients with different infections including otitis media, wound infection, surgical infection, urinary tract infection and upper respiratory infection. The synergistic effect of pyocyanin with antibiotics was examined.

Materials and Methods

Samples collection and identification:

286 samples were collected from different infection sites including wounds, otitis media, burns, surgical infections, urea and sputum, from patients who visited Mosul hospitals. Identification process were initially based on bacterial colony appearance on blood agar as well as the smell of culture. The haemolysis of blood on blood agar were reported as well as pigment production¹⁰. Other standard diagnostic methods were performed according to²². Molecular diagnosis of isolates were performed using 16S rRNA sequencing method. The genomic DNA were extracted using the extraction kit supplied from Geneaid. The genomic DNA were then visualized on agarose gel electrophoresis. The PCR reaction was carried out using the primers 16S RNA-F: AGAGTTTGATCCTGGCTCAG, and 16S RNA-R: AAGGAGGTGATCCAGCCGCA. The reaction was prepared by mixing 50 ng of DNA template with 10 pmol of primer mixture and 10 µl of hot start taq polymerase. The reaction was then topped up to 20 µl. the tubes were then put into the thermocycler and the programe was run. The reaction condition was performed as 1 cycle of: initial denaturation at 95°C for 6 minutes, and 35 cycles of: denaturation at 95°C for 45 seconds, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute, and 1 cycle of: final extension at 72°C for 5 minutes. The amplicons were then visualized on agarose gel electrophoresis. The amplicons were then purified from agarose gel and extracted using DNA extraction kit. The purified PCR products were sent for sequencing using 16S RNA-F and 16S RNA-R. The sequence process was performed at Hitachi Company (Japan). The sequencer 3130 provided from Macrogen Biotechnology Company were used for gene sequencing. The sequence results were then blasted with DNA database using National Center for Biotechnology Information (NCBI) website

(<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Pyocyanin extraction and purification:

P. aeruginosa isolates were grown on King A media to confirm the pigment production and they were then grown on alanine minimal agar glycerol medium to enhance the pigment production. The cultures were incubated at 37° C for 48 hours. The culture were then exposed to light source with incubation at (25° C)⁶. The extraction of pyocyanin were performed according to the method described by Watson et al., 1986 with some modifications.

Antibiotic susceptibility test:

The sensitivity test was performed according to Clinical and Laboratory Standards Institutes (CLSI)⁵. Fresh bacterial culture were spread finely on Muller Hinton agar and left to dry. The antibiotic discs were put on the inoculated plates and incubated at 37 ° C for 24 hours. The antibiotics used in this study were penicillin, Cefixime, peperacillin, nalidixic acid, gentamycin, azithromycin , amikacin, ciprofloxacin, doxycycline, cloxacillin, erythromycin, chloramphenicol, amoxicillin, novobiocin, and tetracycline.

Synergistic effect of pyocyanin with some antibiotics:

The synergistic effect of pyocyanin with some antibiotics was performed according to the method described in¹⁴ with some modifications. the disc diffusion assay was used in this study. The optimal and minimal concentration of pyocyanin and antibiotics were reported against *Staph. aureus* and *E. coli*. The minimal concentrations of the pigment and antibiotics were then mixed to examine the synergistic effect of pseudomonal pyocyanin with antibiotics.

Statistical Analysis

Graphpad Prism software (Graphpad, California, USA) was used for statistical analysis. Means and standard error of means (SEM) were used to analyze the results. Significant difference was assessed at *p* values: * *p*<0.05, ** *p*<0.01.

Results and Discussions

Collecting of sample:

Among 286 samples collected from patients visited Mosul hospitals, we have isolated 76 positive samples of *P. aeruginosa*. The positive samples were confirmed using biochemical tests as well as molecular identification using 16S RNA gene sequencing. All samples tested for 16S RNA showed conserved sequence of the gene confirming that the gene is present in all *P. aeruginosa* isolates. The Figure 1 shows agarose gel electrophoresis of *P. aeruginosa* genomic DNA. This shows the purity and integrity of genomic DNA as the key factor of PCR process success is the purity of DNA used²⁷. The Figure 2 shows agarose gel electrophoresis

of PCR amplicons showing the exact size of gene of interest using the specific primers (16S RNA-F and 16S RNA-R). The amplicons were then purified from the gel and sent for sequencing. The results of sequence were then blasted and aligned with universal gene bank (NCBI). The molecular identification of microorganisms has been extensively used to confirm the bacterial identification. The gene polymorphism analysis is based on conserved sequences present in some housekeeping genes such as 16S RNA³. Although it is widely known that *P. aeruginosa* belongs to genetically diverse group of bacteria, 16S RNA gene is highly conserved in most of species with minor modifications³⁰.



Figure: (1) Agarose gel electrophoresis of *P. aeruginosa* genomic DNA.

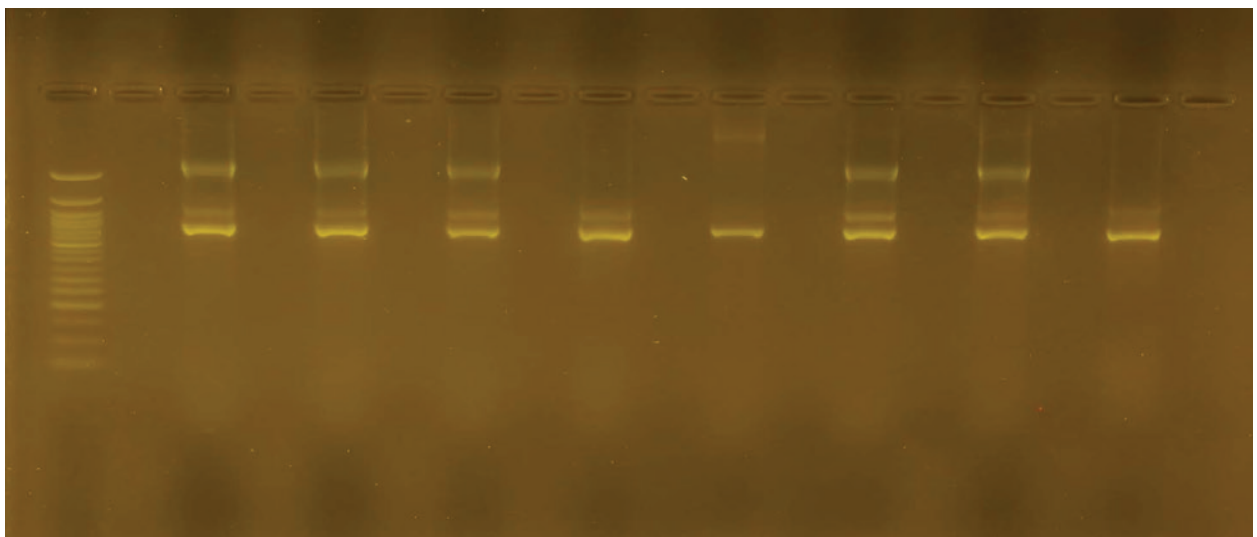


Figure (2) Agarose gel electrophoresis of 16S RNA amplicons with expected size of 650bp DNA. 16S RNA-F and 16S RNA-R primers used to amplify the gene of interest. Lane 1: 100bp DNA ladder, lanes 2-9: DNA amplicons of gene of interest.

Table 1 shows the samples taken and the site of infection as well as the proportion of infections. As can be seen from the Table 1, the highest percentage of infection was 13.28% from surgery infections, whereas the lowest percentage was 0.699% from patients with burns. Surprisingly, *P. aeruginosa* isolated from the respiratory tract infections was relatively low (2.09%) compared to other site of infections. Although many studies have founded that *P. aeruginosa* is a main cause of burn infections with multidrug resistance ^{16,21}, we

found that the burn infections was the lowest. This might be because of the extent of the hospital's concerns with the cleanliness of the hallways and medical equipment as well as the type of detergents and disinfectants that used in hospitals ¹⁵. It has also been reported that *P. aeruginosa* favors the moist and aerobic conditions to cause infections ²³. This explains the reason of the variation of *P. aeruginosa* infections might be affected by the site of infection and environment conditions.

Table (1): The number and percentages of the total samples of *Pseudomonas aeruginosa* isolated from different sites of infection.

Swab site	No. of samples	No. of <i>P. aeruginosa</i>	% for each site	% relative to total samples
Otitis media	17	12	70.58%	4.195%
Burns	2	2	100%	0.699%
Surgical infections	55	38	69.09%	13.28%
Sputum	61	6	9.83%	2.09%
Urinary tract infections	123	11	8.94%	3.84%
Wound infections	28	7	25%	2.44%
Total	286	76	-----	26.57%

Synergistic effect of pyocyanin and antibiotics:

In order to examine the activity of pyocyanin with antibiotics, the optimal concentration of antibiotics that did not show activity against *Staph. aureus* and *E. coli*. Antibiotic resistance test showed diverse activity of antibiotics used in this study. The Table 2 shows antibiotic activity on *Staph. aureus* and *E. coli*. As can be seen from the Table 2, the *Staph. aureus* showed resistance against most antibiotics used. Therefore, we have chosen cloxacillin and novobiocin that showed either intermediate or resistance. Regarding *E. coli*, the results also showed that the microbe was resistant to most antibiotics (Table 2). The antibiotics have chosen for synergistic activity for *E. coli* were ciprofloxacin and nalidixic acid.

Table (2): Antibiotics resistance test showing the effect of antibiotics on *Staph.aureus* and *E. coli*. The antibiotics have chosen according to ⁵ protocol. *: Sensitive; **: Resistant; *: Intermediate**

n.	E.coli			Staph.aureus		
	antibiotics	code	Result	antibiotics	code	Result
1	Pinicillin	P	S*	Doxycycline	DO.10	S
2	Cefixime	CFM	S	Cloxacillin	CX.10	I
3	Piperacillin	PRL	S	Erythromycin	E-10	R

Cont... Table (2): Antibiotics resistance test showing the effect of antibiotics on *Staph.aureus* and *E. coli*. The antibiotics have chosen according to ⁵ protocol. *: Sensitive; **: Resistant; *: Intermediate**

4	Nalidixic acid	NA	R**	Chloramphenicol	C-10	R
5	Gentamicin	CN	S	Amoxicillin	AX.25	R
6	Azithromycin	AZM	S	NovoBiocin	NV	R
7	Amikasin	AK	S	Tetracycline	TE-10	S
8	Ciprofloxacin	CIP	I***	Nalidixic acid	NA	I

Pyocyanin was extracted and purified from the culture to examine its activity against the pathogens used in this study. Different concentrations of pyocyanin used against *Staph. aureus* and *E. coli* to choose the concentrations that have either intermediate or no activity. The reason for choosing these concentrations to find out whether pyocyanin could enhance antibiotics activity. The results showed that *P. aeruginosa* produced pyocyanin in different concentrations (Table 3). The production of pyocyanin is influenced by many factors including environmental and genetic factors. It has also been reported that the site of infection might affect the amount of pyocyanin produced by *P. aeruginosa* ². The production of the pigment might also be affected by other pathogens at the same site of infection. It is proved that *P. aeruginosa* produces high concentrations of pyocyanin to compete other pathogens as the pigment has antibacterial properties ². Although all strains of *P. aeruginosa* have genes that encode to pyocyanin ¹⁸, the expression level of these genes might be influenced by environmental conditions and whether the pathogen requires the pigment during the infection ¹⁷.

Table (3): The percentage of pyocyanin extracted from 10 strains of *P. aeruginosa*. The strains were named as Du to refer to the researchers name.

n	Strain	Concentration mg / 25 ml	percentage%
1	Du1	0.207	0.828%
2	Du2	0.312	1.248%
3	Du3	0.370	1.48%
4	Du4	0.152	0.608%
5	Du5	0.340	1.36%
6	Du6	0.244	0.976%
7	Du7	0.248	0.992%
8	Du8	0.236	0.944%
9	Du9	0.235	0.94%
10	Du10	0.305	1.22%

Synergistic activity showed that *E. coli* became more sensitive to antibiotics when mixed with pyocyanin. *Staph aureus* showed intermediate resistance when antibiotics mixed with pyocyanin. The Figures 3 and 4 shows synergistic activity of pyocyanin with antibiotics against *E. coli* and *Staph. aureus*. As can be seen from the Figure 3, although *E. coli* was intermediate resistant to ciprofloxacin and resistant to nalidixic acid, it became sensitive to the antibiotics when mixed with 100 mg/ml pyocyanin (Table 4). However, ciprofloxacin did not show any activity against *E. coli* when mixed with 10 mg/ml pyocyanin (Figure 3, Table 4). This might be because pyocyanin activity is concentration dependent manner when mixed with antibiotics. Regarding *Staph. aureus*, the results showed that the pathogen became sensitive to novobiocin when mixed with 1 mg/ml pyocyanin (Figure 4, Table 4). It is possible that pyocyanin enhanced the activity of novobiocin in terms of DNA gyrase inhibition. Other explanation might be that pyocyanin might interact with bacterial DNA causing mutations that in favor to novobiocin activity^{12, 29}. However, Cloxacillin did not show any activity when mixed with 1 mg/ml and 6.25 mg/ml pyocyanin, respectively (Figure 4). The limit activity of antibiotics with *P. aeruginosa* pigment reflects the fact that Gram positive bacteria might exhibit resistance strategies against antibiotics, and thus, pyocyanin could not enhance the activity⁹.

It is reported that the ability of inhibition of pyocyanin is increased over the years as a result of the ability of bacteria to adapt to the environment and

produce more efficient virulence factors. This might be due to the development of their genes that encode to pyocyanin more efficiently²⁵. The synergistic activity of pyocyanin with antibiotics might be affected by the cell wall composition. It is clear that peptidoglycan and lipopolysaccharides layer could prevent the penetration of antibiotics as well as pyocyanin^{7,30,31}. This explains why *Staph. aureus* is less affected by pyocyanin and antibiotics as the thickness peptidoglycan layer reduces the penetration. On the other hand, *E.coli* showed less sensitivity against pyocyanin and antibiotics. The peptidoglycan layer in Gram negative pathogens is not thick compared to Gram positive bacteria^{8,20}. Cloxacillin belongs to penicillin antibiotic family that can be broken down by staphylococcal beta-lactamase enzymes¹¹. These enzymes might affect the synergistic activity of cloxacillin with pyocyanin, and thus, did not show any activity.

It is widely known that pyocyanin stimulates the formation of free radicals ($-O_2$ and H_2O_2). This leads to increase the oxidative stress in bacterial cells and makes them more affected by antibiotics²⁶. It is also reported that generation of active oxygen compounds (ROS) due to the exposure to pyocyanin stops all NADPH pathways by excluding electron¹². The gene expression is also affected by ROS and this might be beneficial for antibiotics to stop protein synthesis of bacterial cells⁴. Therefore, pyocyanin activity against pathogens could be beneficial to test whether can be used in antibiotics structure to boost their activity.

Table (4): Synergistic effects of pyocyanin with antibiotics. The concentration of pyocyanin were chosen as they have shown no effect on *Staph. aureus* and *E. coli*. Antibiotics used in this test were chosen because they did not show activity against pathogens.

Staph.aureus					E.coli				
Antibiotics activity		Pyocyanin activity		Synergistic activity	Antibiotics activity		Pyocyanin activity		Synergistic activity
CX.10	I	1mg/ml	I	R	CIR	I	100mg/ml	I	S
NV	R	1mg/ml	I	S	NA	R	100mg/ml	I	S
CX.10	I	6.25mg/ml	R	R	CIR	I	10mg/ml	R	R

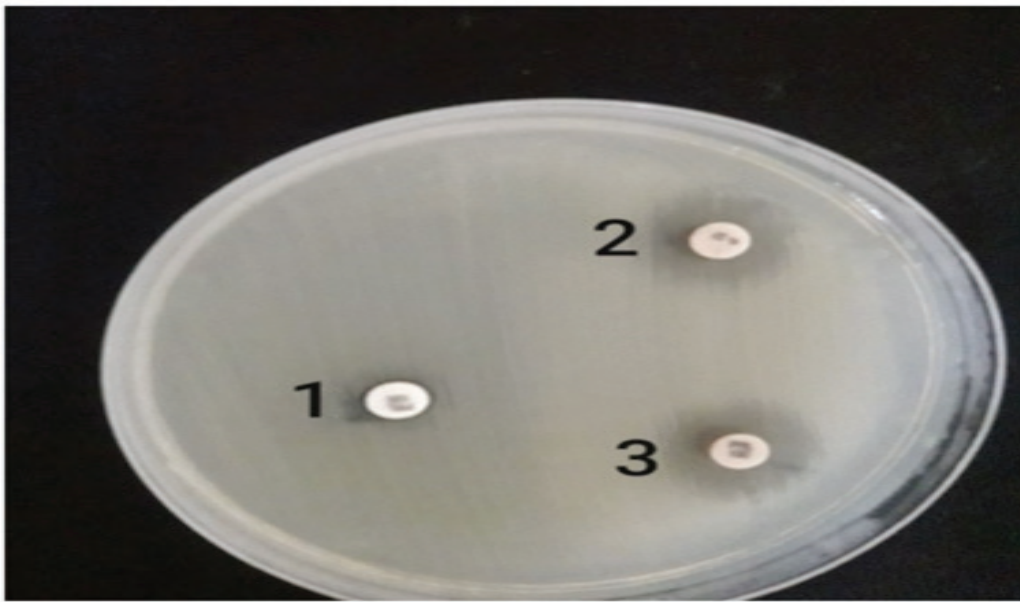


Figure (3): The synergistic activity of pyocyanin and antibiotics against *E. coli*. Muller-Hinton agar plates were used in this test. 1: Ciprofloxacin +10 mg/ml, 2; Nalidixic acid +100 mg/ml, 3; Ciprofloxacin +100 mg/ml.

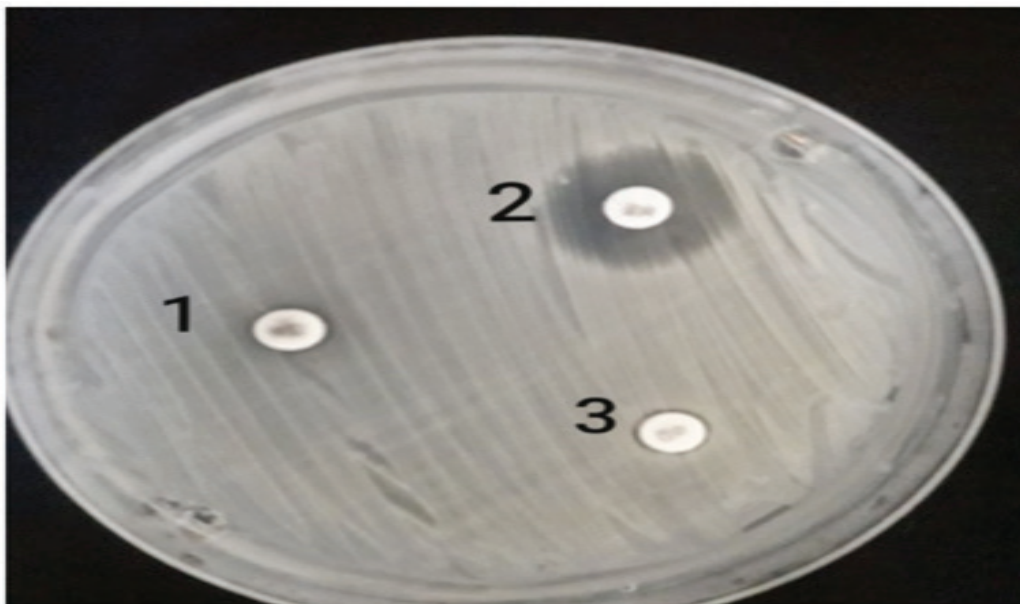


Figure (4): The synergistic effect of pyocyanin and antibiotics against *Staph. aureus*. Muller-Hinton agar plates were used in this test. 1: Cloxacillin + 1 mg/ml, 2; NovoBiocin + 1 mg/ml, 3; Cloxacillin + 6.25mg/ml.

Ethical statement: the project is done according to the ethical standards of Iraqi medical institutions. Ministry of Medicine, Iraq.

Source of Funding: This study was funded by the authors. All article preparation process and publication has done by authors and no other sources involved.

Conflict of Interest: Nil

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