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Study the Antimicrobial and Antifungal Effects on Clinical Isolates, and Molecular Detection of Pseudomonas aeruginosa Pyocyanin

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ABSTRACT

Background: Many substances, including pyocyanin, have antimicrobial, antifungal, and protozoal characteristics. Around 90 to 95% of P. aeruginosa strains generate pyocyanin, the primary phenazine pigment associated with organisms. Pyocyanin displayed powerful antibacterial, antioxidant, and anticancer properties.

Aim of study Pseudomonas aeruginosa Pyocyanin molecular detection and study the antimicrobial and antifungal effects on clinical isolated.

Materials and methods: One hundred patients with urinary tract infections (UTIs) at Babylon, Iraq's Al-Hilla Surgical Teaching Hospital and one hundred patients with burns or wounds at Imam Sadiq Hospital each donated urine samples between November 2023 and January 2024. Through the use of microscopy and bacterial isolation, all samples were cultivated on various mediums. As soon as possible, nutritional agar, MacConkey, and blood were used for aerobic culture. The Compact VITEK-2 System, colony morphology, biochemical testing, and Gram stain are used to identify aerobic bacteria. We looked for virulence genes and identified processes for pyocyanin manufacture, extraction, quantitative testing, and purification. Gene activity of the bacterial isolate pyocyanin.

Results: Vitek 2 found bacteria and fungus in 100 UTI urine samples. E. coli, Staphylococcus aureus, Shigella pneumonia Enterobacter, and E. faecalis. Culturing urine samples to the isolated fungus produced Candida albicans and Cryptococcus spp. In addition, 81% of 100 wound and burn infection samples were positive cultures and 19% negative. With 94% positive cultures on diverse growth conditions, 37% were Ps. aeruginosa-related and 62.9% were other microorganisms. The blue portion of tested agar selected 10 clinical P. aeruginosa isolates for pyocyanin extraction, P. aeruginosa isolate pyocyanin levels. One wound and burn isolate showed the highest pyocyanin concentrations (0.987µg/ml and 0.888µg/ml). Pure pyocyanin (0.987µg/ml) was tested for antibacterial activity against various pathogens. Pyocyanin has amazing biological effects on all creatures. The antimicrobial activity of purified pyocyanin at the concentration (0.987µg/ml) was monitored against different microorganisms. Results indicate that pyocyanin shows high biological activity against all mentioned organisms. Genomic analysis of the phzA1, phzA2 genes were conducted on 30 isolates previously identified as P. aeruginosa, every single isolate showed evidence of having this virulence gene, in contrast to an allelic ladder, there were (232) and (190) bp bands respectively.

Conclusions: Pyocyanin generated by clinical P. aeruginosa isolates showed antibacterial and antifungal action against Gram-positive and Gram-negative bacteria, Candida spp., and filamentous bacteria from UTIs and wound infections.

Keywords: Pseudomonas aeruginosa, Pyocyanin, Antimicrobial activity, fungi

INTRODUCTION

Pyocyanin, a blue redox-active secondary metabolite, is one of several tricyclic phenazine molecules. Its blue hue comes from their discharge into the medium in the later phases of its stationary phase (1). It can be readily removed from culture media since chloroform dissolves it. Pyocyanin is 5-methyl-1-hydroxyphenazine. It may undergo complicated oxidation-reduction reactions (2). Pyocyanin's structure revealed the phenazine nucleus as a natural product. Oxidation or reduction of pyocyanin. Molecular oxygen quickly interacts with reduced form, rendering it unstable. Biotechnological and pharmacological assessment of pyocyanin from marine Pseudomonas otitidis EGY-NIOF-A1 as an antimicrobial agent against clinical pathogens (3). Two stages are needed to produce pyocyanin from phenazine-1-carboxylic acid (PCA). A two-step chemical procedure converts PCA to pyocyanin (4). One enzyme, PhzM, converts PCA to betaine, 5-methylphenazine-1-carboxylic acid. This conversion involves adding methyl to a phenazine-ring N atom. PhzS, a FAD-dependent monooxygenase, hydroxylates 6-methylphenazine-1-carboxylic acid betaine to pyocyanin (5). The peculiar redox potential of pyocyanin caused its substantial antagonistic activity. It can swiftly complete a redox cycle and create a stable anion radical with one electron (6). Pyocyanin reduces when breathing, forming the harmful superoxide radical. Pyocyanin may be antibacterial due to O2- toxicity and increased H2O2 production. Thus, oxygen, superoxide dismutase, and catalase levels affect pyocyanin resistance (7). Antibiotic secondary metabolite pyocyanin may affect how bacteria populations respond to environmental changes. Pyocyanin's antimicrobial actions were shown to hinder metabolic transport by blocking the respiratory chain across cell membranes (8). Pyocyanin was antibacterial, antifungal, and antiprotozoal and had several pharmacological effects on eukaryotic and prokaryotic cells. Pyochelin and pyocyanin, oxygen-rich chemicals, may harm cells and induce resistance when combined (9). Pyocyanin, a water-soluble secondary metabolite, caused most P. aeruginosa strains' antimicrobial properties. Multiple P. aeruginosa strains were treated using a self-assembled portable CAP device (10). Time-dependent CAP sterilization was found. The CAP-treated bacterial culture had less pigmentation than the control group, possibly owing to lower bacterial survival rates (11). Inactivating the phzM and phzS genes had different impacts on P. aeruginosa survival following CAP treatment, with the former reducing and the latter increasing. Pyocyanin and 5-methyl phenazine-1-carboxylic acid (5-Me-PCA) in the bacterial solution protected against P. aeruginosa during CAP sterilization (12). However, adding phenazine-1-carboxylic acid (PCA), another metabolic pathway, and revealed minimal resistance to CAP. This antibiotic worked against Salmonella paratyphi, Escherichia coli, and Klebsiella pneumoniae. Pyrocyanin from P. aeruginosa has antibacterial properties against Listeria monocytogenes and Bacillus cereus, which cause food degradation. Hemolysin, hydrolytic enzymes, and the secondary metabolite were key antibacterial agents. Pyocyanin may impede C. albicans yeast-mycelial transition and Aspergillus fumigatus and Candida growth. Pyocyanin-induced systemic resistance protects tomatoes against Fusarium wilt. Pyrocyanin inhibited A. niger. CF patients' sputum included Aspergillus fumigatus and Candida albicans, which P. aeruginosa pyocyanin decreased (13). Clinical evidence showed that pyocyanin suppressed numerous Candida albicans growth in lung infection patients. Candida albicans returned after suppressing pyocyanin (14). Pyocyanin, pyrrolnitrin, and pseudomonic acid from Pseudomonas aeruginosa have antibiotic actions on Candida species. Pseudomonas aeruginosa N-acyl homoserine lactones (HSLS) reshaped Candida albicans. C. albicans also reduced P. aeruginosa swarming motility. Cultured P. aeruginosa produced pyrocyanin and inhibited Candida albicans growth (15).

OBJECTIVES

This study aimed to molecular detection of pyocyanin genes of Pseudomonas aeruginosa, and study the antimicrobial and antifungal effects on clinical isolates.

METHODS

Bacterial isolation and identification

A hundred urine samples were obtained from patients admitted to the Urology consultant clinic of Al-Hilla Surgical Teaching Hospital in Babylon city (Iraq) with UTIs, and another hundred from patients admitted to and visiting Imam Sadiq Hospital with wounds or burn infections, over a three-month period beginning in November 2023 and ending in January 2024. All of the samples were cultured using various medium according to established protocols for microscopic analysis and bacterial isolation. For the purpose of preventing contamination, specimens were meticulously gathered. The material was quickly put into Blood, MacConkey, and nutrient agar medium for aerobic culture. After incubation at 37oC for 24 hours, the culture was stopped. According to McFadden (16), the aerobic bacteria that were isolated were identified using the following methods: gram stain, colony morphology, biochemical test, and the Compact VITEK-2 System for identification of bacteria.

Ethical Approval

Before being included in the trial, every patient's proper permission was obtained.

Identification of bacterial isolates with Compact VITEK-2 System

To screen and identify all bacterial isolates, the Compact VITEK-2 System (BioMerieux) was used. Biochemical reactions are used to identify isolates in this phenotypic identification approach. For a wide range of fluorescent biochemical analyses, the Vitek-2 card offers 64 individual wells. Twenty of the sixty-four carbohydrate absorption assays included phosphatase, urea, nitrate, and actidione. The card was filled, sealed, and transported to the 35°C incubator by the Vitek-2 machine on its own. A distinct computational approach is used to decipher each report that is generated. The ID-GN, ID-GP database was used to identify the acquired results. The accompanying software automatically proposes the IDs created by these systems. If the first results showed "poor discrimination" or "no ID," then the tests were repeated and the data was analyzed based on the second result. We incubated all of the strains overnight at 37°C after adding them to the culture medium. Following the instructions provided by the manufacturer (BioMerieux), a single isolated colony was identified using the phenotypic VITEK-2 Systems method.

Pyocyanin production, extraction quantitative assay and purification

After incubating each P. aeruginosa isolate for 2-3 days at 37°C, pigment was extracted using nutrient broth. The pigment's transformation into a blue green hue signified its manufacture. Five milliliters of each sample was centrifuged at 3000 revolutions per minute for ten minutes after the color medium became blue (17). Three milliliters of chloroform were then added to the supernatant and mixed until the blue color was restored. The fact that, the blue solution became reddish-pink after adding 0.2 N HCl provided more confirmation. All of the recovered solutions had their absorbance measured at 520 nm. Based on the absorbance of pyocyanin at 520 nm in acid solution, the quantities of pyocyanin were calculated by multiplying the optical density at 520 nm, and the results were reported as micrograms of pigment per milliliter of culture (18). The pink solution was treated with a 0.4 M borate-NaOH buffer (pH=10) until it became blue, and then it was extracted once more using chloroform. Two or three repetitions of this process produced a clear blue solution of pyocyanin in chloroform. The chloroform was evaporated, the pyocyanin powder was weighed, and then 1 cc of sterile distilled water was added for rehydration (19).

DNA extraction of *pseudomonas aeruginosa*:

Genomic DNA was extracted from the pseudomonas aeruginosa isolates according to instruction provided by manufacturer using Genomic DNA purification kit supplemented by (Geneaid, Korea). The isolated DNA was checked by 0.7% agarose gel electrophoresis and viewed using UV-.transilluminator.

Detection of phzA1 and phzA2 Genes

To validate the identity of pseudomonas aeruginosa, the genomic amplification fragments of 232 bp and 190 bp, respectively, were generated using the oligonucleotide primers phzA1 and phzA2. The existence of the phzA1 and phzA2 genes was confirmed using polymerase chain reaction (PCR) with primers that were unique to these genes, as shown in Table 1. It was produced chromosomal DNA for PCR analysis (20).

Table (1): Primers used in this study

Primer	Sequence (5 - 3)	Base pair
phzA1	F: 5'-GACCATGTCGACCCTCTTCG -3 R: 5'-TCAGCGGTTGTTGAGCAGAC -3'	232
phzA2	F: 5'-GAGGACCTGCCGACATCAAT -3' R: 5'-CCTGCTGCTGCTTCTGTTGA -3'	190

Biological activity of Pyocyanin gene on bacterial isolates

The well diffusion method was used to test the antibacterial and antifungal properties of pyocyanin. A petri dish composed of Mueller-Hinton agar was used to create wells that had a diameter of 6mm. All of the harmful bacterial strains that were found in UTI patients' samples were examined. One hundred microliters of a pyocyanin solution (167 mg/ml) was applied to each well after swabbing the growth of each isolate on the surface of nutrient agar. Microorganisms and yeasts were cultured in petri dishes at 37ºC for a period of one to two days, while other types of fungus were left to grow for as long as a month. To quantify the biological activity of pyocyanin, the inhibition zone in each well was measured using a ruler in millimeters (21).

RESULTS

The Vitek 2 system was used to identify the bacteria and fungi isolated from 100 urine samples collected from patients with UTIs. The samples were cultured on various media to isolate the microorganisms, and the results showed that all of the isolates were confirmed with an ID massage confidence level ranging excellent (probability percentage from 94 to 99.7 percent). This technique was known for its rapid detection of bacteria. In 33 cases (33%) of E. coli, 19 cases (19%) of Staphylococcus aureus, 13 cases (13% of the total), 12 cases (12%) of Shigella pneumonia and Enterobacter, and 4 cases (4% of the total) of E. faecalis were identified. The findings showed that 4 (4% of samples) had Candida albicans and 3 (3% of samples) had Cryptococcus spp. after cultivating the urine samples to the isolated fungus. These results were shown in Table (2).

Table 2: Identification of bacterial and fungal isolates from urine samples according to gram stain, culture media and Vitek 2 system

Type of bacteria	**Number of isolates**	**%**
E. coli	33	33%
Staphylococcus aureus	19	19%
Shigella pneumonia	12	12%
Enterobacter	12	12%
E. faecalis	4	4%
Candida albicans	4	4%
Cryptococcus spp	3	3%
Total	100	100%

Furthermore, from a total of 100 samples obtained from wound and burn infections, 81% yielded positive cultures and 19% yielded negative cultures, as seen in Figure (1). Results demonstrated that out of 81(94%) positive cultures on various growth media, only 30 (37%) of the samples) were associated with *Pseudomonas aeruginosa*, while 51 (% of the samples) were associated with other microbial agents. The findings are shown in Table (3). The automated Compact Vitek-2 system was used, together with GN-ID cards that comprised 64 biochemical tests, to identify the bacterial isolates. With a confidence level ranging from good (probability percentage from 94 to 99.7 percent), the findings showed that all thirty isolates were verified by ID massage. This approach was characterized by quick bacterial identification. The biochemical test confirmed the presence of *Pseudomonas aeruginosa* in all of the isolates.

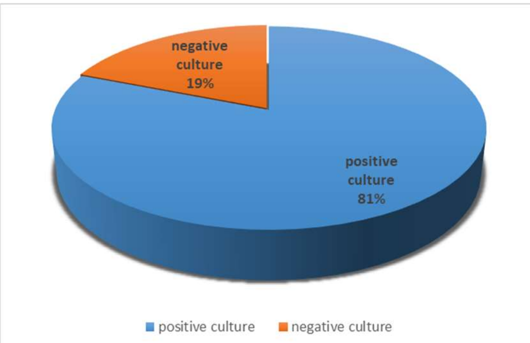

Figure 1: positive and negative culture from wounds and burns infection

Table 3: Identification of Pseudomonas aeruginosa isolates from wound and burns samples according to gram stain, culture media and Vitek 2 system

Type of bacteria	Number of isolates	%	No culture
Pseudomonas aeruginosa	30	37.1%	19
Other types of bacteria	51	62.9%	
Total	81	100%	

The level of blue coloration on tested agar was used to select 10 clinical P. aeruginosa isolates for pyocyanin extraction. The quantity of pyocyanin extracted from each P. aeruginosa isolate, categorized by their clinical source, is shown in Figure 2. One wound and burn isolate had the highest concentration of pyocyanin (0.987µg/ml and 0.888 µg/ml) respectively.

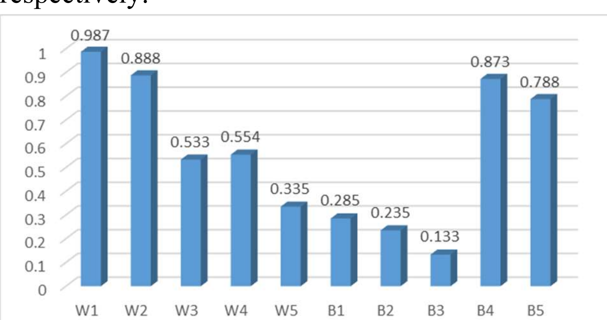

Figure 2: Pyocyanin quantity (concentration (µg/ml) from selective 10 *P. aeruginosa* isolate

The antimicrobial activity of purified pyocyanin at the concentration (0.987µg/ml) was monitored against different microorganisms as shown in Table 4. Results indicate that pyocyanin shows high biological activity against all mentioned organisms.

Table 4: Diameter of antimicrobial activity of pyocyanin to various tested microorganism

Type of bacteria	Number of isolates	Properties	Inhibition zone (mm)
E. coli	33	-ve	21

Staphylococcus aureus	19	+ve	23
Shigella pneumonia	12	-ve	25
Enterobacter	12	-ve	21
E. faecalis	4	-ve	22
Candida albicans	4	Yeast	16
Cryptococcus spp	3	mold	17

Genomic analysis of the *phzA1* gene was conducted on 30 isolates previously identified as *P. aeruginosa*. Without exception, every single isolate showed evidence of having this virulence gene. Figure 3 shows that, in contrast to an allelic ladder, there were (232) bp bands, which was a good sign.

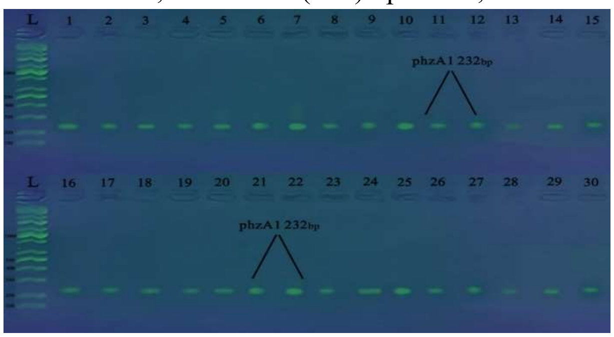

Figure 3: Agarose gel electrophoresis at 70 volt for 50 min for phzA1 specific gene products visualized under U.V light at 301 nm after staining with ethidium bromide. Lanes were positive for P. aeruginosa, the size of product is (232 bp).

PCR analysis confirmed the presence of the phzA2 gene in clinical isolates of P. aeruginosa, with a product size of 190 pb, as shown in Figure (4). All thirty isolates examined in this study yielded positive results for this gene when tested by PCR (Figure 4).

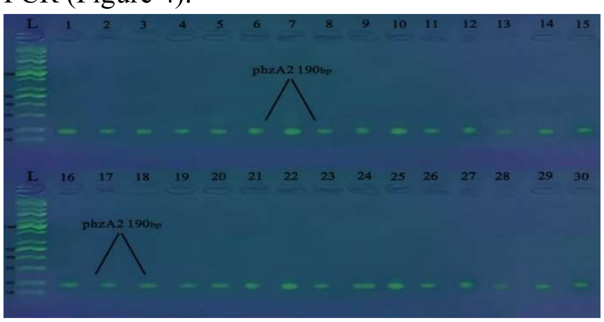

Figure 4: Agarose gel electrophoresis at 70 volt for 50 min for phzA2 specific gene products visualized under U.V light at 301 nm after staining with ethidium bromide. Lanes were positive for P. aeruginosa, the size of product is (190 bp).

DISCUSSION

Many immunocompromised persons die from Pseudomonas aeruginosa. Colonizing epithelial surfaces, weakening host defenses, producing systemic toxicity, and increasing the risk of sickness and death make Pseudomonas aeruginosa harmful. This research corroborated Serra et al., (22), who found Pseudomonas spp. caused 52% of wound infections. According to Khosravi et al., (23), 60% of Pseudomonas spp. isolates entered via wounds. Puca et al., (24) isolated 24% of Pseudomonas spp. from wounds. Elmanama et al., (25) found 28.3% of Pseudomonas spp. isolates had burn-in rates. Burn samples had the most Pseudomonas spp. (37.1%), according to Sleem et al., (26). Although the skin is the body's first line of protection against germs, Pseudomonas spp. needs a broad cut or puncture to infect. Trauma, surgery, massive burns, and other conditions may circumvent skin or mucosal barriers (27). Burns caused by Pseudomonas spp. were prevalent. Pseudomonas spp. infections may be isolated from many sites, however Spernovasilis et al., (28) found them most commonly in burn, surgical wound, urine, and ear discharges. Infections produced by Pseudomonas spp. often result from the patient's local flora or ambient bacteria colonizing the burn site. Pseudomonas spp.-infected burn patients died more and stayed longer in the hospital. Antibiotic prices rose due to surgical treatments. A prevalent and widespread bacterial infection in hospitals and other healthcare facilities was Pseudomonas spp., Pseudomonas spp., which may cause fatal infections, spreads despite antimicrobials and surgical and medical advances. Both catalase and oxidase tests are positive (29). Pseudomonas also emitted vivid yellow-green pyroverdin when iron was lacking. C ertain Pseudomonas species may produce their own colors. Pyocyanin (blue), quinolobactin (yellow and dark green), pyorubrin (red), and pyomelanin are siderophores. Blood agar may hemolyze, according to Pattnaik et al., (30). Current study studied local clinical P. aeruginosa isolates' pyocyanin pigment production, purification, characterization, and bioactivity. Pseudomonas aeruginosa produces redox-active blue pigment pyrocyanin. P. aeruginosa's unique pyocyanin production may increase on King's A medium, which includes potassium and magnesium salts at the correct amounts to inhibit fluorescein synthesis (pyoverdine) (31). Pyocyanin becomes red at acidic pH and blue in neutral and alkaline pH. The color of pyocyanin may alter with pH (30). The strain-to-strain difference in pyocyanin production may be due to environmental factors such light intensity (32). Under certain lighting conditions, pyocyanin concentration reduced. This intensity- and wavelength-dependent decrease was produced by ultraviolet and violet light (33). Pyocyanin, which inhibited gram-positive and gram-negative bacteria, yeast (Candida spp.), and filamentous fungus, was generated by all clinical isolates. Phenazine antibiotics (chemicals related to pyocyanin) show antibacterial activity against Bacillus subtilis and Candida albicans strains, as Jameel et al., (34) discovered. P. aeruginosa produced pyocyanin, a greenish blue pigment, on royal A media. All isolates examined in this study did this. The bacteria Pseudomonas aeruginosa produce pyocyanin. Bacteria's virulence and antibacterial effect depend on this pigment. Pyocyanin produces superoxide and hydrogen peroxide. ROS may damage DNA, proteins, and lipids, killing competing bacteria (35). Pyocyanin may disrupt the electron transport system, limiting other bacteria and fungi's energy production and growth. Pyocyanin regulates quorum sensing, a population density-related stimulus-response mechanism (36). Quantum sensing regulates biofilm-forming and virulence-producing genes. Due to biofilm protection, Pseudomonas aeruginosa becomes more resistant to antimicrobials and immunological responses (37). Pyocyanin provides iron for bacterial growth and metabolism. Pyocyanin chelates iron, preventing competing bacteria from growing. Research suggests that pyocyanin may inhibit fungal, Gram-positive, and Gram-negative bacteria development (38). While inhibitory techniques vary, oxidative damage and cellular process disruption are prevalent. P. aeruginosa may infect persons with compromised immune systems or chronic fibrosis by generating pyocyanin (39). Biofilms may become antibiotic-resistant, making P. aeruginosa infections difficult to treat. Pyocyanin, which is involved in biofilm production, is antibacterial (40). Pyocyanin production or its effects have been targeted to understand its role in P. aeruginosa infections, decrease its pathogenicity, and increase antibiotic efficacy (41). Pyocyanin may be used in biotechnology as a natural antimicrobial due to its antibacterial properties (42). Pseudomonas aeruginosa's phenazine biosynthesis involves pyocyanin-producing genes. Two important genes, phzA1-G1 and phzA2-G2, encode enzymes that produce phenazine-1-carboxylic acid (PCA), a precursor to pyocyanin (43). Additional modifying genes convert PCA to pyocyanin. Pyocyanin redox cycling in cells generates ROS such superoxide and hydrogen peroxide. These ROS may induce oxidative stress and cell death in fighting microbes (35). Phz operons encode redox-active enzymes. Pyocyanin may inhibit the development or kill other bacteria and fungi by interfering with their electron transport chains, which affects their respiration and energy production (42). Phz genes produce enzymes that convert PCA to pyocyanin, which affects respiration. Pyocyanin helps bacteria obtain iron, a crucial mineral (15). Because it chelates iron, it denies other micro-organisms nourishment. Several genes enhance iron intake, but the phz operons' pyocyanin production aids it (44). Pyocyanin protects P. aeruginosa by producing biofilms. Biofilms were impermeable to antimicrobials and immunological reactions; thus, bacteria may survive within them (45). Quorum sensing systems like las and rhl govern biofilm development by interacting with pyocyanin genes (46). Antimicrobial and antifungal pyocyanin may inhibit Gram-positive and Gram-negative bacteria and fungi. P. aeruginosa broad-spectrum antibacterial activity helps it outcompete other microorganisms. Pyocyanin, an antibacterial molecule, requires the phzA1-G1 and phzA2-G2 operons (47-49).

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