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Detection of Pyocin Multi-Drug Resistance *Pseudomonas aeruginosa* in Clinical Samples Collected from Patients and Study the Effects of CFSs against the Bacterium

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Abstract: *Pseudomonas aeruginosa* have emerged that are resistant to all widely prescribed antimicrobial medicines, treating *Pseudomonas* infections has grown more challenging. The aim of this study is the detection of some resistance genes in pyocin MDR-*P. aeruginosa* and examine the effect of specific concentrations of *Lactobacillus Acidophilus* Cell-Free Supernatants (CFSs) on *P. aeruginosa*. The 60 *P. aeruginosa* isolates were gathered from clinical sources from Baghdad hospitals. the disk diffusion method was used to examine the antibiotics' sensitivity and lowest inhibitory concentration (MIC). The resistance genes were discovered by PCR, and the effects of CFSs on the growth of *P. aeruginosa* were studied by using a specified concentration. Obtaining Pyocin Multi-Drug Resistance isolates which carry some resistance genes, and determines the CFSs concentration effects on *P. aeruginosa* growth. It was concluded that use of (CSFs) as an alternative therapy for infections by Multi-Drug Resistance *P. aeruginosa* is thus conceivable in the future.

Keywords: *Pseudomonas aeruginosa*, pyocin S, MDR, resistance gene, CFSs.

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Introduction

Pseudomonas aeruginosa is a small rod gram-negative, aerobic. It is an opportunistic, motile one or more polar flagella, a non-spore pathogen. they appear as single bacteria, in pairs, and sometimes in short chains. It can grow at 40-41°C which is the primary characteristic of *P. aeruginosa* (1). The *P. aeruginosa* is prevalent in many natural environments such as soil and water and is a significant cause of human infection. This is due to their ability to survive and even thrive under a wide range of temperatures, on different nutrient sources and strains that are harmful and resistant to antibiotics, disinfectants, and other antimicrobial compounds (2). Serious diseases, such as cancer, HIV, and cystic fibrosis (CF), may be associated with a high death ratio in immunocompromised patients. Significant morbidity and mortality are frequently caused by these diseases, Additionally, these bacteria can cause severe, sometimes fatal infections in people with cystic fibrosis (CF), endocarditis, skin injuries, artificial implants, and burns (3). The pathogenicity of *P. aeruginosa* is associated with a wide range of

virulence factors and adaptation, as well as a variety of resistance mechanisms, and gene expression is crucial for tightly organizing all of these activities(4).

The United states Centers for Infectious Disease Control and the World Health Organization have identified it as a serious infection type linked to many forms of antibiotic resistance (5). Due to the rise in drug resistance, traditional antibiotic regimens against *P. aeruginosa* were becoming increasingly ineffective. The clinical study of antibiotic resistance in the various *P. aeruginosa* strains is developing quickly. Multidrug-resistant *P. aeruginosa* (MDRPA) isolates were defined as those that were resistant to at least three different antimicrobial classes, including cephalosporins, quinolones, aminoglycosides, carbapenems, and anti-*pseudomonas* penicillin (6).

Most *P. aeruginosa* strains produce different types of bacteriocins (pyocins). Bacteriocins are a large family of functionally and ecologically diverse ribosomal protoxins produced by archaea, bacteriophages, and bacteria for competition within and between species which are produced as a secondary metabolite by many bacteria and can oxidize and reduce other molecules, exhibiting lethal or growth-inhibiting activity (7). *P. aeruginosa* bacteria produce a wide range of secondary metabolites to protect them from other fungi and competing bacteria. As they live in all environments, from aquatic to wild, from soil to distilled water, and from plants to humans, there are two main groups of pyocin produced by *P. aeruginosa* S-type pyocins and Tailocins (8).

Pyocin S type is similar to colicin (*Escherichia coli* bacteriocin), a small water-soluble heat-sensitive protease. These bacteriocins are secreted as binary protein complexes consisting of a protein with lethal activity (9).

Bifidobacterium and lactic acid bacteria (LAB) form the majority of the microorganisms utilized as probiotics. LAB "lactobacilli species" are the most widely used class of bacteria due to their potential probiotic benefits. It is well known that many harmful bacteria are suppressed by these bacteria's antagonistic activity (10).

The role of *Lactobacillus* spp. play in preventing and treating various diseases is well created. It is known that *Lactobacillus* spp. contributes to the prevention and treatment of certain infections. *Lactobacillus* bacteria are found commensally in the human body, The ability to secrete antibacterial substances like "lactic acid, and hydrogen peroxide" to prevent the spread of bacteria has been identified as one of the benefits (11).

Probiotics are regarded as highly safe, natural treatments and preventatives for many illnesses, including urinary tract infections. Because members of the Lactobacillus genus most usually have the safe or generally recognized as safe (GRAS) status, probiotics were described as "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" (12).

This study discovered local isolates of *P. aeruginosa*, which are resistant to antibiotics and capable of producing pyocin. antibiotics resistance genes and genes responsible for the production of pyocin were also detected, and the ability to use probiotics as alternative treatments against *P. aeruginosa* infections.

Materials and methods

Collection and identification of bacterial isolates

Three hundred and fifty clinical samples of (burns, wounds, sputum, ear swabs, and urine) were collected from patients in Baghdad hospitals (Al-Yarmouk Teaching Hospital, Baghdad Teaching Hospital, Al-Karama Hospital, and Al-Kadumia Medical City), from the period between December/2021 to June/2022.

Samples were collected under sterile conditions and cultured in suitable media for the isolation of *P. aeruginosa* isolates and were identified according to colonies morphological structure, microscopic examinations, and biochemical tests (13).

Using sterilized swab sticks, samples of *Lactobacillus* species were obtained. The Man-Rogosa_sharpe broth-filled sterile screw cap bottles containing these sterile swabs were then transferred to the lab while being kept chilled. The material was subsequently grown on MRS agar (14).

The VITEK® 2 Compact system is dedicated to the identification of *P. aeruginosa* and *Lactobacillus* species.

Antibiotics susceptibility test

The antibiotics sensitivity tests on the isolates to 15 antibiotics (Liofilchem, Italy) and (Bioanalys, Turkey) by Kirby-Bauer0 disk diffusion method. Amikacin 10µg, Aztreonam 30µg, Cefepime 30µg, Ceftazidime 30µg, Ciprofloxacin 5µg, Ceftriaxone 30µg, Gatifloxacin 5µg, Gentamicin 10µg, Imipenem 10µg, Levofloxacin 5µg, Meropenem 10µg, Netilmicin 30µg, Norfloxacin 10µg, Piperacillin 100µg, Tobramycin 10µg, were determined on Mueller-Hinton agar by the Kirby Bauer disk diffusion method. The zone diameter of inhibition was measured and the results were translated based on guidelines from the Clinical and Laboratory Standards Institute (15).

Extraction of bacterial genomic DNA and molecular detection

The DNA genome of *P. aeruginosa* was isolated from bacterial growth by the Qubit Kit's instructions, and electrophoresis was performed, Then PCR was conducted in optimal laboratory conditions (16). The primer designs used in this inquiry were based on the global genome website National Center for Biotechnology Information, as stated in (Table 1), and the *P. aeruginosa* genome database.

Table (1): Primer Pairs, Sequences, and Expected Size

Gene	Primer	Sequence	Size (bp)
16SrRNA	**Forward** **Reverse**	AGGGCCATGATGACTTGACG TCGTGTCGTGAGATGTTGGG	143
PyoS	**Forward** **Reverse**	GAGCTGTTGAGTGACCTGCT GCTCAATGCTGAAACCGACC	124
Resistance genes			
bla_{oxa}	**Forward** **Reverse**	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	814
Par C	**Forward** **Reverse**	CATCGTCTACGCCATGAG AGCAGCACCTCGGAATAG	267
Gyr A	**Forward** **Reverse**	GTGTGCTTTATGCCATGAG GGTTTCCTTTTCCAGGTC	287
Gyr b	**Forward** **Reverse**	ATGAGTCGATCACTGTCCGC GTGTTGTCGTCGAACTTGCC	127
TEM	**Forward** **Reverse**	TGATAACACTGCGGCCAACT TTCATTCAGCTCCGGTTCCC	124
SHV	**Forward** **Reverse**	GAAACCGCACGTATCAACCT CCTGTTTCAGCGAACCATTT	129

Detection of PyoS and resistance genes

P. aeruginosa DNA genome was isolated from bacterial growth according to the protocol of Qubit™ Kit, and electrophoresis was carried out according to (14). The PCR has occurred in optimal laboratory conditions.

The primers used in this study were designed based on the *P. aeruginosa* genome database as a reference. These primers were supplied in a lyophilized form by Macrogen Company. The PCR conditions for the 16S rRNA gene are shown in (Table 2).

while the PCR Program of the PyoS and resistance genes is shown in (Table 3).

Table(2): The PCR conditions for the 16S rRNA gene

Cycle No.	Stage	Temperature	Time
1	Initial Denaturation	94 C	5 min.
38x	Denaturation	94 C	30 sec.
	Annealing	57 C	45 sec.
	Extension	72 C	45 sec.
1	Final Extension	72 C	7 min.

Table (3): PCR Program for PyoS and resistance genes.

Cycle No.	Stage	Temperature	Time
1	Initial Denaturation	94 C	5 min.
35x	Denaturation	94 C	30 sec.
	Annealing	48 C	45 sec.
	Extension	72 C	45 sec.
1	Final Extension	72 C	7 min.

***Lactobacillus acidophilus* supernatant preparation**

The *L. acidophilus* strain was also cultivated in MRS broth. overnight incubation in an air-filled condition at 37 °C. centrifuged at 5000 rpm for 30 minutes, filtered through sterile filter paper with a 0.22 m pore size, and then combined with crude Cell-Free Supernatants (CFSs) (17).

CSFs' minimum inhibitory concentration (MIC)

The MIC is the minimum concentration of a test sample that inhibits observable growth in broth. By using the Agar dilution procedure, the CSFs with the inhibitory activity determine the MIC. established the lowest inhibitory concentration for *P. aeruginosa* under the influence of *L. acidophilus* probiotics (CSFs) (18).

Results and discussion

Isolation of *P. aeruginosa*

Of the 350 samples collected from hospitals, 60 isolates were successfully diagnosed as *P. aeruginosa* representing 17% of the total samples, and the highest percentage of *P. aeruginosa* was obtained from burn samples, 26 isolates (43.3%) while the lowest percentage was obtained of the samples of urine tract infections 4 isolates (6.7%). *P. aeruginosa* in a community may occur because of increased numbers of immune-compromised patients due to contaminations of the hospital environment and in special patients with long stays in the hospital. This agrees with several studies conducted by (19).

Identification of *P. aeruginosa*

In the laboratory, *P. aeruginosa* can grow on a nonselective agar including nutrient agar and broth, blood agar, and MacConkey agar. On MacConkey agar medium the colonies of *P. aeruginosa* isolates appeared 2-3 mm, flat, smooth, non-lactose fermenting colonies with regular margins. In Blood agar *P. aeruginosa* produces mucoid-type colonies with a typical metallic sheen with a clear zone around the colonies due to B hemolysis. and in Cetrimide agar *P. aeruginosa* colonies are medium-sized colonies and characterized by irregular growth (20).

According to biochemical testing, they behaved positively to the indole, methyl red, and Voges Proskauer tests, but negatively to the oxidase, catalase, urea, citrate utilization, and gelatin hydrolysis tests. They convert glucose, mannose, and xylose into other sugars. Maltose, lactose, and sucrose, on the other hand, were tolerable to them. On the triple sugar iron (TSI) agar medium, *P. aeruginosa* formed a red butt and slant without releasing H2S and that was also described previously by (21).

Identification of *P. aeruginosa* and *L. acidophilus* by Vitek2 system

Which is a new tool for the identification of bacteria from clinical specimens. The identification was performed by an automated Vitek2 system using GN-ID cards containing (64) biochemical tests. The results showed that all (60) isolates for *P. aeruginosa* and (2) isolates of *L. acidophilus* were confirmed with ID message (the percentage was 95%-99%). This system is distinguished by its ability to quickly identify bacteria without the need for many culture media, and its ability to reduce culture contamination. (21).

Antibiotics susceptibility test

The antibiotics susceptibility test (Kirby-Bauer disk diffusion method) exhibited that a total of the 60 isolates obtained in this study have a resistance to antibiotics, with the highest resistance percentage (90%) observed against tetracycline, (86.7%) against Aztreonam, (85%) against Erythromycin, (66.7%) against to Ceftriaxone and (65%) against to Ceftazidime. Also, results in a high percentage of *P. aeruginosa* isolates were sensitive to Imipenem (85%), Ciprofloxacin (80%), Meropenem (78.3%), and (66.7%) against Levofloxacin.

Several studies showed a relationship between resistance to multiple antibiotics and pyocin production, as pyocin affects alterations in LPS, which impedes the permeability of the outer membrane of antibiotics (22). For this reason, antibiotic-resistant isolates were chosen to test the pyocin productivity of *P. aeruginosa*.

Detection of PyoS and resistance genes extraction of DNA

The most antibiotic-resistant 10 isolates were selected to detect the presence of PyoS and resistance genes(obtained from growing on Brain Heart infusion). and the DNA of these isolate *P. aeruginosa* was extracted and purify by DNA extraction kit. The result was detected by electrophoresis on 1% agarose and exposure to ultraviolet light in which the DNA appears as compact bands (Figure1).

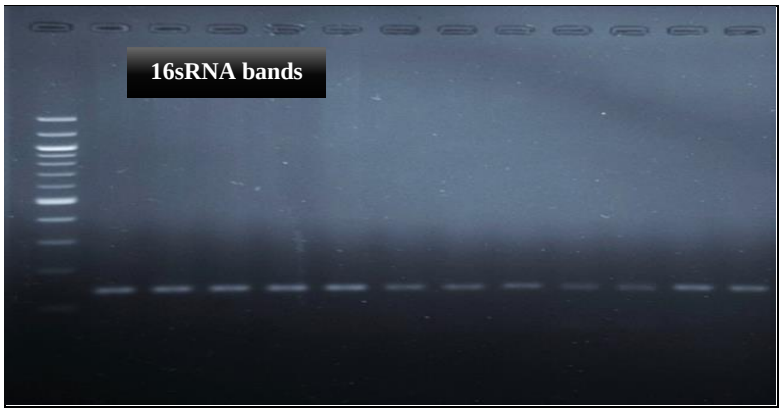

Figure (1): 16sRNA bands.

The results showed that 10/10 (100%) isolates were positive for the pyocin S gene. the results similar to (23).

Detection of pyocin S genes

The most drug-resistant isolates were subjected in PCR to DNA amplified to detect the pyoS gene. The PCR amplification results were confirmed by electrophoresis analysis. after analysis, the DNA strands that resulted from successful binding between the selected pyoS primer basis and the extracted DNA template appear as a single band under UV light. Light using ethidium bromide as a specific form of the DNA dye (24) (Figure 2)

Figure (2): Amplification results of pyocin S primers in *P. aeruginosa* sample.

The results showed that 8/10 (80%) isolates were positive for the pyocin S gene. the results differed with (7), in which the pyocin S gene was 22%, and the results were almost identical to the study were showed that (50%) isolates were positive for the pyocin S gene.

Detection of resistance genes

P. aeruginosa isolates chosen as the most drug-resistant isolates; the resistance gene's presence was examined.By amplifying the isolate's DNA using PCR, the resistance genes (bla_{oxa}, ParC, gyrA, gyrB) were found. Electrophoresis analysis verified the PCR amplification results. The effective binding of the chosen primers to the extracted DNA template causes a single band of DNA strands emerged under UV light. light employing a particular kind of DNA dye called ethidium bromide (23). as seen in (Figure 3).

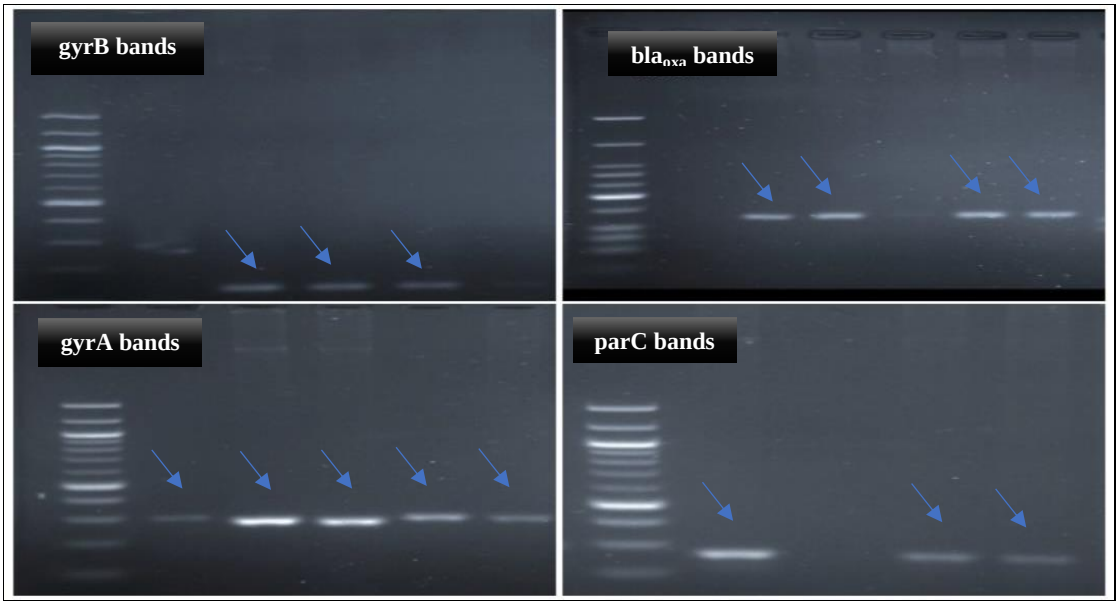

Figure (3): Amplification results of available resistance genes(gyrB, gyrA. parC, bla$_{Oxa}$)

The results showed that 6/10 (60%) of different isolates were positive for the parC, gyrB gene, 8/10 (80%) of isolates were positive for the bla$_{oxa}$ resistance gene,10/10 (100%) of all investigative isolates were positive for gyrA, and all 10 isolates were negative for SHV and TEM resistance genes. In addition to the pyocin gene, the presence of resistance genes explains why bacteria are resistant to antibiotics.

Determination of minimal inhibitory concentration (MIC) for CFSs

To determine the minimal inhibitory concentration, 6 antibiotic-resistant isolates whose virulence genes had been identified were examined. The MIC was established as the lowest concentration of CFSs that, after a 24-hour incubation period, can inhibit *P. aeruginosa* from developing visibly. The agar dilution method was used to calculate the minimal inhibitory concentrations (MIC), and the susceptibility of the *P. aeruginosa* isolates to CFSs was investigated. This was accomplished via dilutions of various increasing CFS concentrations (5, 10, 15, 20, 25, and 30 ml) with cetrimide agar and the placement of the mixtures in Petri dishes. The research's findings indicate that CFSs suppressed *P. aeruginosa* isolates at fixed MIC values of 10 and 15, as reported in (Table 4).

Tables (4): The MIC results for CFSs in *P. Aeruginosa;(+) mean inhibition of growth of P. aeruginosa,* (-) mean growth of *P. aeruginosa.*

Isolate	CFSs concentrations					
	5	10	15	20	25	30
P9	-	+	+	+	+	+
P17	-	-	+	+	+	+
P32	-	-	+	+	+	+
P38	-	+	+	+	+	+
P55	-	-	+	+	+	+
P59	-	-	+	+	+	+

The results of probiotic treatment (CSFs) to *P. aeruginosa* bacteria were that samples P9 and samples P38 had an inhibitory concentration of 10 ml. While the samples P17, P32, P55, and P59 recorded the degree of inhibition at 15 ml. Thus, the effect of lactic acid bacteria (*L. acidophilus*) extracts inhibited their growth. Similar results to the results of the current study were recorded in terms of growth inhibition and obtaining the minimum inhibitory concentration (MIC) in studies (25).

Conclusion

The study investigates local isolates of *P. aeruginosa* that can produce pyocin and were resistant to antibiotics. 80% of resistant isolates could produce pyocin and genes for antibiotic resistance were found in more than 50% of study isolates, Also the antibiotics susceptibility test for isolates revealed the efficacy of Tetracycline, Aztreonam, and Erythromycin in inhibiting *P. aeruginosa*. Consequently, new therapy modalities must be used. Alternative *P. aeruginosa* treatments are necessary. CSFs from *L. acidophilus* had an impact by inhibiting its growth. Consequently, perhaps in the future, we will be able to employ (CSFs) as a substitute treatment for infections caused by *P. aeruginosa*.

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