Original Article

Effect of the antibiotic levofloxacin on the ability of Pseudomonas aeruginosa to form biofilm and produce pyocyanin pigment

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Abstract

The current study included a group of (150) clinical swabs for patients suffering from burns, wounds, diabetic ulcers, and urinary tract infections for (69) males and (81) females, their ages ranging from 1- 68 years, for the period of November 2021 until March 2022, from Hospitals in Mosul. 75 samples of P. aeruginosa were isolated, representing (50%) of the total clinical samples, and the rate of isolation of them from samples of burns, wounds, diabetic foot ulcers, and urinary tract infections was 75% (42/56), 41.1% (23/56), 53.3% (8/15) and 8.7% (2/23), respectively. The antimicrobial susceptibility test was performed by the disk diffusion method, and it was found that the isolates are multi-antibiotic resistant. The percentage of resistance shown by the isolates was 70.7% for Piperacillin, 89.3% for Amikacin, 74.7% for Aztreonam and Levofloxacin, 81.3% for Gentamicin, Ceftazidime, and Cefepime, 76% for Ciprofloxacin, 80% for Netilmicin and Tobramycin, and 45.3% for Imipenem. The MIC values for the antibiotic Levofloxacin were determined using Macrodilution method, the results showed that the MIC value ranged between (0.5-512) µg/ml.

The biofilm formation was assayed by the microtiter plate. Pyocyanin was extracted from the culture supernatants, and the absorbance values were measured using a spectrophotometer.

The results showed that the antibiotic Levofloxacin at the MIC and Sub-MIC concentrations has an inhibitory effect to varying degrees on biofilm formation and pyocyanin pigment production.

Keywords: Pseudomonas aeruginosa, Pyocyanin, Biofilm, Burn infections, PhzM, PslA.

1. INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen . These bacteria can invade the bloodstream, causing bacteremia and then septicemia, and infect immunodeficiency patients such as patients with leukemia, diabetes, an Acquired immunodeficiency syndrome (AIDS), severe burn patients, and those who have undergone surgery (Hassuna et al.,2015, Jalil et al.,2017, Obaid et al.,2016).

Pseudomonas aeruginosa is one of the main causes of nosocomial infection, causing (10-15%) of these infections. It is an opportunistic pathogen with the ability to cause various infections such as urinary tract infections, meningitis, bone infection, eye infection, middle ear infections, and respiratory infections, including pneumonia, especially those with cystic fibrosis, and inflammation of the digestive system (Pedersen et al.,2018, Abdulwahhab and Atiyea, 2021).

P. aeruginosa is Gram-negative bacteria that cause diseases due to its possession of many virulence factors that affect its pathogenesis, including flagellum, Pili IV, exoenzyme S that helps it to attach, protease, hemolysin, Phospholipase, and elastase (Ochoa et al., 2013), toxins such as Exotoxin A and Endotoxin responsible for the fever and shock associated with septicemia, and the production of pigments such as Pyocyanin It is soluble in chloroform and a member of the tricyclic compounds "phenazine" (Sudhakar et al., 2015).

P. aeruginosa has two homologous operons (phzA1B1C1D1E1F1G1 and phzA2B-2C2D2E2F2G2) to be coded for phenazine-1-carboxylic acid. two phenazine genes (phzS and phzM) responsible for converting the enzymes of phenazine-1-carboxylic acid to pyocyanin (Khadim et al., 2019). as well as The presence of an alginate layer and a biofilm increases its resistance to antibiotics.

Biofilm formation is another important characteristic of P.aeruginosa that contributes to chronic infections as they reduce susceptibility to antibiotics and as a result, treatment options are reduced (Bo Fu et al., 2017). Biofilm is defined as an assembly of microbial cells bound (not removed by gentle rinsing) to a surface and covered with an exopolysaccharide matrix (slime). (Bose et al., 2009).

Bacteria within biofilms can escape host immune responses and resist antimicrobial treatments up to 1,000 times more than their planktonic cells. P. aeruginosa can produce biofilms, making it an excellent model for studying biofilm formation (Crespo et al., 2018). The flexible biofilm is a critical weapon for bacteria to compete, survive and control in the lung of cystic fibrosis patients (Oluyombo et al., 2019).

2. Materials and Methods:

2.1. Specimen collection:

A total of 150 specimens were collected from patients admitted to several hospitals in Mosul between November 2021 and March 2022.

2.2. Bacterial identification:

The Vitek-2 automated system by using Gram-Negative cards according to the manufacturer's instructions was used for diagnosis. The isolated bacteria were stored in trypticase soy broth (TSB) with 40% glycerol at -20°C until used.

2.3. Antibiotic Susceptibility Testing:

The susceptibility of isolates to different antibiotics was tested using the Kirby-Bauer disk diffusion method following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020). Using antibacterial agents included: Gentamicin (CN), Tobramycin (Tob), Amikacin (AK), Ciprofloxacin (CIP), Levofloxacin (LEV), Piperacillin (PRL), Ceftazidime (CAZ), Cefepime (FEP), Netilmicin (NET), Aztreonam (ATM), and Imipenem (IMP). On Mueller- Hinton agar plate (Himedia, India) using overnight culture at McFarland standard 0.5 followed by incubation at 35°C for eighteen hours (Al-najar et al.,2020).

2.4. Biofilm Formation

2.4.1 Detection of Biofilm Formation (Smith et al.,2008)

The biofilm formation assay was carried out as follows, the bacterial culture was grown in brain heart infusion broth (BHIB) at 37°C for 24 hours. at the end of the incubation period; Bacterial culture was diluted in (BHIB) with 1% glucose at a 1:50 dilution and then 200 µl of the diluted bacteria were distributed into a sterile 96-wells polystyrene microtiter plate and incubated under constant conditions at 37°C for 24 hours. Biofilms were detected as follows:

- After incubation, the contents of the wells were removed by aspiration with a pipette, and the wells were washed with phosphate buffer saline or distilled water three times to remove the unattached cells. (Note: Control negative are three wells that contain only the culture media without bacteria).
- A volume of 200 µl of 0.1% solution of crystal violet dye was added to each hole of the Microtiter plate and incubated at room temperature for 10-15 minutes.
- The plate was rinsed 3-4 times with distilled water by immersion in water, shaken vigorously, and cleaned on a stack of paper towels to rid the plate of all excess cells and dye.
- The Microtiter plate was turned upside down and dried for a few hours or overnight.
- A volume of 200 µl of 33% glacial acetic acid was added to each hole of the Microtiter plate to dissolve the crystal violet. The plate was incubated at room temperature for 10 minutes.
- A volume of 200 µl of dissolved crystal violet was transferred to a new flat-bottomed Microtiter plate.
- The absorbance was measured in a microtiter plate reader at 630 nm, and 33% acetic acid was used as blank.

The amount of biofilm was calculated as follows:

Biofilm = OD1 – Odc

The optical density (O.D) of each well was measured at 630 nm by an ELISA reader (Paramedical, Italia). These tests are performed in triplicate and the average is calculated. The cut-off optical density (O.Dc) was determined as 3 standard deviations (SD) higher than the mean optical density (O.D) for the negative control. The bacterial strains can be classified as follows:

- The isolation does not form a biofilm if the optical density of the control is greater or equal to the optical density of the bacterial strain (ODc ≥ OD).

- A Weakly adherent isolate if the optical density of the isolate is greater than the optical density or less than or equal to twice the optical density of the control (ODc<OD≤2xODc).
- A Moderately adherent isolate if the average optical density of the isolate is greater than twice the optical density of the control or less than or equal to four times the control (2xODc<OD≤4xODc).
- A Strong adherent isolate if the rate of the optical density of the isolate is greater than four times the optical density of the control (OD>4xOD).

2.4.2. The effect of the antibiotic Levofloxacin on the formation of biofilms.

The same previous steps were performed to test biofilm formation in paragraph 2.4.1 with a slight change, which is to take 20 µl of incubated culture (several 24 hour-old colonies with 10 ml of BHIB medium) and dilute it with 1000 µl of BHIB with 1% glucose With a certain concentration of the antibiotic Levofloxacin (which is the MIC and Sub MIC for each bacterial isolate) to get a 1:50 dilution, meaning the bacteria are treated with the antibiotic.

Then 200 µl of the culture diluted in the previous step is distributed to each well of the 96-microtiter plate. Then the plate was incubated at 37 °C for 24 hours. Then the other steps of staining and quantification are completed to show the effect of the antibiotic on the formation of biofilms.

Biofilm inhibition was studied by using the antibiotic Levofloxacin at the concentrations MIC and Sub MIC by comparing the measurement of the wavelength at 630 nm before and after treatment with the antibiotic according to the following equation: (Namasivayam et al., 2013).

$$\text{Biofilm inhibition} \% = \frac{\text{Wavelength before treatment} - \text{Wavelength after treatment}}{\text{Wavelength before treatment}} * 100$$

2.5. pyocyanin pigment

2.5.1 Phenotypic Detection of Pyocyanin Production

The test was carried out without using the stimulants as described before (Hong et al., 2016), where a 5 ml tube of Nutrient broth was inoculated with one of the multi-antibiotic resistant bacterial isolates and incubated for 24 hours at 37 °C, then the tubes were placed in a centrifuge at 4000 rpm. For 10 min, during this period, Mueller Hinton agar medium was brought into Petri dishes, two pits were made in each dish and cultured with activated bacteria Staphylococcus aureus, and incubated for 24 h. After centrifugation of the tubes containing P. aeruginosa isolates, 100 µl of the filtrate was taken and placed in one pit and 100 µl of the precipitate was placed in the second pit using a micropipette, then the dishes were incubated for 24 hours at 37 °C. , in the case of an inhibitory region around the pit, these bacteria produce Pyocyanin and in the absence of an inhibitory diameter, it means that the bacteria do not produce Pyocyanin Pigment.

2.5.2. The Quantitative Pyocyanin Assay and the effect of Levofloxacin on the production of pyocyanin pigment

Pyocyanin production has been determined as described (Husain et al., 2013) with some modifications. We take 5 ml of Nutrient broth without or with the antibiotic Levofloxacin at MIC and Sub-MIC, the media was inoculated with 100 µl of P. aeruginosa and incubated for 72 hours at 37°C. Then the bacteria were removed by centrifugation (5000 rpm, 15 min) and the filtrate was used to extract the pyocyanin. Briefly, 3 ml of chloroform was added to 4 ml of the filtrate. The solution was thoroughly mixed for 2 minutes and centrifuged at 5000 rpm for 15 minutes.

The pyocyanin-containing layer was transferred to a new tube and 2 ml of 0.2 M HCl was added. Then the resulting mixture was centrifuged at 3000 rpm for 5 minutes, and the red and pink layer was used to measure the absorbance at 520 nm. Finally, the pyocyanin concentration was determined by multiplying the A520 values by 17,072 and the results were expressed in µg/ml.

2.6. Statistical Analysis.

Data are represented as the average value obtained from three independent experiments. A t-test was applied to analyze significant differences between control (before antibiotic treatment) and treated (after antibiotic treatment) values measured under the same conditions (linked samples T-test). A probability (P) less than 0.05 was considered statistically significant. SPSS (version 22; SPSS Inc.) and Microsoft Excel 2010 were used.

3. Results and Discussion

3.1. Bacterial Isolates

A total of 150 specimens were included in the study. P. aeruginosa was present in 75 of various clinical specimens, representing (50%) of the total clinical samples, and the rate of isolation of them from samples of burns, wounds, diabetic foot ulcers, and urinary tract infections was 75% (42/56), 41.1% (23/56), 53.3% (8/15) and 8.7% (2/23), respectively.

The incidence of P. aeruginosa infection was higher in men than in females, 42 (56%) and 33 (44%) respectively. The results of the current study agree with the study of (AL-Shamaa et al., 2016) of bacteria isolated from burn patients, which showed that the percentage of males was (77%) and females (23%), and also agree with the study (Yolbaş et al., 2013), where This study showed infection rate for males (70%) and females (30%).

3.2. Antimicrobial Susceptibility of P. aeruginosa isolates

The results of the current study showed that all P. aeruginosa isolates had a difference in resistance to the antibiotics used in this study, Figure (1). It was noted from the results of the current study that the highest antibiotic resistance was to the antibiotics Amikacin, Gentamicin, Ceftazidime, Cefepime, Netilmicin, and Tobramycin; 89.3%, 81.3%, 81.3%, 81.3%, 80%, 80% respectively.

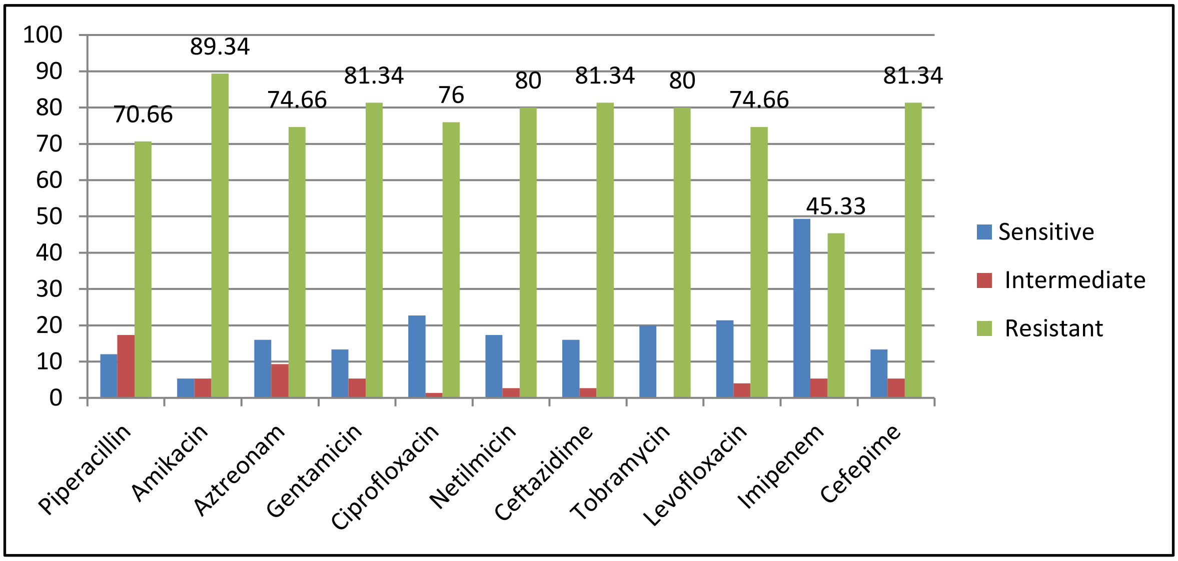


Figure 1: Percentage of resistance of P. aeruginosa isolates to antibiotics

The results of the current study are consistent with the results of (Al-Shwaikh and Alornaaouti, 2018). Whereas, P. aeruginosa isolates showed high resistance to Ceftazidime (81%), Cefotaxime (78%), Piperacillin (76%), and were resistant to Ciprofloxacin, Tobramycin (74%), and Gentamicin (72%). Amikacin and Meropenem (70%), Ofloxacin (66%), and Imipenem resistant (65%).

Wounds and burns of hospital patients can be contaminated with P. aeruginosa, due to the presence of this antibiotic-resistant bacterium in the hospital environment, and it can also be found in tap water, latrines, as well as hospital care workers; Thus, bacteria are transmitted from one patient to another. The cause of antibiotic resistance may be attributed to the concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (eg, mexXY) and reduced permeability of bacterial cell membranes. Besides intrinsic resistance, P. aeruginosa develops easily acquired resistance either by chromosomal mutations in chromosomal-encoded genes or by the process of gene transfer for determinants of antibiotic resistance. Resistance to antibiotic aminoglycosides is caused by P. aeruginosa's production of modified enzymes such as phosphotransferase and N-acetyl-transferase, the genes for these enzymes being transferred on a plasmid or chromosome (Khademi et al., 2021).

3.3. Detection of the ability of P. aeruginosa to form biofilms

The ability of P. aeruginosa isolated from samples of wounds, burns, diabetic foot, and urinary tract infections to form biofilms was tested using the Microtiter plate method (MTP) (Figure 2).

The results show that 74 samples were biofilm-producing at a rate of 98.7% in different degrees compared to the negative control. After applying the steps and equations in paragraph (2.4.2), 5 isolates appeared with a percentage of 6.7% are weakly adherent, 34 isolates with a percentage of 45.9 % are moderately adherent, and 35 isolates with a percentage of 47.3% are strong adherent (Figure 3),(Table 1).

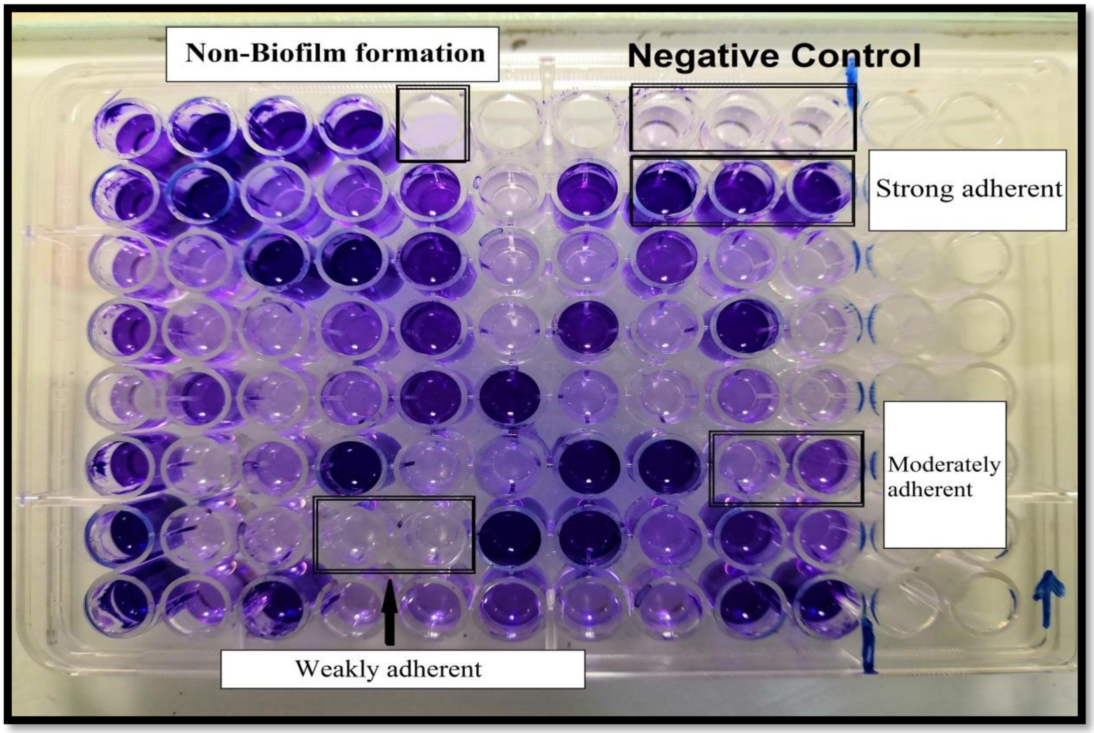


Figure 2: Biofilm formation test for 75 isolates of P. aerugenos using microtiter plate method (MTP)

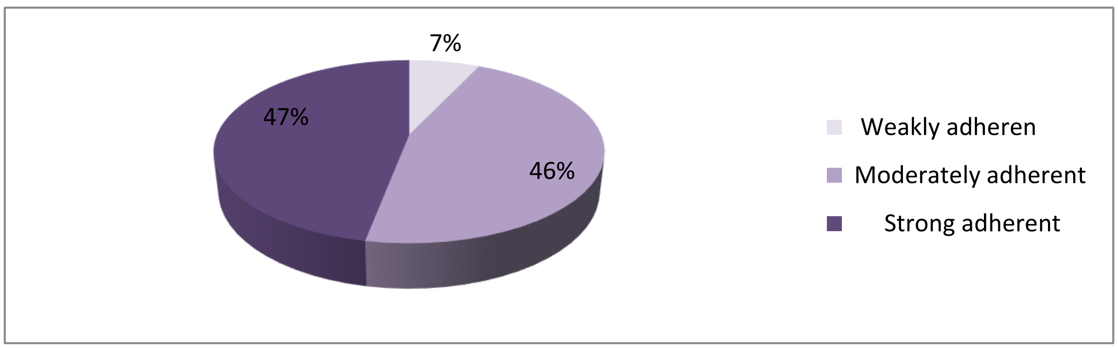


Figure 3 : Percentage of biofilm production in P.aeruginosa bacteria.

Table 1: OD readings using ELIZA reader at wavelength 630 nm in the microtiter plate method (MTP) test for biofilm formation.

	1	2	3	4	5	6	7	8	9	10
A	**PA135** 0.182	**PA136** 0.482	**PA137** 0.327	**PA138** 0.312	**PA141** 0.04			**C.N 0.095**	**C.N 0.098**	**C.N 0.119**
B	**PA117** 0.222	**PA119** 0.591	**PA121** 0.322	**PA125** 0.155	**PA127** 0.211	**PA129** 0.093	**PA130** 0.233	**PA132** 0.366	**PA133** 0.268	**PA134** 0.297
C	**PA87** 0.114	**PA88** 0.111	**PA90** 0.51	**PA91** 0.443	**PA92** 0.235	**PA97** 0.09	**PA107** 0.093	**PA108** 0.145	**PA115** 0.095	**PA116** 0.083
D	**PA60** 0.183	**PA63** 0.128	**PA67** 0.098	**PA71** 0.133	**PA74** 0.198	**PA76** 0.088	**PA77** 0.208	**PA78** 0.09	**PA80** 0.283	**PA86** 0.086
E	**PA33** 0.105	**PA39** 0.185	**PA42** 0.086	**PA43** 0.102	**PA45** 0.177	**PA46** 0.312	**PA49** 0.087	**PA51** 0.082	**PA56** 0.107	**PA57** 0.088
F	**PA23** 0.215	**PA24** 0.079	**PA25** 0.109	**PA26** 0.448	**PA27** 0.079	**PA28** 0.093	**PA29** 0.315	**PA30** 0.279	**PA31** 0.096	**PA32** 0.15
G	**PA13** 0.701	**PA14** 0.086	**PA15** 0.08	**PA16** 0.077	**PA17** 0.065	**PA18** 0.526	**PA19** 0.297	**PA20** 0.102	**PA21** 0.203	**PA22** 0.128

H	PA1 0.408	PA2 0.146	PA3 0.295	PA4 0.122	PA5 0.13	PA6 0.162	PA7 0.118	PA8 0.116	PA9 0.243	PA11 0.288
C.N = Control negative , PA = *P.aeruginosa*										
Non-biofilm formation) ODc ≥ OD) .(ODc = 0.04(										
Weakly adherent (ODc < OD ≤ 2xODc) =(0.04 < OD ≤ 0.08)										
Moderately adherent)2xODc < OD ≤ 4xODc) = (0.08 < OD ≤ 0.16(										
Strong adherent OD > 4xOD) = (OD > 0.16) (										

The results of the current study agreed with the researcher (Al-Naimi, 2015) whom she determined that 100% of the strains of P.aeruginosa were biofilm producers, and the researcher (Al-Saray, 2016) determined that 95.56% of the P.aeruginosa isolates were biofilm producers. While The researcher (Vasiljevic et al., 2014) determined that 97.55% of the isolates of P. aeruginosa bacteria are capable of producing biofilms.

This microtiter plate method (MTP) is necessary for knowing and studying the early stages of biofilm formation and classifying biofilm-producing isolates into low, medium, and high biofilm production, Where the production of P. aeruginosa biofilm is one of the important virulence factors because it provides protection from phagocytic cells and other environmental factors, as it is the first stage of disease occurrence and infection initiation, as well as the important role of biofilms in adhesion of bacteria to hard surfaces, especially in a hospital environment that causes Hospital-acquired infection (Pedersen et al., 2018).

In the current study, the high percentage of biofilm production by P. aeruginosa may indicate a rise in the resistance of bacterial isolates towards all antibiotics, as the biofilm plays a prominent role in the pathogenesis and resistance of these bacteria because it is the mucous layer that provides Conditions suitable for bacterial growth and increased resistance to treatment, and this in itself is a major problem (Algburi et al., 2017).

3.4. Effect of the antibiotic levofloxacin on biofilm formation

The effect of the antibiotic Levofloxacin on biofilm formation was studied under laboratory conditions by monitoring the binding of the dye Crystal violet to adherent cells, which indicates the effective ability to influence biofilm production. Treating the biofilm-forming bacteria P.aeruginosa with the antibiotic caused a decrease in the ability of the bacteria to produce biofilms. Table (2) and Figures (4,5) show the inhibition results for MIC and Sub-MIC in µg/ml.

Table 2: Measuring the wavelengths of ten samples before treatment with the antibiotic and after treatment at two concentrations, MIC and Sub MIC, and indicating the percentage of inhibition of biofilm formation.

%inhibition at Sub-MIC	Average wavelength at Sub-MIC	%inhibition at MIC	Average wavelength at MIC	Average wavelength before treatment	Sample No.
75%	0.004	18.75%	0.013	0.016	1
31.8%	0.15	0	0.22	0.22	2
-41.1%	0.27	-31.57%	0.25	0.19	3
99.3%	0.001	98%	0.003	0.15	4
99.3%	0.001	99.3%	0.001	0.16	5
92.5%	0.003	82.5%	0.007	0.04	6
99.1%	0.002	98.2%	0.004	0.22	7
98%	0.001	96%	0.002	0.05	14
22.38%	0.052	85.07%	0.01	0.067	15
50%	0.01	65%	0.007	0.02	16

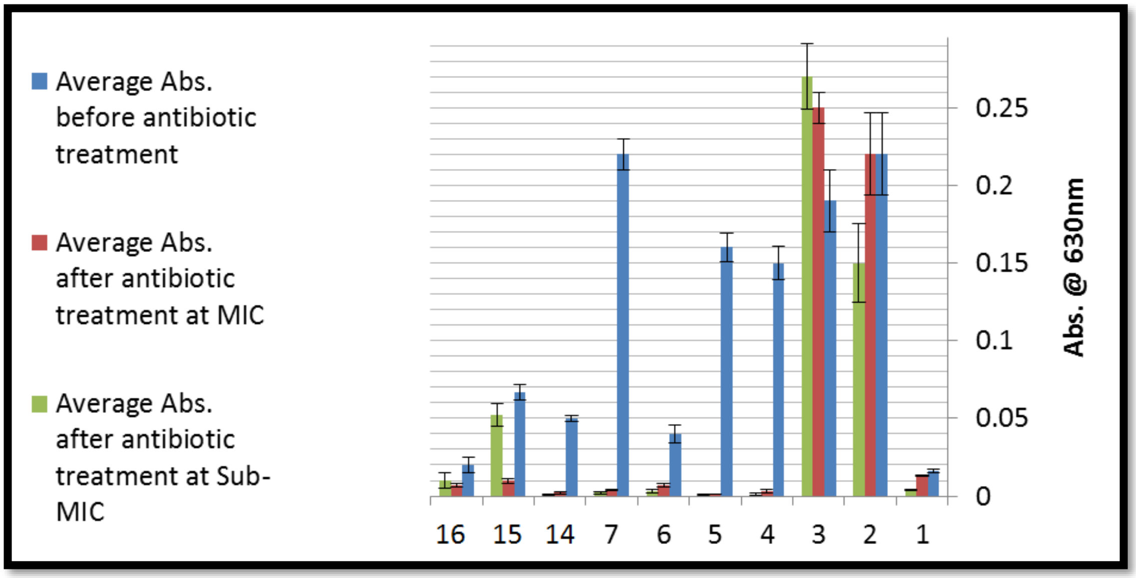


Figure 4: Average absorbance of biofilm at 630nm wavelength for ten bacterial samples before antibiotic treatment and after treatment at MIC and Sub MIC concentrations, (significant level, $p < 0.05$). Error bars represent SD from three independent experiments.

We note from the results a discrepancy in the percentage of inhibition of biofilm formation between isolates, which may be due to the source of the isolate and the strain, as well as the lack of effect of increasing the concentration of the antibiotic Levofloxacin on the inhibition of biofilm formation for the two concentrations MIC and Sub-MIC, as the percentage of inhibition of biofilm formation was more At the concentration of the antibiotic Sub-MIC, Figure (4).

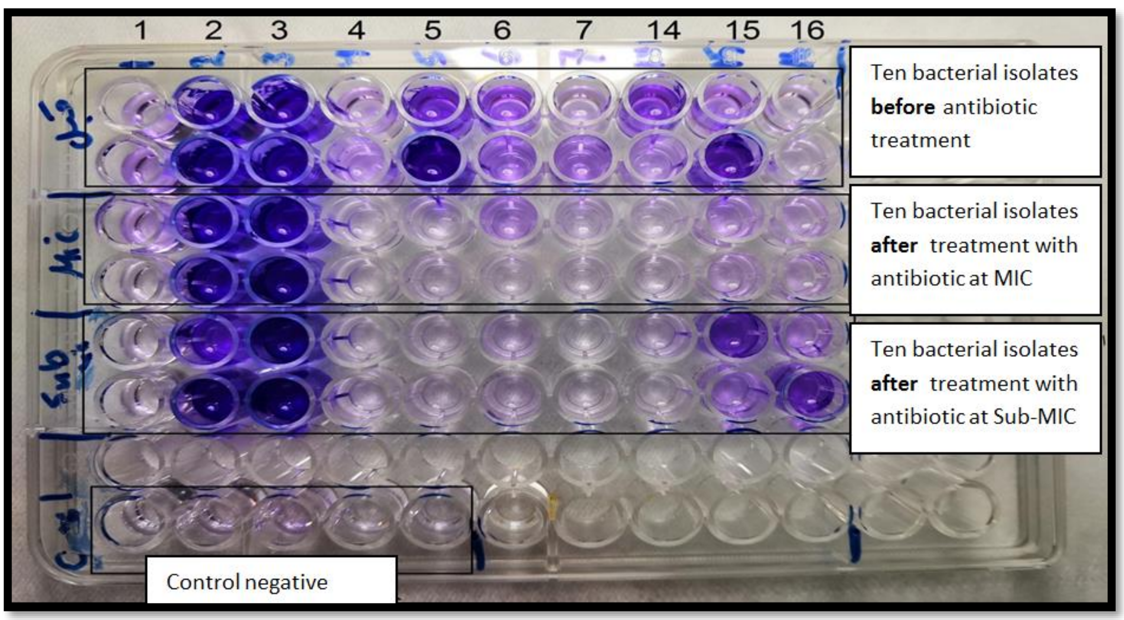


Figure 5: The inhibitory effect of isolates of P.aerugenosa after treatment with the antibiotic Levofloxacin using the Microtiter plate method (MTP).

When conducting a T-test for paired samples (before and after antibiotic treatment), we assume the following hypotheses:

The null hypothesis H0: There are no differences between the pre and post-measurements.

The alternative hypothesis H1: There are statistically significant differences between the pre and post-measurements.

The T-test value, degrees of freedom (n-1), and level of significance (Sig.) were calculated, Since the level of significance (Sig.) In the MIC and Sub-MIC concentrations are (0.047, 0.049) respectively, and the T value calculated in the MIC and Sub-MIC concentrations is (2.296, 2.274) respectively. So the level of significance (Sig.) is Less than 0.05 and the calculated T value is greater than the tabular T value which is (2.26) at the 0.05 level of significance. Thus, we reject the null hypothesis and accept the alternative hypothesis H1, which states that there are statistically significant differences between the mean measurement of biofilm absorbance before treatment with the antibiotic Levofloxacin and after treatment with the antibiotic in the concentrations MIC and Sub-MIC and the occurrence of a process of inhibition of biofilm production.

3.5. Detection the ability of P. aeruginosa to produce pyocyanin pigment.

The test results showed the presence of (55 isolates, 73.3%) that produce Pyocyanin, while (20 isolates, 26.7%) showed their inability to produce Pyocyanin, and the highest Pyocyanin - producing isolates were in urinary tract infections (2 isolates, 100%). While the least productive isolates were diabetic foot ulcer samples (5 isolates, 62.5%), as shown in Figure (6) and Table (3).

Table 3: Detection of the ability of P. aerugenosa isolates to produce the pyocyanin pigment.

Samples that did not produce pyocyanin	Pyocyanin -producing samples	Total No. of samples	Sample source
(%26.2)11	31) 73.8(%	42	burn samples
(%26.1)6	17) %73.9(	23	Wound samples
(%37.5)3	5) %62.5(	8	Diabetic foot ulcer samples
(%0) 0	2) 100(%	2	UTI samples
(26.7)20	55) 73.3(	75	Total

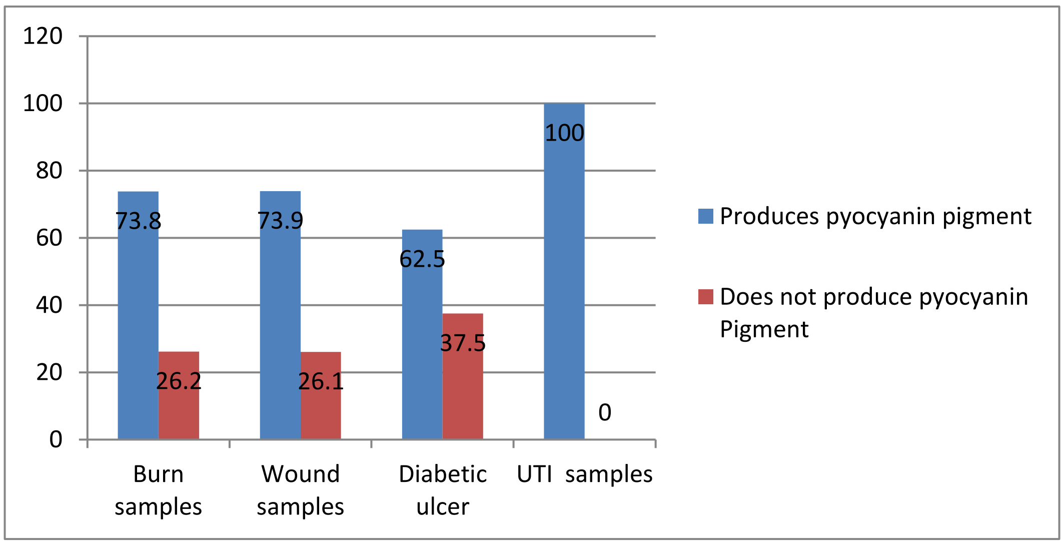


Figure 6: Percentage of pyocyanin-producing bacteria in isolates from different sources.

The inhibitory effect of Pyocyanin from precipitate and filtrate on the growth of S.aureus indicates the ability of P.aeruginosa to produce Pyocyanin pigment (Qasim et al., 2009). As shown in Figure (7).

This result is close to that of (Iwalokun et al., 2006) who obtained 81.5% of their Pyocyanin-producing isolates. While (Sharp et al., 2017) confirmed that Pyocyanin is produced by more than 85% of P.aeruginosa strains. Our study showed this productivity, which is high for the production of pyocyanin in isolates of P. aeruginosa resistant to antibiotics. The diameters of the inhibitors ranged between (5-88 mm) indicating their great importance in the protection, virulence, and antibiotic resistance of P. aeruginosa. (Fan et al., 2019) recently revealed the role of pyocyanin in antimicrobial properties and its contribution to resistance to the antibiotic Ciprofloxacin.

The characteristic of Pyocyanin production is genetically determined by chromosomes, and the decrease in production may be due to the presence of genetic mutations that lead to a change in production. (Gupte et al., 2021). (Vrenna et al., 2021) indicated that Pyocyanin plays an increasingly prominent role in a variety of serious hospital patient infections and is the main cause of pneumonia and cystic fibrosis, and confirmed that Pyocyanin is more frequent in the pus of clinical isolates than in the environment.

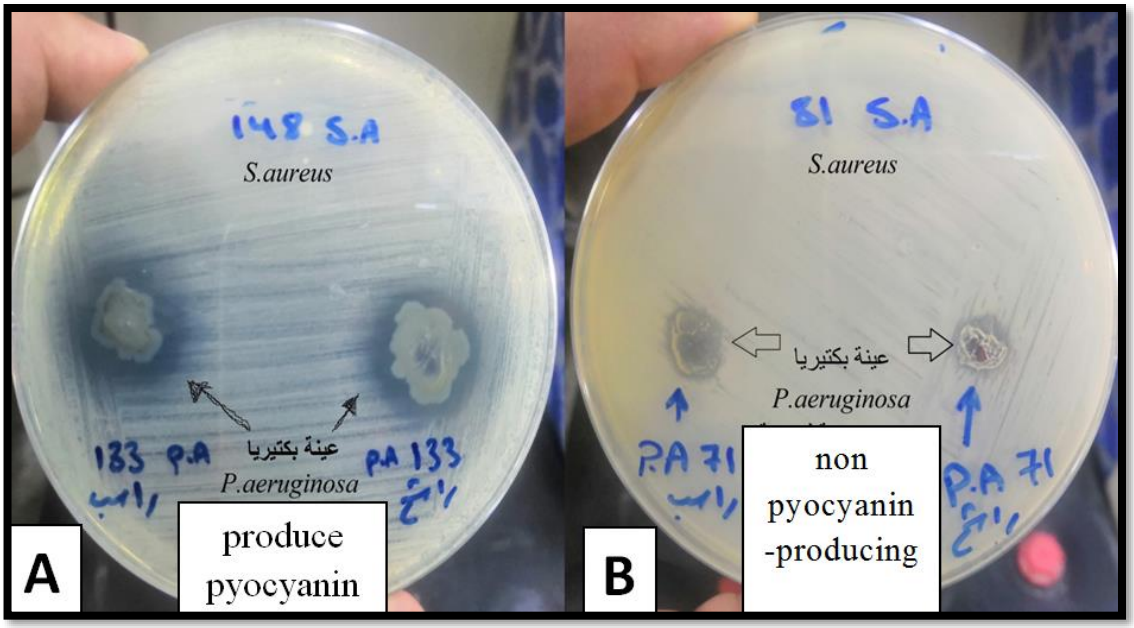
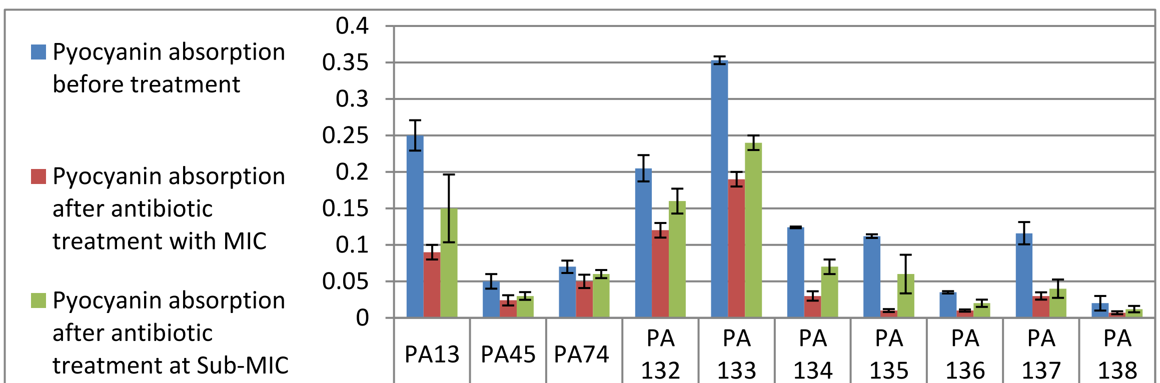


Figure 7: detection of the ability of P. aeruginosa to produce pyocyanin pigment , (A) pyocyanin-producing bacteria inhibit the growth of Staph.aureus bacteria, (B) P.aeruginosa bacteria are not pyocyanin-producing bacteria because there is no inhibition zone for staphylococcal bacteria.

3.6. Effect of the antibiotic levofloxacin on pyocyanin production

After using the method of extracting pyocyanin with chloroform from bacterial samples as in paragraph (2.5.2), the effect of the antibiotic Levofloxacin on the production of pyocyanin in the ten selected isolates of P. aeruginosa is shown in Figure (8). The pyocyanin production was significantly decreased (ranging from 30% to 80%) with MIC and Sub-MIC concentrations of Levofloxacin compared with P. aeruginosa isolates not treated with antibiotics, And the mean inhibition of pyocyanin pigment was (58.5%, 36.9%) after treating bacterial samples with the antibiotic at the MIC and Sub-MIC concentrations, respectively ($P < 0.05$).



	PA13	PA45	PA74	PA 132	PA 133	PA 134	PA 135	PA 136	PA 137	PA 138
Pyocyanin absorption before treatment	0.25	0.05	0.07	0.205	0.353	0.124	0.112	0.035	0.116	0.02
Pyocyanin absorption after antibiotic treatment with MIC	0.09	0.024	0.05	0.12	0.19	0.03	0.01	0.01	0.03	0.007
Pyocyanin absorption after antibiotic treatment at Sub-MIC	0.15	0.03	0.06	0.16	0.24	0.07	0.06	0.02	0.04	0.012

Figure 8: Effect of MIC and MIC Sub- of the antibiotic Levofloxacin on pyocyanin production by 10 clinical isolates of P. aeruginosa and comparison before and after treatment with the antibiotic. (Significant level, $p < 0.05$), error bars represent SD from three independent experiments.

The mean, standard deviation (SD), and standard error (SE), of the pyocyanin absorbance scale, were measured at 520 nm wavelength for the ten samples before and after treatment with the antibiotic Levofloxacin, and the data were represented as a diagram in Figure (9).

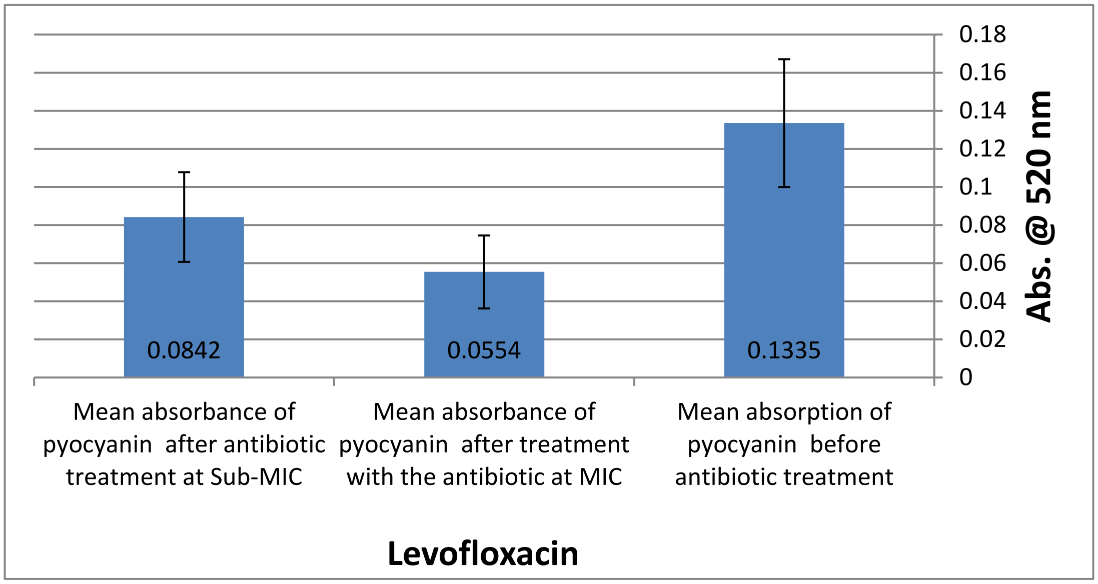


Figure 9: The average absorbance of pyocyanin Pigment at 520nm is shown for the ten selected bacterial samples before and after treatment with the antibiotic.

When conducting a T-test for paired samples (before and after antibiotic treatment), we assume the following hypotheses:

The null hypothesis H0: There are no differences between the pre and post-measurements.

The alternative hypothesis H1: There are statistically significant differences between the pre and post-measurements.

The T-test value, degrees of freedom (n-1), and level of significance (Sig.) were calculated, Since the level of significance (Sig.) In the MIC and Sub-MIC concentrations it is (0.001, 0.002) respectively, and the T value calculated in the MIC and Sub-MIC concentrations is (4.498, 4.154) respectively. So the level of significance (Sig.) is Less than 0.05 and the calculated T value is greater than the tabular T value which is (2.26) at the 0.05 level of significance. Thus, we reject the null hypothesis and accept the alternative hypothesis H1, which states that there are statistically significant differences between the mean absorbance measurement of pyocyanin Pigment before treatment with the antibiotic Levofloxacin and after treatment with the antibiotic in the concentrations MIC and Sub-MIC and the occurrence of inhibition of pyocyanin pigment production.

The researcher (Najafi et al., 2021) measured the effect of silver nanoparticles on the production of pyocyanin pigment by P. aeruginosa bacteria, and it was found through his results that silver nanoparticles affect the production of pyocyanin pigment.

Researchers (Ugurlu et al., 2021) used phenolic compounds and studied their effect on the production of pyocyanin pigment by bacteria. The results showed the effect of phenolic compounds that inhibited the production of pyocyanin pigment by 9-21% compared to isolates not treated with phenolic compounds.

The results of the current study agreed with the studies that were conducted on the effect of antibiotics on the production of pyocyanin pigment, where researchers from Egypt (Ali et al., 2021) used the antibiotic Levofloxacin and Ciprofloxacin at a concentration lower than the minimum inhibitory concentration Sub-MIC, and the inhibitory effect was also found for these antibiotics to produce the pigment pyocyanin.

Also, researchers (Ricci et al., 2017) found that one of the quinolones, which is Ciprofloxacin, also has an inhibitory effect on the production of pyocyanin pigment.

REFERENCES

1. Abdulwahhab, M. A., & Atiyea, Q. M. (2021). Isolation and Diagnosis of Escherichia Coli Causing Urinary Tract Infections from People of Different Ages in Mosul City. Annals of the Romanian Society for Cell Biology, 7017-7025.
2. Algburi, A; comito,N; kashtanov, D; Dicks, LM.T and chikindase, ML.(2017) control of Biofilm formation; Antibiotics and Beyond. applid and Environmental Microbiology. 83(3) :1-16pp.
3. Ali, G. H. (2021). Effect of subinhibitory concentrations of selected antibiotics and propolis on pyocyanin and biofilm production among Pseudomonas aeruginosa isolates in Alexandria, Egypt. Egyptian Journal of Medical Microbiology, 30(4), 129-137.

4. Al-najar, F. M., Atiyea, Q. M., & AL-Azzawie, A. F. (2020). DETECTION the GENETIC EFFECTS of ANTIBIOTICS and PLANT EXTRACTS on E. COLI BACTERIAL ISOLATED FROM UTI PATIENTS USING RAPD MARKERS. European Journal of Molecular & Clinical Medicine, 7(09).
5. Al-Saray, Zahraa Abdul Kareem Kadhem (2016). Effect of Gama Irradiation on some virulence factors and Multidrug Resistance of Bacteria Isolated from Burn and Wound Infections. M.Sc. thesis, College of Science, AL Mustansiriyah university. 103.
6. AL-Shamaa, N. F., Abu-Risha, R. A., & AL-Faham, M. A. (2016). Virulence genes profile of Pseudomonas aeruginosa local isolates from burns and wounds. Iraqi journal of biotechnology, 15(3).
7. Al-Shwaikh, R., & Alornaaouti, A. (2018). Detection of tox A gene in Pseudomonas aeruginosa that isolates from different clinical cases by using PCR. Ibn AL- Haitham Journal for Pure and Applied Science, 26 30. doi:10.30526/2017.IHSCICONF.1767.
8. Bo Fu, Qiaolian Wu, Minyan Dang, Dangdang Bai, Qiao Guo,Lixin Shen, and Kangmin Duan.2017.Inhibition of Pseudomonas aeruginosa Biofilm Formation by Traditional Chinese Medicinal Herb Herba patriniae. BioMed Research International; Volume 2017, Article ID 9584703, 10 pages.
9. Bose, S., Khodke, M., Basak, S., & Mallick, S. K. (2009). Detection of biofilm producing staphylococci: need of the hour. Journal of clinical and diagnostic research, 3(6), 1915-1920.
10. Crespo, A.; Blanco-Cabra, N.; Torrents, E. Aerobic Vitamin B12 Biosynthesis Is Essential for Pseudomonas aeruginosa Class II Ribonucleotide Reductase Activity During Planktonic and Biofilm Growth. Front. Microbiol. 2018, 9, 986.
11. Fan, Z.; Chen, H.; Li, M.; Pan,X.; Fu, W.; Ren, H.; Chen, R.; Bai, F.; Jin, Y.and Cheng, Z. Pseudomonas aeruginosa polynucleotide phosphorylase contributes to ciprofloxacin resistance by regulating PrtR. Front Microbiol , 2019; 10: 1762.
12. Gupte, A., Jyot, J., Ravi, M., & Ramphal, R. (2021). High pyocyanin production and non-motility of Pseudomonas aeruginosa isolates are correlated with septic shock or death in bacteremic patients. Plos one, 16(6), e0253259.
13. Hassuna , N. A.; Ibrahim, A. H.; AboEleuoon, S. M. and Rizk, H. A. W. (2015). High prevalence of Multidrug Resistant Pseudomonas aeruginosa recovered from Infected Burn Wounds in children. Med. Pub. J., 6(4):1 7.
14. Hong, J. S.; Yoon, E-J.; Lee H.; Jeong, S. H.and Lee, K.Clonal dissemination of Pseudomonas aeruginosa sequence type 235 isolates carrying bla IMP-6 and emergence of bla GES-24 and bla IMP-10 on novel genomic islands PAGI-15 and -16 in South Korea. Antimicrob Agents Chemother., 2016; 60: 7216–7223. doi:10.1128/AAC.0160116.
15. Iwalokun, B.A.; Akinsinde, K.A.; Lanlenhin, O.and Onubogu, C.C.Bacteriocinogenicity and production of pyocins from Pseudomonas species isolated in lagos, Nigeria .African.Journal of Bacteriology, 2006;5(11):1072-1077.
16. Jalil, M. B.; Abdul-Hussein, Z. R. and AL-Hmudi, H. A. (2017). Isolation and identification of multi drug resistant biofilm prpducer with Burn Wound infection in Basra Provincel/ Iraq IJDR 7:11 pp.
17. Khademi, F., Ashrafi, S. S., Neyestani, Z., Vaez, H., & Sahebkar, A. (2021). Prevalence of class I, II and III integrons in multidrug-resistant and carbapenem-resistant Pseudomonas aeruginosa clinical isolates. Gene Reports, 25, 101407.
18. Khadim, M., & Marjani, M. (2019). Pyocyanin and biofilm formation in Pseudomonas aeruginosa isolated from burn infections in Baghdad, Iraq. Biological, 12(1), 131.
19. Najafi, M., Moghaddam, M. N., & Yousefi, E. (2021). The Effect of Silver Nanoparticles on Pyocyanin Production of Pseudomonas aeruginosa Isolated From Clinical Specimens. Avicenna Journal of Medical Biotechnology, 13(2), 98.
20. Namasivayam, S. K. R., & Roy, E. A. (2013). Anti biofilm effect of medicinal plant extracts against clinical isolate of biofilm of Escherichia coli. Int. J. Pharm. Pharm. Sci, 5(2), 486-489.
21. Obaid, S. S., Atiyea, Q. M., & Qattal, S. M.(2016) Comparison between bacteremia in burned and nonburned patients.
22. Ochoa, S.A.; Lopez-Montiel, F.; Escalona, G.; Cruz-Cordova, A.; Davila, L.B.; Lopez-Martinez, B.; Jimenez-Tapia, Y. ; Giono, S.; Eslava, C.; Hernandez-Castro, R. and Xicohtencati-Cortes, J. (2013) . Pathgenic characteristics of Pseudomonas aeruginosa strians resistance to carbapenems associated with biofilm formation. J. Bol. Med. Hosp. Infant., 70(2): 133-144 .
23. Oluyombo, O.; Penfold, C.N.; Diggle, S.P. Competition in Biofilms between Cystic Fibrosis Isolates of Pseudomonas aeruginosa Is Shaped by R-Pyocins. mBio 2019, 10, e01828-18.
24. Pedersen, S. S; Hoiby, N.; Espersen, F. and Koch, C.H. (2018). Role of alginate in infection with Mucoid Pseudomonas aeruginosa in cystic fibrosis. 47: 6-13.
25. Qasim, S. S.; the narrator, Z.F.;Zain al-Abidin, S.S.Z. Isolation of Pseudomonas aeruginosa and its resistance to some specialized antibiotics and its ability to produce biocene. Anbar University Journal of Pure Sciences, 2009; 1: 1991-8941.
26. Ricci, A., Coppo, E., Barbieri, R., Debbia, E. A., & Marchese, A. (2017). The effect of sub-inhibitory concentrations of rifaximin on urease production and on other virulence factors expressed by Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa and Staphylococcus aureus. Journal of Chemotherapy, 29(2), 67-73.
27. Sharp, C.; Bray, J.; Housden, N.G.; Maiden, M.D.J.and Kleanthous, C. Diversity and distribution of nuclease bacteriocins in bacterial genomes revealedusing Hidden Markov models. PLoS. Comput. Biol., 2017; 13:e1005652. https:// doi.org/10.1371/journal.pcbi.1005652.
28. Smith, K.; Perez, A.; Ramage, G.; Lappin, D.; Gemmell, C. G. and Lang ,S. (2008). Biofilm formation by Scottish clinical isolates of Staphylococcus aureus. Journal of Medical Microbiology , 57: 1018–1023.
29. SudhaKar, T.; Karpngam, S. and Premkumer, J. (2015). Biosynthesis antibacterial activity of pyocyanin pigment ced produced by pseudomonas aeruginosa SU1. JCPRG5. 7(3):921-924.
30. Ugurlu, A., Yagci, A. K., Ulusoy, S., Aksu, B., & Bosgelmez-Tinaz, G. (2016). Phenolic compounds affect production of pyocyanin, swarming motility and biofilm formation of Pseudomonas aeruginosa. Asian Pacific journal of tropical biomedicine, 6(8), 698-701.
31. Vasiljevic, Z.; Jovcic, B.; and Dukic, S. (2014). An examination of Potential differences in biofilm production among different genotypes of Pseudomonas aeruginosa.Arch. Biol. Sci., 66(1): 117-121.
32. Vrenna, G., Artini, M., Ragno, R., Relucenti, M., Fiscarelli, E. V., Tuccio Guarna Assanti, V., ... & Selan, L. (2021). Anti-virulence properties of coridothymus capitatus essential oil against pseudomonas aeruginosa clinical isolates from cystic fibrosis patients. Microorganisms, 9(11), 2257.
33. Yolbaş, İ.; Tekin, R.; Kelekçi, S.; Selçuk, C. T. ; Okur, M. H.; Tan, İ.and Uluca, Ü. , (2013).Common pathogens isolated from burn wounds and their antibiotic resistance patterns, Dicle Medical Journal . 40 (3): 364-368.