A study on Quorum Sensing gene gen luxS and Mixed biofilm dynamics of *Proteus mirabilis* and *Candida albicans* in Urinary Tract Infections

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ABSTRACT

The role of biofilm formation is to act as protective defences to harbour bacteria, aids them to avoid the host immune system as well as the exposure to antibiotics. This is especially relevant to the case of urinary tract infections (UTIs), where biofilm forming bacteria causes recurring and persistent infection. This study was founded to identify the quorum- sensing gene luxS which is related to the formation of biofilm and to evaluate the antibiotic resistance profile of *Proteus mirabilis*, and finally to isolate *Candida albicans* yeast using urinary samples of women with UTIs. 270 midstream urine samples were obtained after collecting female patients aged 15 to 70 years at Kirkuk hospital, Kirkuk, Iraq, over the period April, to October, 2024. Culture was done on appropriate media after the samples were examined microscopically. Among the whole, 20 samples (7.4%) gave growth of bacteria that was characterised as *Proteus*. *mirabilis* and 30 isolated as *Candida albicans*. The results of an antibiotic susceptibility test were low sensitivity to ampicillin (20%), high sensitivity to vancomycin (80%), levofloxacin (90%), and total sensitivity (100%) to imipenem and amikacin. Eighty percent were positive in biofilm growth, i.e., 16 out of 20 isolates. In molecular analysis, 100 percent or all the 20 isolates had luxS gene which is involved in the production of signaling molecules in quorum sensing, and is given significant role in pathogenicity and biofilm. Such results imply that luxS gene is distributed widely among P. mirabilis isolates and can influence antibiotic resistance, thus being one of the targets in the future treatment strategies.

1. Introduction

Urinary tract infections or UTIs are among the most popular forms of bacterial infections in the world, and millions of people are affected by it, especially women. Depending on the affected urinary tract portion (from the urethra to the kidneys), these infections are usually accompanied by both bacteriuria and pyuria [1]. UTI ranks second after respiratory tract infections in regards to their prevalence and is a major cause of morbidity, particularly in hospitalized patients or those with a urinary catheter [2]. proteus mirabilis is a Gram-negative, motile bacillus which possesses the capability of producing urease and can cause opportunistic urinary tract infection in its opportunistic pathogenesis. It is very important in as far symptomatic and asymptomatic urinary tract infections are concerned especially among those who have complicating health factors like advanced age, or chronic diseases like diabetes mellitus [3] [4]. The process that allows the growth of Proteus mirabilis in such a way is that the organism has an array of virulence attributes as production of fimbriae and flagella to facilitate the motility and adhesion, urease to alter the urinary environment, hemolysis to damage the host tissues, and great propensity to form biofilm [5] The reason behind the pathogenicity of the Proteus mirabilis lies in its property staying attached to a biofilm that renders it highly resistant to antibiotics and persistence Biofilms are well grouped communities of micro-organisms surrounded by a self-secreted covered layer that surmounts and permanently dwells on both living and inert surfaces [6]. In molecules, the gene luxS is part of the quorum sensing (QS) pathway that supports a set of bacterial cells to communicate with others by using the signaling compound autoinducer-2 (AI-2). Such channels of compliance affect various collective activities such as the production of biofilms, virulence characteristic, and antimicrobial resistance [7]. Considering the increase observed in the number of reported cases of the multidrug resistant P. mirabilis strains, the present study was developed to study the luxS gene infection prevalence in clinical isolates, to determine their biofilm-forming capacities, and the pattern of their antibiotic resistance.

2. Experimental

2.1. Sample Collection and Processing

A total of 270 midstream urine samples were collected from female patients, aged 15 to 70 years, who presented with symptoms indicative of urinary tract infections at Kirkuk General Hospital, Iraq, during the period from April to October 2024. The sterile urine collection cones were used to collect the samples under aseptic condition and these were run to the microbiology laboratory immediately. Each of these samples was observed under microscope and those that

appeared significant were then grown on selective and differential media namely blood agar and MacConkey agar (Oxoid, UK) and Sabouraud Dextrose Agar (SDA) . The bacteria were incubated aerobically at 37°C for 24 hrs; the fungi 30°C for 48–72 hrs.

2.2 Bacterial Isolation and Identification

The bacterial isolates were first determined by colony morphology, Gram stain and the usual biochemical testing. Confirmatory identification was done by VITEK 2 Compact (bioMrieux, France) with GN ID cartridges, the manufacturer procedure was followed. The isolates that were not confirmed as Proteus mirabilis with > 99 % identification probability were eliminated and not subjected to further analysis.

2.3 fungal Isolation and Identification

Diagnosis of *Candida albicans* was done through a mixture of morphological and biochemical tests. First of all, clinical isolates were grown in Sabouraud Dextrose Agar (SDA) and CHROMagar Candida. *C. albicans* usually grew bright green shades of colonies, after 24 - 48 h of incubation at 37ºC on CHROMagar. The germ tube test was used in presumptive identification by placing a small amount of yeast in human serum and incubating it at 37ºC to 2 - 3 hours, and drop was observed under microscope; the identification of the presence of germ tubes i.e. short non-septate extensions of hyphae indicated *C. albicans*. Further microscopic morphology was also done by sub cultivating the isolates on either Cornmeal Agar plate or Rice Extract Agar that had been inoculated with Tween 80 and incubated at 35ºC for 48 hours. They were also found to have pseudohyphae, chlamydospores as well as blastoconidia but these are typical characteristics of *C. albicans*, which were observed under the microscope [8]. Further, the assimilation and fermentation of sugar were determined by API 20C AUX system or similarly other biochemical kits, with which *C. albicans* produces glucose fermentation and maltose fermentation, as well as galactose assimilation, sucrose assimilation, and glucose assimilation, therefore, it can be identified as *C. albicans* [9].

2.4 Biofilm Formation Assay for *Candida albicans* Isolates

The formation of biofilm in *Candida albicans* isolates was evaluated by the conventional Microtiter Plate Biofilm Assay. The isolates were incubated in Sabouraud Dextrose Agar (SDA) with incubation at 37ºC after 24 hours. A single pure colony was streaked out in 10 mL Sabouraud Dextrose Broth (SDB) and incubated in a shaker incubator at 120 rpm at 37ºC and left to incubate at 18 hours.

The resulting suspension of yeast was then brought to an optical density (OD) of OD600 ≈ 0.5 , which coincided with a value of about 1×10^6 CFU/mL , with sterile Phosphate Buffered Saline (PBS). Subsequently, this was standardized, then 100 UL of such standardized yeast suspension was inoculated in each well of a 96-well microtiter plate (preferably triplicate) and cultured at 37 o C for 24 - 48 hrs in static condition to form biofilms.

The wells were then aspirated gently after the incubation and the planktonic cells were removed but the formed biofilm was not disturbed. The non adherent cells were then eliminated by washing the wells three times with sterile PBS. Then, 150 µL absolute methanol (99–100%) was used to fix the adherent cells and added to each well, and kept at 15 minutes. Methanol was evaporated, and the plate dried at air.

Then, 150 µL of 0.1% crystal violet was put in each of the well and was left to absorb 15min, to color the biofilm. The distilled water was then gently washed on the wells three times to discard any excess stain. Lastly, 200 L of 95% ethanol (or 80:20 ethanol/acetone mix) was added to each sample and left to incubate in room temperature (15 minutes) in order to dissolve the bound dye. A microplate reader (ELISA reader) measurement was conducted at the wavelength of 570 nm to obtain absorbance [10].

2.5 Biofilm Formation Assay for *Proteus mirabilis* isolates

A method described by [11] and slightly modified was used to determine biofilm production; Congo Red Agar (CRA). CRA medium was prepared by combining in 900 mL of distilled water brain heart infusion broth (37 g/L), sucrose (50 g/L), and agar (10 g/L). On its own, 0.8 g of Congo red dye was dissolved in 100 mL of distilled water, filtered and added to the cooled medium at 50°C after which Petri dishes are filled. Biofilm-forming colonies had black, dry crystalline appearance after incubation at 37°C during 24-48 hours whereas non-producers were red or pink.

Biofilm production was determined using Congo Red Agar (CRA) as described by [11] albeit in slightly modified form. An equal volume of brain heart infusion broth (37 g/L), sucrose (50 g/L), agar (10 g/L) and 900 mL of distilled water was added to CRA media. Each of the dyes contained 0.8g of Congo red dye in 100 ml of distilled water, sterilized and inoculated in cooled medium of 50ºC Petri dishes. After incubation at 37ºC in a 24-48 hr period, biofilm producer colony looked like black, dry crystalline colony and non producers looked red or pink in color

2.6 Biofilm Detection Method for *Proteus mirabilis* Isolates (Crystal Violet Assay) :

To determine the formation of biofilm by the isolates of *Proteus mirabilis*, the technique based on crystal violet stain was applied. First, 20 microliters of bacterial cultures were inoculated into LB broth to create a nutrient-rich environment upon which bacteria can grow and form biofilms as possible. Bacterial suspension was transferred in 96-well micro-titer plates after incubation and incubated at 37ºC over 24 hours without shaking so that the bacteria settled on the surfaces of the wells and formed biofilms.

It was incubated and after that the liquid broth was poured very lightly so that the planktonic (free floating) cells could also be washed away and the wells were then washed thoroughly with phosphate-buffered saline (PBS) so as to leave just the adherent biofilm. The biofilm was subsequently immobilized through the application of methanol in order to stop any form of shifting in the staining and washing processes.

A dye of crystal violet was then added to stain the biofilm matrix. This stain can bind with the cells of bacteria and also with the extracellular polymeric substances in biofilm thus making them countable. Remaining stain was also removed by washing it away and the solubilization of the bound dye done with ethanol.

Lastly, a micro-plate reader was used to determine optimal density (OD) at 570nm of each well. Higher OD570 values mean that more of the biofilm is produced and that translates into the adherence and aggregation abilities of the isolates, which are associated with the resistance to treatment and the pathogenic qualities [12].

2.7 Antibiotic Susceptibility Testing:

Antimicrobial susceptibility was determined by the disk diffusion standard method on Mueller Hinton agar (Oxoid, UK), as per guidelines of Clinical and Laboratory Standards Institute (CLSI, 2023). The following types of antibiotic discs were present: ampicillin (10 µg), vancomycin (30 µg), clindamycin (2 µg), trimethoprim (5 µg), ceftazidime (30 µg), cefotaxime (30 µg) cefepime (30 µg), gentamicin (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), azithromycin (15 µg), and amikacin (30 µg). The sensitivity, responsive, or resistant results were in terms of measurements of the diameters of the inhibition zones.

2.8 Genetic Study

2.8.1 Isolating Genomic DNA from Gram Negative Bacteria :

Overnight cultures of P. mirabilis were used as the source of genomic DNA, and were extracted using the Wizard 1 genomic DNA purification kit (Promega, USA), as per the manufacturers recommendations of the Gram-negative bacteria. Purity and concentration of DNA was analysed in a NanoDrop^{TM} spectrophotometer (Thermo scientific, USA), and samples were frozen at -20^{o} C until analysis .

2.8.2 PCR Amplification of the luxS Gene:

The PCR amplification procedure for the genetic level to detecting luxs of P. mirabilis, Final volume for PCR mixture was 25 µl (12.5 of Master Mix 2x, 5 µl template DNA, 1 µl primers (table: 1) for each forward and reverse primer, finally, 5.5 µl nuclease free water) in uniplex PCR Eppendorf tubes mixed briefly via vortex then been placed in thermal cycler. The program used for PCR mixture was illustrated in the table

Table 1 : Primers Sequences of luxs gene.

Primer	Sequences (5'_3')	Product size/bp	Reference
luxs	ACGTATGTCTGCACCTGCG CCATAGCTGCCTTCCATGCA	290	(16)

Table 2 : The optimum condition of detection luxs**.**

Phase	Tm (0C)	Time	No. of cycle
Initial denaturation	94	5 min	1 cycle
Denaturation-2	94	1 min	30 cycle
Annealing	58	30 sec	
Extension	72	45 sec	
Final extension	72	5 min	1 cycle

2.8.3 Agarose Gel Electrophoresis

Electrophoresis was performed with a 1.5% agarose gel that was stained with RedSafe nucleic acid stain (Intron, Korea) in 1× TBE buffer in order to visualize PCR products. Molecular size Maker was 100 bp DNA ladder (Thermo Scientific, USA). The gels were run at 70 V during 1.5 hours and their observation was performed under the UV trans illuminator (Bio-Rad, USA).

2.8.4 Preparation of sample

Once 5 ul of the alleged DNA (electrophoresis Loading Dye) is mixed with 3 ul of the loading buffer (processor) of Intron / Korea, the loading is conducted to the wells of the gel. The electric current of 70 V \ CM2 was applied (at the rate of one to two hours) until the tincture reached the other side of the gel. The gel was tested through UV 336 nm source following its insertion in a pool consisting of 500 ml distilled water and 30 ul of red safe nucleic acid staining solution.

2.9 Preparation of Mixed Cultures of *Candida albicans* and *Proteus mirabilis*:

After incubating them at 37ºC and 24 hours, fresh pure colony of *C. albicans* and *P. mirabilis* were cultured on Sabouraud Dextrose Agar (SDA) and MacConkey Agar respectively.

These were then suspended as bacterial and fungal suspensions by transferring a single colony of each of the organisms into 5mL of RPMI-1640 medium.

A spectrophotometer was used to adapt the optical density of suspension to give a final concentration of OD600 ≈ 0.1 of *C. albicans* (≈1×10⁶ CFU/mL) and OD600≈ 0.2 of P. mirabilis (≈1x 10 8 CFU/mL).

A mixed culture was then harvested via adding volumes of the standardized *C. albicans* and P. mirabilis suspensions equal (1 mL each) into a fresh sterile tube. To obtain adequate mixing of the mixture without rupturing the cells by a mechanical action, it was vortexed gently [13].

2.10 Detection of Biofilm Formation in Mixed Cultures:

The capacity to form biofilms under the mixed cultures was also determined with the help of standard Microtiter Plate Biofilm Assay. To the same well of a 96-well plate, 50 L in each of the suspensions (*P. mirabilis* + *C. albicans*) were added to the wells keeping the final concentrations equal. All the samples were run in three replicates.

The plates were incubated at 37ºC over a 24 hrs period without shaking. Following incubation the wells were carefully aspirated to remove any non-adherent cell and washed three times with sterile phosphate buffer saline (PBS).

Fixing of biofilm cells was done by placing 100 µL of methanol in each well and letting it remain there for 15 minutes. Drying of plates by air was done. To each well, 100 µL of 0.1% crystal violet stain was then added after which a 15-minute incubation period proceeded. Washing was done by the addition of distilled water in the wells to eliminate excess stain followed by air drying of the plates.

To determine the biofilm 150 µL of 95% ethanol was added in each well to dissolve the bound dye. The absorbance was thereafter determined on a microplate reader against a 570 or 590 nm wavelength. The larger values of absorbance define the larger amounts of biofilm [14].

2.11 Statistical Analysis

ANOVA and the post hoc test (Tukey) has been performed on data . P- value < 0.05 as being significant. All the experiments were carried out in triplicates and the mean looked as mean + SD.

3. Results and Discussion

3.1 Bacterial Isolation and Identification: Out of 270 midstream urine samples collected from women with UTI symptoms, 20 (7.4%) yielded significant bacterial growth on blood and MacConkey agar (table 3 fig 1) . The isolates were identified as Proteus mirabilis based on characteristic swarming motility, non-lactose fermenting pale colonies on MacConkey agar, and Gram-negative rod morphology on microscopy. Confirmatory identification using the VITEK® 2 Compact system revealed a high confidence level of ≥99.99% for *P. mirabilis*. These findings are consistent with a study by [15] , who also reported a high identification accuracy of P. mirabilis using the VITEK-2 system in clinical isolates from Iraq. Globally, similar isolation rates and identification accuracy have been reported in studies from North America [16].

Figure 1 : Colonies of *P. mirabilis* on Macconkey agar**.**

The relatively low prevalence (7.4%) of P. mirabilis in this study may be attributed to geographical variation, patient demographics, and specimen selection criteria, as highlighted in

previous work by [17]. Moreover, the anatomical proximity of the urethral and anal regions in females enhances the risk of ascending infections by uropathogens such as *P. mirabilis* [1].

3.2 Antibiotic Susceptibility Patterns: The antimicrobial susceptibility profile of the *P. mirabilis* isolates revealed high resistance to ampicillin (70%) and ciprofloxacin (70%), whereas complete sensitivity (100%) was observed toward imipenem and amikacin. High sensitivity was also recorded against levofloxacin (90%) and vancomycin (80%) (Table 4).

Table 3 : Antibiotic susceptibility test of *P. mirabilis*

Antibiotics	Sensitive %	Intermediate %	Resistant %
Ampicillin	20.0	10.0	70
Vancomycin	80.0	0.0	20.0
Clindamycin	40.0	30.0	30.0
Trimethoprim	40.0	30.0	30.0
Ceftazidime	60.0	20.0	20.0
Cefotaxime	40.0	0.0	60.0
Cefepime	50.0	10.0	40.0
Gentamicin	70.0	0.0	30.0
Imipenem	100.0	0.0	0.0
Ciprofloxacin	20.0	10	70.0
Levofloxacin	90.0	0.0	10.0
Azithromycin	60.0	10.0	20.0
Amikacin	100.0	0.0	0.0

These resistance trends are in line with findings from [18], who reported high resistance to β-lactams and fluoroquinolones in P. mirabilis from European urinary isolates. Locally, [19] documented similar resistance profiles in isolates from catheterized patients in Iraq, with marked resistance to ampicillin and third-generation cephalosporins. The high susceptibility to imipenem in our study aligns with the hypothesis that restricted use of carbapenems in Iraq may help preserve their efficacy [20].

The development of multidrug resistance (MDR) in P. mirabilis may be associated with chromosomal and plasmid-mediated resistance mechanisms, and the organism's ability to produce biofilms further complicates antibiotic penetration and effectiveness [5].

3.3 Biofilm Production

Out of the 20 *P. mirabilis* isolates, 16 (80%) demonstrated positive biofilm formation on Congo Red Agar (table 5). Biofilm-forming colonies appeared as black, dry, and crystalline, indicative of exopolysaccharide production (fig: 2).

Table 4: number and percentage of biofilm formation.

Isolated bacteria	Biofilm positive	Biofilm negative	No. of isolated bacteria
P. mirabilis	16(80.0%)	4(20.0%)	20(100.0%)

Figure 2 : Colonies positive and negative biofilm for *P.mirabilis* using CAR method.

These results are comparable to previous findings in Iraq by [21] and [22], where over 85% of isolates were biofilm producers. [23] similarly confirmed biofilm formation in the majority of P. mirabilis strains isolated from chronic UTIs. Biofilm production is a critical survival mechanism, enabling bacteria to persist in hostile environments, evade host immune responses, and develop tolerance to antimicrobials [24]. The strong association between biofilm formation and antibiotic resistance in our isolates supports findings by [25], who reported enhanced resistance in biofilm-forming E. coli strains from UTIs.

Biofilm formation capability of *Proteus mirabilis* isolates was assessed by crystal violet assay and the absorbance determined at 570 nm (OD570). The findings indicated that 80 % (16 out of 20) of the isolates tested biofilm positive with biofilms of different intensities being positive, whereas 4 isolates were biofilm negative (20 %). The absorbance was high in 3 isolates (15%) (0.82, 0.78, and 0.76), so these isolates displayed the formation dense and complex biofilm, which can be a substantial protection cause against antibiotics and environmental stressors. Moderate isolates (35%) with $0.40 \leq OD < 0.75$ indicated that seven isolates could lead to chronic or recurrent infections especially in the case of UTIs. Weak biofilm growth (OD, less than 0.40) was observed in 6 isolates (30%), and these isolates had poor adherence and poor community structure, which possibly makes them susceptible to external factors [26]. These

findings are evidence that most clinical isolates of *P. mirabilis* achieve the capacity to form biofilms in varying intensities. This trait is one of the major virulence factors, particularly in UTIs, where the formation of biofilms provides protection to the bacteria, making antibiotics less effective and promoting the increased resistance of bacteria to the reacting host immune system.

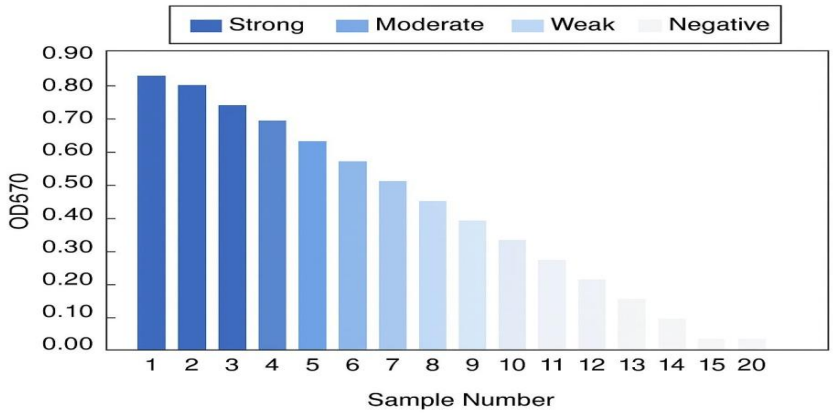


Figure (3) Biofilm Formation Strength of *Proteus mirabilis* Isolates Based on OD570 Values

3.4 Results of Biofilm Evaluation for *Candida albicans* Isolates :

The findings revealed that the percentage of *Candida albicans* isolates with moderate to high biofilm forming capacity was more than 80%. In particular, 12 isolates (40%) depicted an optical density (OD) ≥ 0.75 at 570 nm, which endorsed the production of a dense and thick biofilm. This is an indication of a high adaptation ability to adhere to the surface, and build complicated structures within the cell, which can result in severe infection and the difficulty of treatment. 9 (30%) out of the 30 isolates were moderate biofilm-forming isolates with OD values of 0.40 and 0.74. Such isolates can lead to milder infections yet have a comparative resistance against antifungal agents [27].

The weak biofilm-forming isolates comprised only 6 (20%) isolates whose OD values were < 0.40 and hence showed the lower potential to persist or invade the chronic infection.

Lastly, there were 3 isolates (10%) which were negative towards biofilm formation since it displayed no significant optical density readings and no apparent biofilm production is evident.

Table (5) Biofilm Formation Ability of *Candida albicans* Isolates Based on OD570

Absorbance OD at 570 nm	Biofilm Formation Strength	Percentage (%)	Number of Isolates
0.75 ≤	strong	40	12
0.74 – 0.40	Moderate	30	9
0.40 >	weak	20	6
0.2–0.1~	Non-producer	10	3

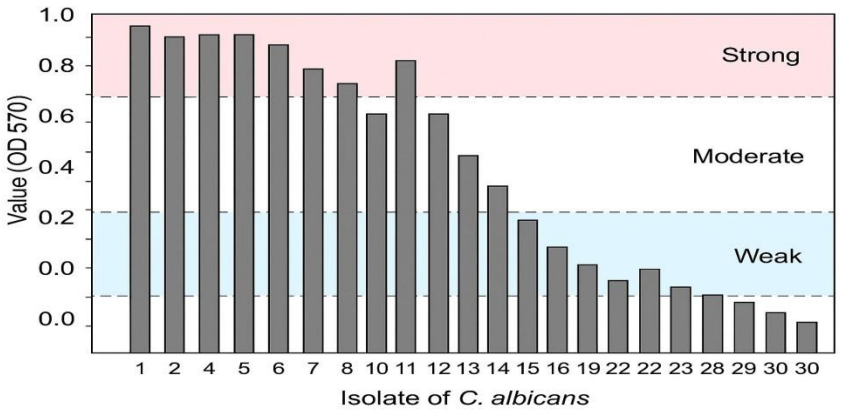


Figure (4) Results of the biofilm formation test for *Candida albicans* isolates

3.5 Correlation Between Biofilm Formation and Resistance Patterns:

The production of biofilm in isolates of Proteus mirabilis is also associated with increased resistance to antibiotics. The present table is a comparison of the antibiotic-resistance profiles of biofilm-forming isolates of *P. mirabilis* and non-biofilm-forming isolates. The isolates were prepared in two groups where 16 biofilm-forming isolates were prepared and 4 non-biofilm forming isolates were prepared. Each panel was compared with an array of antibiotics and results tabulated as percents of sensitive (S), intermediate (I), or resistance (R) isolates with respect to every drug. Isolates that make biofilm were more resistant to majority of antibiotics compared to non-producers. There was a marked elevated ampicillin resistance among the biofilm makers (93.7%) and it declined to 25% in the non-makers. Similarly, the rate of vancomycin resistance was 81.2% in the biofilm group compared to 25% in the non-biofilm group.

The above results also showed that multi-drug resistance among biofilm-producing bacteria strains is also observed with clindamycin, trimethoprim, ceftazidime and cefotaxime indicating that biofilm formation confers resistance to these agents. On the other hand, some antibiotics worked much better against non-biofilm isolates and, in this case, primarily, imipenem and levofloxacin. Interestingly, all the isolates that did not form biofilms were still sensitive to imipenem but only 43.8 % of the biofilm producers had the same result. Amikacin exhibited even further differences to biofilm- and non-biofilm-producing isolates; biofilm-producing isolates were found to be resistant to following percentages; 68.7% compared to full sensitivity of even non-biofilm-producing isolates (100%).

Table (6): Antibiotic resistance pattern based on biofilm reaction in *P. mirabilis.*

	biofilm production (No: 16)			Nil biofilm production (No: 4)		
Antibiotics	S No.(%)	I No.(%)	R No.(%)	S No.(%)	I No.(%)	R No.(%)
Ampicillin	0 (0.0%)	1 (6.3%)	15 (93.7%)	2 (50.0%)	1 (25.0%)	1 (25.0%)
Vancomycin	2 (12.5%)	1 (6.3%)	13 (81.2%)	2 (50.0%)	1 (25.0%)	1 (25.0%)
Clindamycin	5 (31.3%)	2 (12.5%)	9 (56.2%)	2 (50.0%)	0 (0.0%)	2 (50.0%)
Trimethoprim	6 (37.5%)	0 (0.0%)	10 (62.5%)	1 (25.0%)	1 (25.0%)	2 (50.0%)
Ceftazidime	7 (43.8%)	2 (12.5%)	7 (43.8%)	1 (25.0%)	0 (0.0%)	3 (75.0%)
Cefotaxime	5 (31.3%)	0 (0.0%)	11 (68.7%)	2 (50.0%)	1 (25.0%)	1 (25.0%)
Cefepime	8 (50.0%)	1 (6.3%)	7 (43.7%)	2 (50.0%)	1 (25.0%)	1 (25.0%)
Gentamicin	4 (25.0%)	1 (6.3%)	11 (68.7%)	2 (50.0%)	0 (0.0%)	2 (50.0%)
Imipenem	7 (43.8%)	0 (0.0%)	9 (56.2%)	4 (100.0%)	0 (0.0%)	0 (0.0%)
Ciprofloxacin	2 (12.5%)	1 (6.3%)	13 (81.2%)	2 (50.0%)	0 (0.0%)	2 (50.0%)
Levofloxacin	14 (87.5%)	1 (6.3%)	1 (6.3%)	2 (50.0%)	1 (25.0%)	1 (25.0%)
Azithromycin	9 (56.3%)	2 (12.5%)	5 (31.2%)	2 (50.0%)	0 (0.0%)	2 (50.0%)
Amikacin	5 (31.2%)	0 (0.0%)	11 (68.7%)	4 (100.0%)	0 (0.0%)	0 (0.0%)

These findings align with [28] , who demonstrated a significant increase in antibiotic resistance among biofilm-producing *Pseudomonas aeruginosa* isolates. The resistance is likely due to the reduced diffusion of antibiotics through the biofilm matrix and altered metabolic states of biofilm-embedded cells.

3.6 Genetic study

3.6.1 Detection of the luxS Gene

PCR analysis confirmed the presence of the luxS gene in all 20 *P. mirabilis* clinical isolates (100%), with a 290 bp amplicon visualized by agarose gel electrophoresis (Figure 3). This gene encodes an essential enzyme involved in the AI-2–dependent quorum sensing pathway, which governs bacterial communication, the expression of virulence factors, and the formation of biofilms [7].

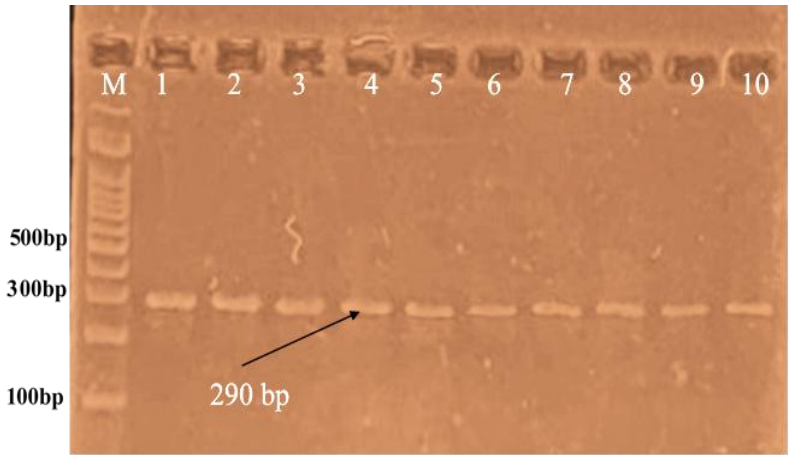


Figure (5): PCR amplification of 290bp Luxs gene by 1.5% agarose gel electrophoresis. Ladder:M, Lane (1-10): PCR product of 10 *P.mirabilis* isolates from urine samples.

The results of this study align with those reported by [29] , who identified the luxS gene in the majority of *P. mirabilis* isolates from UTI cases. Similarly, [30] observed luxS in 95% of isolates capable of biofilm formation in Iraq. This study further reinforces the high prevalence of the luxS gene among *P. mirabilis* isolates and suggests its pivotal role in regulating both virulence and antimicrobial resistance mechanisms.

3.6.2 The Relationship Between the luxS Gene and Biofilm Formation

A consistent and significant correlation was found between the presence of the luxS gene and biofilm production in *P. mirabilis isolates* (fig: 4).

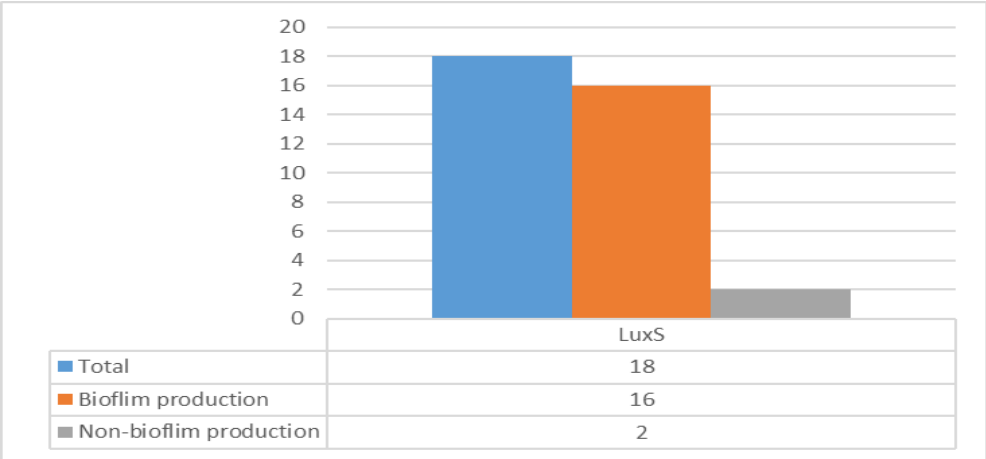


Figure (6): LuxS gene frequency among Biofilm and None Biofilm forming of *P. mirabilis* isolates.

All 20 isolates (100%) tested positive for the luxS gene, with 16 isolates (80%) also showing phenotypic biofilm formation. This strong association suggests that luxS-mediated quorum sensing plays a crucial role in regulating genes related to biofilm formation and the

establishment of bacterial communities on both living and non-living surfaces.These findings are in agreement with those of [29] , who identified a significant link between the luxS gene and the biofilm-forming capacity of P. mirabilis clinical isolates from UTI cases. Similarly, [30] reported that 95% of biofilm-producing isolates contained the luxS gene, further supporting its role in virulence and persistence within the urinary tract. The luxS gene encodes S-ribosylhomocysteinase, an enzyme involved in the synthesis of autoinducer-2 (AI-2), a universal signaling molecule found in both Gram-negative and Gram-positive bacteria. AI-2 regulates cell-density–dependent behaviors such as biofilm maturation, motility, and antibiotic resistance mechanisms [7]. In *P. mirabilis*, AI-2 signaling has been shown to activate regulatory proteins that govern adhesion and the structural formation of biofilms [31]. Despite the presence of the luxS gene in all isolates, 20% did not exhibit visible biofilm formation, indicating that additional environmental or regulatory factors may be required to fully induce biofilm expression. These observations suggest that while the luxS gene is an essential factor, biofilm formation is a multifactorial process influenced by factors such as gene expression levels, nutrient availability, and host conditions.

3.7 Biofilm production in mixed cultures of *Candida albicans* and *Proteus mirabilis*

The crystal violet staining and 570 nm absorbance were used to check the biofilm production of mixed cultures of *Candida albicans* and *Proteus mirabilis*. As indicated in the table (8) the mean absorbance values were differed considerably ($P < 0.01$) with mixed cultures and that of the single species cultures. The values of OD570 in all the mixed samples were higher than in the separate cultures and showed a synergistic relation between *C. albicans* and *P. mirabilis* in enhancing biofilm formation. This interaction can be a possible reason why drug resistance in mixed infection is usually astounding making it difficult to treat the disease.

The values of OD570 in the mixed cultures were between .0.59-0.88 as indicated in Figure (7). The maximum was obtained in isolate M8 (0.88), which indicates a very strong formation of biofilms, and the minimum was observed in M10 (0.59), which indicates weak formation of the biofilms. Majority of the isolates (7/10) had the capacity to produce a high amount of biofilm (OD 0.80) and this highlights the cooperation between the two microorganisms. M1-M9 and M2-M3 had moderate growth and M10 grew weakly. Based on these researches, the interaction between *C. albicans* and *P. mirabilis* could be more versatile and persistent in the biofilm than either microbe alone, which can increase resistance to external stress and antimicrobial agents and create a clinical challenge [27].

Table (7) Optical Density (OD570) of mixed cultures of *C. albicans* and *P. mirabilis*

Biofilm Formation Intensity	Optical Density (OD570)	Sample No
Moderate	0.68	1
Moderate	0.73	2
Moderate	0.78	3
Strong	0.81	4
Strong	0.83	5
Strong	0.84	6
Strong	0.86	7
Strong	0.88	8
Moderate	0.75	9
Weak	0.59	10

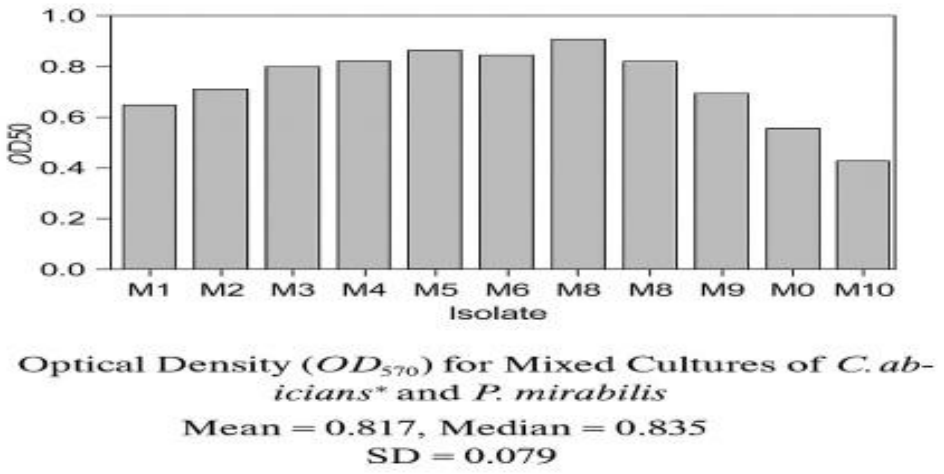


Figure (7) Biofilm Strength in Mixed Cultures of *C. albicans* and *P. mirabilis*

Table (8) The average biofilm production (OD570).

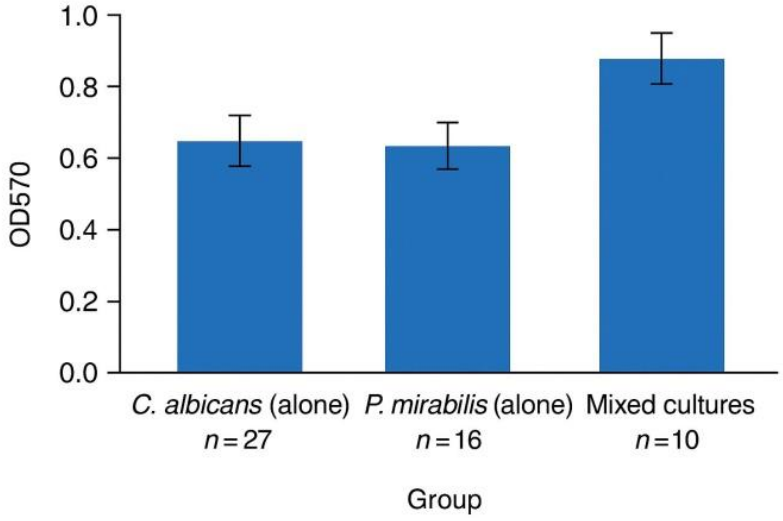
Minimum Value	Maximum Value	Standard Deviation (SD)	Mean OD570	Sample Size (n)	groupe
0.15	0.97	0.18	0.69	27	*C. albicans* (alone)
0.12	0.89	0.22	0.61	16	*P. mirabilis* (alone)
0.43	0.92	0.08	0.82	10	Mixed cultures

The table (9) indicated that the mean biomass of purified biofilms (OD570) was too high in the parallel cultures (0.82) than in the individual cultures of *C. albicans* (0.69) and *P. mirabilis*

(0.61). The standard deviation was also reduced in the mixed cultures which implied that the behavioral response of the men was relatively homogeneous across the samples.

The interaction between the yeast *C. albicans* and the bacterium *P. mirabilis* likely enhances biofilm production through mutual molecular signaling mechanisms, such as quorum sensing. P. mirabilis may secrete factors that accelerate the hyphal transition in *C. albicans*, promoting the formation of a more complex biofilm structure. This cooperation provides a more protective environment against external factors, reflected in the increased thickness and stability of the biofilm [32].

Such interaction may also facilitate stronger adhesion to the substrate surface and the formation of a denser extracellular matrix.



Figure(8) Biofilm Formation (OD570) of *C. albicans*, *P. mirabilis*, and Mixed Cultures

3.8 Comparison of Antibiotic Resistance profiles of Single Bacterial vs Mixed (Bacteria + yeast) Biofilm-Forming Isolates

Comparative findings indicate an increase in resistance to antibiotics by the mixed isolates (bacteria + yeasts) that produced biofilm than the bacteria isolates as indicated in Table (10).

As an example, 82.3 % of the mixed isolates were identified to be resistant towards Ampicillin as opposed to the 33.2 % of the bacterial-only isolates. In the same way, the resistance to Vancomycin was 90% in mixed isolates and 77.7% in bacterial isolates. In case of Trimethoprim, only 33.3 % were resistant relating to *P. mirabilis* but when mingled with other isolates, this number jumped to 85 %.

The sense of it, contrariwise, was usually higher within the single *P. mirabilis* isolates indicating that coexistence with *Candida albicans* would boost bacterial resistance. This may be because

of an expansion of the production of biofilms or a common expression of the genes between the two organisms in combination cultures.

Table(9): Antibiotic Resistance Patterns in Single Bacterial and Mixed (Bacteria + Yeasts) Biofilm-Producing Isolates Comparison

Antibiotic	*P. mirabilis* Biofilm Producers (n=16)			Mixed Biofilm Producers (n=10)		
	(%) S	(%) I	(%) R	(%) S	(%) I	(%) R
Ampicillin	31.8	35	33.2	10	8	82
Vancomycin	16.7	5.6	77.7	5	5	90
Clindamycin	61.1	11.1	27.8	10	10	80
Trimethoprim	63.7	3	33.3	5	10	85
Ceftazidime	38.9	11.1	50.0	14.4	10	75.6
Cefotaxime	53.3	20	27.8	6.5	5.5	88
Cefepime	50	5.6	44.4	20	10	70
Gentamicin	27.8	10	62.2	15.2	11.3	73.5
Imipenem	33	12.6	54.4	7.4	4.6	88
Ciprofloxacin	73.3	10	16.7	10	10	80
Levofloxacin	11.1	10	78.9	5	15	80
Azithromycin	33.3	11.1	55.6	30	10	60
Amikacin	17.5	13.6	68.9	12	13	75

A more resistant impact has been attributed to the fact of increased density and complexity of the mixed biofilm formation as a result of which the antibiotics struggle to penetrate and the concentration of extra cellular polymer substances (EPS) surges. Moreover, the mixed biofilm offers dual resistance to an immune system and antibiotics.

These data corroborate the hypothesis of increased treatment difficulty caused by mixed infection, with mixed biofilms depicting high levels of antibiotic resistance and therefore indicating the necessity of more efficient and specific treatment regimens. Yeasts can also be the causes of biofilm thickness and the decrease of antibiotic penetration.

This characterizes clinical risk of mixed-species biofilms, particularly within chronic infections, like urinary tract infections (UTIs), and the necessity of treatment of mixed biofilms, instead of strategies that attack individual organisms. The key questions should be answered in the background statement of the same writing [33].

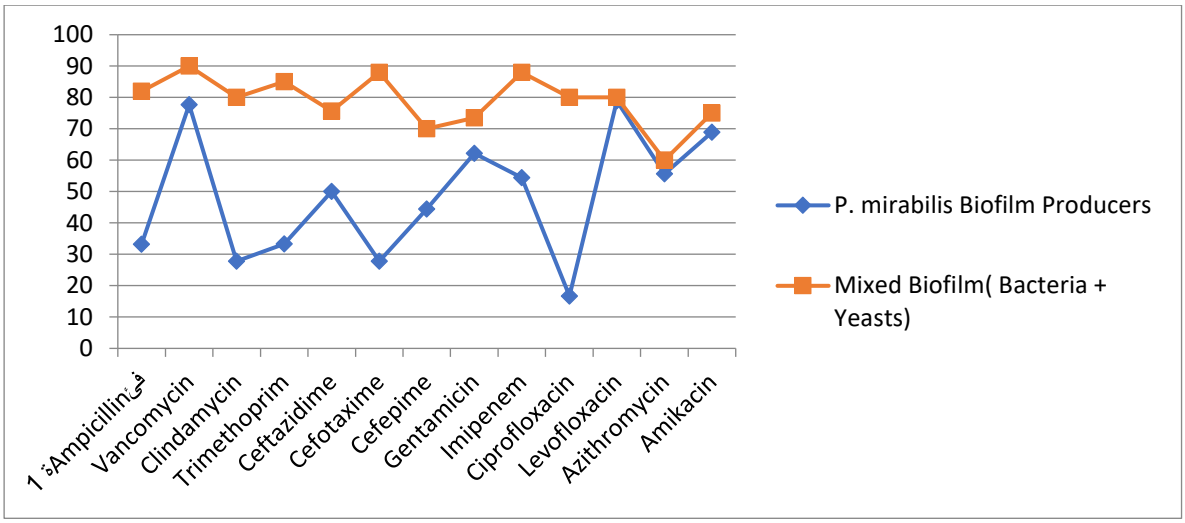


Figure (9) Antibiotic Resistance in Single vs. Mixed Biofilm-Producing Isolates

4. Conclusions

1. Isolates of *Proteus mirabilis* showed high rate of biofilm forming capability such that 80 % of the isolates were biofilm positive, meaning that they have the potential to produce chronic and hard to cure infections.

2. 100% of *P. mirabilis* isolates were positive to luxS quorum-sensing gene and therefore indicated that luxS might play a major role in modulating the biofilm formation, virulence, and antimicrobial resistance.

3. There was also a high rate of biofilm-producing ability by *Candida albicans* isolates, which is more than 80 percent of moderate to strong production.

4. *P. mirabilis* and *C. albicans* form mixed biofilms which were found to be much thicker than the ones single-species cultures form showing that there were synergistic interactions that increase complexity and protection of the biofilm.

5. Isolated biofilms were much more resistant to antimicrobials than single species isolates, providing some indication of the clinical significance of such co-infections.

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دراسة حول جين استشعار النصاب gen luxS وديناميكيات الأغشية الحيوية المختلطة لـ *Proteus mirabilis* و *Candida albicans* في التهابات المسالك البولية

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المستخلص

دور تكوين الأغشية الحيوية هو العمل كدفاعات وقائية لإيواء البكتيريا، ومساعدتها على تجنب الجهاز المناعي للمضيف وكذلك التعرض للمضادات الحيوية. وهذا مهم بشكل خاص في حالة التهابات المسالك البولية(UTIs) ، حيث تسبب البكتيريا المكونة للأغشية الحيوية عدوى متكررة ومستمرة. تأسست هذه الدراسة لتحديد جين استشعار النصاب luxS والذي يرتبط بتكوين الأغشية الحيوية وتقييم ملف مقاومة المضادات الحيوية لـ*Proteus mirabilis* ، وأخيرًا لعزل خميرة *Candida albicans* باستخدام عينات بولية من النساء المصابات بالتهابات المسالك البولية. تم الحصول على 270 عينة بول منتصف مجرى البول بعد جمع مريضات تتراوح أعمارهن بين 15 و 70 عامًا في مستشفى كركوك، كركوك، العراق، خلال الفترة من أبريل إلى أكتوبر 2024. تم إجراء الزراعة على بيئات مناسبة بعد فحص العينات مجهريًا. من بين المجموع، أعطت 20 عينة (7.4٪) نموًا للبكتيريا التي تم وصفها بأنها *Proteus. mirabilis* و30 عزلة منها على أنها فطريات *Candida albicans*. أظهرت نتائج اختبار حساسية المضادات الحيوية حساسية منخفضة للأمبيسيلين (20%)، وحساسية عالية للفانكومايسين (80%)، والليفوفلوكساسين (90%)، وحساسية كلية (100%) للإيميبينيم والأميكاسين. أظهرت 80% من العزلات نتائج إيجابية في نمو الأغشية الحيوية، أي 16 من أصل 20 عزلة. في التحليل الجزيئي، احتوت جميع العزلات العشرين، أو جميعها، على جينluxS ، الذي يشارك في إنتاج جزيئات الإشارة في استشعار النصاب، وله دور مهم في الإمراضية وتكوين الأغشية الحيوية. تشير هذه النتائج إلى أن جين luxS منتشر على نطاق واسع بين عزلات *P. mirabilis*، ويمكن أن يؤثر على مقاومة المضادات الحيوية، مما يجعله أحد الأهداف في استراتيجيات العلاج المستقبلية.

الكلمات المفتاحية : المتقلبة الرائعة، استشعار النصاب، تكوين الغشاء الحيوي، جينluxS ، مقاومة المضادات الحيوية، عدوى المسالك البولية.