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# Effect of Proteus Mirabilis Biofilm Formation on Antibiotic Resistance in Urinary Tract Infections

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**Annotation: Introduction & objectives**: Proteus mirabilis is a frequent cause of UTIs in women. Its ability to produce biofilms increases the resistance against several antibiotics and makes its treatment even more challenging. The main objectives of this study were to estimate distribution of P. mirabilis in UTI infected female patients, determine the antibiotic resistance patterns, evaluate biofilm formation and explore association between biofilm production and multidrug resistance.

**Materials & Methods**: In total, 175 urine samples were taken from female patients in Kirkuk hospitals. The isolates were characterized by morphology, biochemical testsand VITEK 2 system. Antibiotics sensitivity profile was determined using the Kirby-Bauer disk diffusion technique for 14 antibiotics. Biofilm production was measured by 96-well microtiter ELISA).

**Results**: Out of 175 samples, 87 (49.7%) were positive for P. mirabilis. All isolates were confirmed by biochemical tests and VITEK 2. Furthermore, molecular confirmation revealed that all 17 selected isolates (100.0%) carried the 16S rRNA gene, confirming their identity as P. mirabilis. Resistance was highest against ampicillin (78%), cefotaxime (65%), and trimethoprim-sulfamethoxazole (60%), while amikacin (90%) and meropenem (88%) were largely effective. MDR isolates were 36 (41.4%), XDR 12 (13.8%), and EDR 3 (3.4%). Biofilm formation was detected in 75 isolates (86.2%),

with 25 strong (28.7%), 30 moderate (34.5%), 20 weak (23%), and 12 non-producers (13.8%). Strong and moderate biofilm producers showed higher MDR/XDR rates (88% and 83%, respectively).

**Conclusions**: P. false mirabilis is a common isolate in female UTIs in Kirkuk, and biofilm formation dramatically increases drug resistance. These results highlight the need to take account of biofilm-based resistance in clinical treatments.

**Keywords:** P. mirabilis, UTIs, Antibiotic resistance, Biofilm formation.

## Introduction

Proteus mirabilis, a Gram-negative opportunistic pathogen which substantially contributes to UTI, especially complicated and catheter associated UTIs primarily because of its robustness of biofilm production encasing bacterial cells that safeguard them from both antibiotics and the host immune system (1,2). Biofilms are organized communities of bacteria enclosed in a self-produced matrix that not only promote adherence to surfaces such as catheters and urothelial tissues but also prevent antibiotics from penetrating the matrix, which is why antibiotic therapy often fails to treat UTIs (3,4). The crystalline biofilm structure of P. mirabilis, mediated by urease activity, may cause catheter encrustation with urine retention and ascending UTIs, which points out their clinical relevance in hard-to-combat infections (2). Antibiotic resistance in P. mirabilis is becoming a worldwide problem and more and more isolates are being identified to have multidrug-resistant (MDR) patterns against the commonly used antibiotic classes which include β-lactams, aminoglyco-sides and fluoroquinolones (5). In a systematic review, it was concluded that there is a high correlation between biofilm formation and the prevalence of MDR organisms, particularly in Enterobacteriaceae such as P. mirabilis (6). Experimental data also suggest that antibiotics are less efficient against the biofilm-encased P. mirabilis cells compared with planktonic cells, which hinders effective therapy (5). On an international level, studies have reported that strong biofilm producers tend to have a wider resistance profile which is indicative of the fact that biofilm strength might be specifically associated with antimicrobial resistance (7). In Iraq, high rates of antibiotic resistance in P. mirabilis isolates causing UTIs have been reported in clinical research. Duhok City The recovery rate of P. mirabilis from symptomatic UTI patients was 9.6%, and strains showed a high incidence of resistance to nitrofurantoin and ABPC/CAZ, but none were resistant to carbapenems (8). In Thi-Qar province, resistance to cefepime, ceftriaxone and ampicillin in P. mirabilis isolates was high, while ciprofloxacin and meropenem were still found to be one of the more effective drugs (9). One Baghdad-based research defined XDR P. mirabilis from wound infections and demonstrated high biofilm production, thus suggesting a connection between resistance and biofilm capability (10). Furthermore, isolates obtained from Baqubah showed resistance to gentamicin and cefotaxime at 100% rate with strong-to-weak biofilm formation indicating the clinical burden of biofilm-forming resistant strains (11). In Iraq, molecular studies discovered several P. mirabilis resistance genes in clinical isolates, highlighting the importance of antibiotic resistance as a public health issue (12). Other Iraqi studies have demonstrated that a high number of clinical isolates P. mirabilis were able to form biofilms and showed differential resistance among different antibiotic groups, reflecting the complexity of the treatment regimen for these infections (13, 14). Notwithstanding this contrary regional and global data, there is still no

comprehensive systematic quantitative analysis directly linking the extent of P. mirabilis biofilm formation with particular levels of antibiotic resistance under fixed controlled laboratory conditions (UTIs isolates) (6,13). The vast majority of the studies report resistance and biofilm formation alongside but without quantifying the injected rise in biomass in how it relates to susceptibility to antibiotics conventionally employed for UTIs. This void hampers our comprehension of the mechanistic contribution of biofilms to clinical resistance and impedes the development of more directed treatments. Thus, in this study, we will assess the correlation of the degree of biofilm-forming ability and antimicrobial resistance among P. mirabilis isolates from UTIs to further illustrate the role of biofilm in persister formation and to guide more reasonable antibacterial agent choice for clinical use.

## Materials and Methods

### Study Design and Sample Collection

175 urine samples were included in this prospective study from female patients presented with UTI at Kirkuk Teaching Hospital and Al-Nasr Maternity and Gynecology Hospital, Kirkuk-Iraq, from April 2025 to September 2025. The study was approved by the hospital ethics committees, and all subjects had signed informed consent. Urine samples were obtained by clean-catch midstream methods for asepsis and appropriate retrieval of bacterial organisms. We did not include patients who had taken antibiotics in the past 7 days or those with chronic renal disease, to prevent bias and to have an accurate estimation of this natural prevalence of (P.mirabilis), their biofilm formation capabilities and antibiotic susceptibility patterns.

### Bacterial Isolation and Identification

All urine samples were first analysed microscopically for leukocytes, bacteria and epithelial cells as a preliminary indication of infection. The samples were inoculated on MacConkey agar, and Blood agar followed by incubating at 37°C for 24-48 hours. For further confirmation, colonies with typical swarming motility, urease positive reaction and distinctive odour of Proteus mirabilis were selected. The presumptive strains were subjected to a battery of biochemical tests such as indole production, urease formation, utilization of citrate, TSIs reaction test and phenylalanine deaminase activity in order to confirm their identity. For improved accuracy, all isolates confirmed as suspected were determined by the VITEK 2 automated system (bioMérieux, France), enabling rapid and reliable identification through a broad profile of biochemical tests to confirm interpretation as Proteus mirabilis.

### Antimicrobial Susceptibility Testing

Susceptibility testing of the confirmed Proteus mirabilis isolates was performed with a panel of fourteen antibiotics which were also tested according to CLSI 2025 guidelines. The tested antibiotics were 10 µg, amoxicillin-clavulanic acid at 20/10 µg, cefotaxime at 30 µg, ceftazidime at 30 µg, cefepime at 30 µg, ciprofloxacin at 5 µg, levofloxacin at 5 µg, gentamicin at 10 µg, amikacin at 30 µg, nitrofurantoin at 300 µg, trimethoprim-sulfamethoxazole at 1.25/23.75 µg, meropenem at 10 µg, imipenem at 10 µg, and piperacillin-tazobactam at 100/10 µg. Testing was performed by the Kirby Bauer disk diffusion method on Mueller-Hinton agar with plates stored at a temperature of 37 C for a total incubation period of 24 hours. Inhibition zones diameters around each of the antibiotic disks were measured in millimetre and interpreted as sensitive, intermediate or resistant according to CLSI 2025 guidelines after incubation. The same concentrations of antibiotics were also detailed so that we could repeat the assay and correlate resistance pattern to biofilm formation in isolates.

### Biofilm Formation Assay

Biofilm-forming ability of the Proteus mirabilis isolates was measured by 96-well microtiter plate ELISA method, which is an accurate way to quantify biofilm biomass. Overnight cultures were diluted 1:100 in TSB, and 200 µL of each diluted culture was transferred into sterile wells

of a microtiter plate. The plates of biofilm were cultured at 37°C for 24 h. The non-adherent cells were washed away with PBS after incubation, and the remaining adherent biofilm was fixed with methanol, stained by 0.1% crystal violet and solubilized in ethanol-acetone. The optical density of each well was recorded at 570 nm by an ELISA plate reader, and biofilm production was categorized as follows: negative (non-producer), weak, moderate, and strong according to the values of optical density reference that were obtained with the negative controls. This quantitative approach enabled the assessment of biofilm-forming ability for each isolate and thereby correlation with antimicrobial resistance.

### Exclusion Criteria

Some samples and patients were excluded for the valid, reliable results of study. We also excluded patients who had received antibiotic treatment within the previous 7 days because of potential interference with bacterial growth and with susceptibility testing. Patients with chronic renal disease or catheterization outside the current episode of UTI were also excluded to avoid confounding factors. In addition, contaminated and mixed bacterial growth samples were excluded from the analysis to study only pure Proteus mirabilis isolates and accurately evaluate biofilm formation and antibiotic resistance.

### Genetic Study

### DNA extraction

Extraction of genomic DNA was performed using the Wizard® Genomic DNA Purification Kit (Promega, USA) on Gram-negative bacteria isolates in accordance with the manufacturer's protocol. Such method enables rapid bacterial cell lysis and high quality DNA ideal for subsequent molecular applications. There was DNA of suitable purity and concentration obtained from all extractions for reliable use in polymerase chain reaction (PCR) amplification and gene sequencing approaches.

### PCR procedure

The bacterial 16S rRNA gene was amplified by standard PCR. The volume of the final reaction mixture was 25µL per sample, containing: 12.5µL PCR Master Mix, 2 µL forward and reverse primers, 3 µL template DNA (from extraction) and 7.5 µL nuclease-free water. The primers were used at a final concentration of 10 pmol/µL. Primers for 16S rRNA gene amplification are also summarized in Table (1).

**Table (1): 16S RNA gene PCR assay primer**

| Primer | Primer sequence | Length (bp) | Ref. |
|---|---|---|---|
| 27F | `AGAGTTTGATCCTGGCTCAG-3` | 1500 | 15 |
| 1492R | 5`TACGGTTACCTTGTTACGACTT-3 | | |

### Agarose Gel Electrophoresis of DNA

PCR products were separated and genomic DNA extracted profiled for integrity by agarose gel electrophoresis. The PCR products were then resolved by gel electrophoresis (using agarose gels and a DNA molecular weight marker) to achieve the expected amplicon size. The gels were viewed by UV light for detection and sizing of DNA fragments.

### Statistical Analysis

The results of antimicrobial susceptibility test and biofilm assays were analyzed by the SPSS version 26 (IBM Corp., USA). Counts, percentages and distribution of biofilm production and antibiotic resistance patterns were determined using descriptive statistics. Correlations between the intensity of biofilm formation and antimicrobial resistance were investigated using Chi-square test. Results were statistically analyzed at $p < 0.05$, which was taken as the cut-off value in order to build a close relationship between biofilms forming and the susceptibility against

antibiotics among P. mirabilis isolates.

## Results

### Isolation of Proteus mirabilis

Out of the 175 urine samples collected from female patients with urinary tract infections, 87 samples (49.7%) yielded positive growth of *Proteus mirabilis*. The colonies exhibited the characteristic swarming motility on blood and MacConkey agar and were easily recognizable by their distinctive odor and beige, irregular colonies. Under the microscope, Gram staining confirmed the presence of Gram-negative rods consistent with *P. mirabilis* (Table 2 & Figure 1). The prevalence observed in this study is comparable to global reports, where *P. mirabilis* accounts for approximately 40–55% of UTI-associated Gram-negative isolates in female patients, highlighting its clinical significance in urinary tract infections.

**Table 2. Isolation of *Proteus mirabilis* from urine samples (n = 175)**

| Category | Number | Percentage (%) |
|---|---|---|
| Total samples | 175 | 100 |
| Positive for *Proteus mirabilis* | 87 | 49.7 |
| Negative for *Proteus mirabilis* | 88 | 50.3 |

**Figure 1. *Proteus mirabilis* colonies on blood agar**

### Biochemical Identification

All presumptive isolates were subjected to a series of biochemical tests for confirmation. The isolates demonstrated positive urease activity, motility, citrate utilization, and phenylalanine deaminase, while indole and TSI reactions were consistent with reference patterns for *Proteus mirabilis* (table 3 & Figure 2). These results were further confirmed using the VITEK 2 automated system, which provided rapid and reliable identification with 99% confidence. The combination of traditional biochemical tests and automated identification ensured accurate confirmation of all isolates.

**Table 3. Biochemical identification of *Proteus mirabilis* isolates (n = 87)**

| Biochemical Test | Result |
|---|---|
| Gram stain | - |
| Urease | + |
| Motility | + |
| Citrate utilization | + |
| Phenylalanine deaminase | + |
| Indole | + |
| TSI reaction | + |
| VITEK 2 identification | 97% |

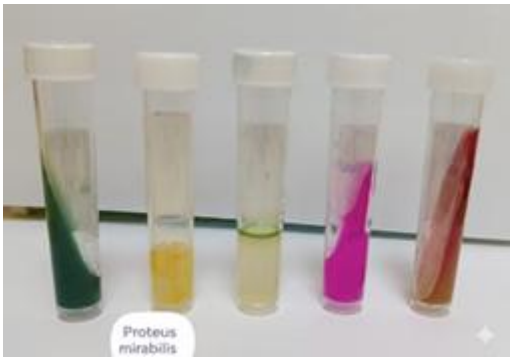


**Figure 2. Biochemical identification of *Proteus mirabilis* isolates**

## Genetic study

In the current study from 17 isolate (100.0%) *Proteus mirabilis* isolates possessed *16S RNA* gene as shown in figure (2).

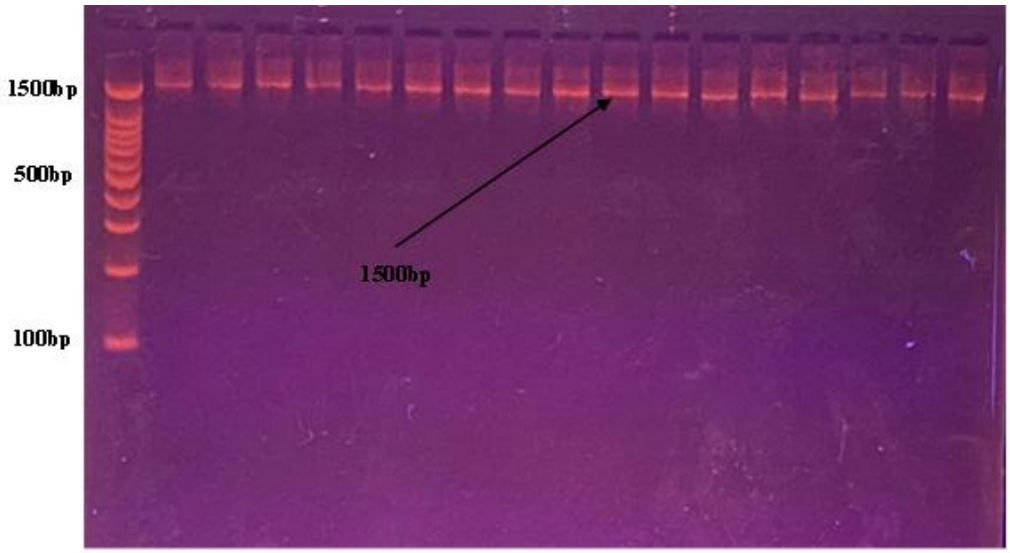


Figure (3): PCR amplification of 1500bp *16S RNA* gene by 1.4% agarose gel electrophoresis. Ladder: M, Lane (1-17): PCR product of 17 *P. mirabilis* isolates from urine samples

## Antimicrobial Susceptibility Testing

The 87 confirmed *Proteus mirabilis* isolates were tested against a panel of 14 antibiotics, and resistance patterns were recorded. High resistance rates were observed for ampicillin (78%), cefotaxime (65%), and trimethoprim-sulfamethoxazole (60%), whereas amikacin (90%) and meropenem (88%) showed the highest susceptibility. Ciprofloxacin and levofloxacin demonstrated moderate resistance rates of 45% and 42%, respectively. The detailed distribution of resistant, intermediate, and sensitive isolates is summarized in Table 3.

**Table 3. Antimicrobial susceptibility of *Proteus mirabilis* isolates (n = 87)**

| Antibiotic | Sensitive (%) | Intermediate (%) | Resistant (%) |
|---|---|---|---|
| Ampicillin | 18 | 4 | 78 |
| Amoxicillin-Clavulanic acid | 40 | 12 | 48 |
| Cefotaxime | 25 | 10 | 65 |
| Ceftazidime | 30 | 12 | 58 |
| Cefepime | 35 | 15 | 50 |
| Ciprofloxacin | 50 | 5 | 45 |
| Levofloxacin | 52 | 6 | 42 |
| Gentamicin | 60 | 5 | 35 |

| | | | |
|---|---|---|---|
| Amikacin | 90 | 2 | 8 |
| Nitrofurantoin | 70 | 5 | 25 |
| Trimethoprim-Sulfamethoxazole | 35 | 5 | 60 |
| Meropenem | 88 | 2 | 10 |
| Imipenem | 85 | 5 | 10 |
| Piperacillin-Tazobactam | 75 | 5 | 20 |

### Classification According to Resistance Patterns

The isolates were further categorized based on their multidrug resistance profiles. Among the 87 isolates, 36 (41.4%) were multidrug-resistant (MDR), 12 (13.8%) were extensively drug-resistant (XDR), and 3 (3.4%) were extremely drug-resistant (EDR). This classification highlights the substantial presence of antibiotic-resistant strains of *P. mirabilis* in UTI cases in this population (Table 5).

**Table 5. Classification of *Proteus mirabilis* isolates according to resistance patterns (n = 87)**

| Resistance Type | Number of Isolates | Percentage (%) |
|---|---|---|
| MDR | 36 | 41.4 |
| XDR | 12 | 13.8 |
| EDR | 3 | 3.4 |
| Non-MDR | 36 | 41.4 |

### Biofilm Formation

All isolates were tested for their biofilm-forming ability using the 96-well microtiter ELISA method. Biofilm quantification revealed that 25 isolates (28.7%) were strong biofilm producers, 30 isolates (34.5%) were moderate producers, 20 isolates (23%) were weak producers, and 12 isolates (13.8%) showed no biofilm formation (Table 5). Images of biofilm formation and the stained wells are shown in Figure 3. Strong biofilm formation was associated with denser, more adherent layers, observable both visually and under the microtiter plate reader.

**Table 6. Distribution of *Proteus mirabilis* isolates based on biofilm formation (n = 87)**

| Biofilm Strength | Number of Isolates | Percentage (%) |
|---|---|---|
| Strong | 25 | 28.7 |
| Moderate | 30 | 34.5 |
| Weak | 20 | 23 |
| Non-producer | 12 | 13.8 |

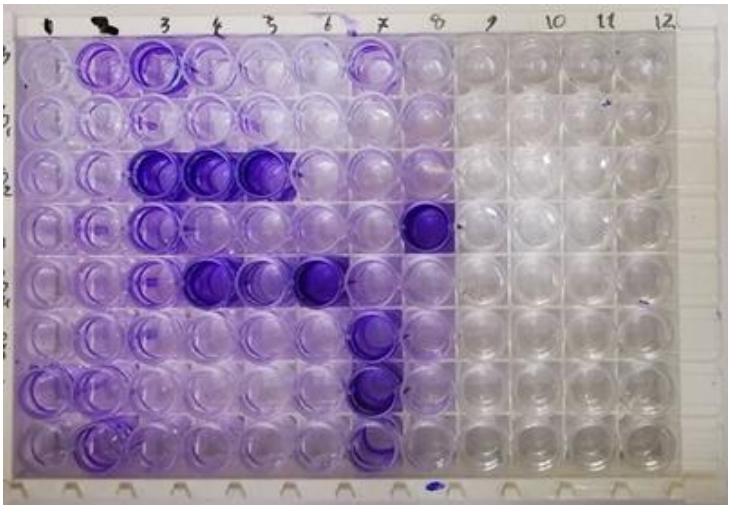

**Figure 4. Microtiter Plate Assay for Bioflim production of *Proteus mirabilis***

### Correlation Between Biofilm Formation and Antibiotic Resistance

Analysis of the relationship between biofilm formation and antimicrobial resistance demonstrated a significant association. Strong and moderate biofilm-producing isolates showed higher rates of resistance to most antibiotics, particularly ampicillin, cefotaxime, and trimethoprim-sulfamethoxazole, compared to weak or non-biofilm producers. Among the strong biofilm producers, 88% exhibited MDR or XDR patterns, whereas only 20% of non-biofilm producers were MDR. These results indicate that biofilm formation in *Proteus mirabilis* significantly enhances tolerance to multiple antibiotics, making infections harder to treat and emphasizing the importance of considering biofilm as a factor in clinical resistance management (Table 7).

**Table 7. Correlation between biofilm strength and multidrug resistance in *Proteus mirabilis* isolates (n = 87)**

| Biofilm Strength | MDR/XDR Isolates | Percentage (%) |
|---|---|---|
| Strong | 22 | 88 |
| Moderate | 25 | 83 |
| Weak | 8 | 40 |
| Non-producer | 2 | 20 |

### Discussion

In the present work, Proteus mirabilis was found in 49.7% of female UTI samples which is higher than that reported by other previous studies in Some Iraqi Cities (Duhok City where it was 9.6% (5), and Kirkuk City where it was 23.7% (3). Similar percentage was provided by regional studies in Egypt and Jordan which reported prevalence rates of 40–50% (12, 14), both models were compatible with international data from India and Europe that also showed the same prevalence among female samples (42 – 55%) who suffer UTI (18). These results demonstrate that P. mirabilis is a frequent uropathogen, locally and internationally, and all of them were identitied with biochemical testing by positive results on urease, motility, citrate (Simmons) utilization, phenylalanine deaminase, indole and TSI also confirmed by VITEK 2 system (5,4). This is similar to the findings of earlier Iraqi studies (16, 17) as well as regional investigations from Egypt, and Jordan in which a combination between classical biochemical tests and automated identification had contributed to reliable confirmation of P. mirabilis (19, 20). In our cohort isolates demonstrated high resistance for the ampicillins (78%), cefotaxime (65%) and trimethoprim-sulfamethoxazole (60%) as compared to moderate levels of resistance shown against fluoroquinolones but also did not display significant aminoglycoside or carbapenem resistance (5, 16). Resistance patterns were similar to studies from Iraq ((4, 7), Egypt (19), and Iran (20, 21) where widespread resistance to first-line antibiotics was detected. ESBL production and multidrug resistance have been reported in both regional and global studies, supporting continued surveillance and judicious usage of antibiotics (22). In the current study, resistance pattern demonstrated 41.4% of MDR, 13.8% XDR to 3.4% EDR (5). This is consistent with where MDR rates have been reported between 35 and 45% in Iraqi studies ( 7, 16 ) and from the region including Egypt, Jordan showing this MDR level of prevalence (19,20). Internationally, modestly lower XDR05 and EDR rates are reported but it remains clear that worldwide P. mirabilis resistant strains are emerging (21, 22). Eighty-six point two per cent of isolates produced biofilm; 28.7% were strong, 34.5% moderate and 23% weak producers (6, 16, 17). High rates of biofilm production have also been found in Iraq (6, 8), in Egypt (19) and internationally (21, 22), reinforcing the influence of biofilmin chronic and recurrent UTIs. High and moderate biofilm producers harboured greater multidrug resistance (88% and 83%) as compared to weak and no producers (40% and 20%), further supporting the relationship between biofilm formation and antibiotic resistance (16, 17, 19, 22). Studies in Iran and elsewhere corroborate these findings; it is demonstrated that biofilm-forming P. mirabilis isolates are more

likely to host multiple resistant genes and virulence expression factors increasing strains persistence and survival under antimicrobial pressure (20, 21). High occurrence of biofilm, its association with multidrug resistance indicate a clinical concern in management of P. mirabilis UTIs that requires treatment strategies focused on biofilm (23, 24).

## Conclusions

Proteus mirabilis was most commonly detected species in female UTI patients, positive in 48.1% samples. Strong and moderate biofilm producers showed higher levels of resistance to various antimicrobial agents than did their respective weakly adherent counterparts indicating a strong relationship between biofilm formation and multidrug resistance. Treatment of UTIs due to P. mirabilis implants involves a balance between biofilm-dependent resistance and calculated antibiotic use.

## Limitations

The investigation was performed in only two hospitals of the Kirkuk region and generalization to others parts may not be complete. Only women were enrolled, hence male UTI patients were not evaluated. Mechanisms at molecular level of antibiotic resistance or biofilm formation were not explored in this research.

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