RESEARCH ARTICLE

The role of biofilm in *Proteus mirabilis* as antibiotic resistance biomarker in patients with urinary tract infections

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***Proteus mirabilis,* a urease-producing bacterium, is a frequent culprit in urinary tract infections (UTIs). Most Gram-negative bacteria are characterized by their ability to produce biofilms outside their bodies, which usually adhere to solid surfaces. Biofilm formation is a significant concern in UTIs as it can hinder the efficacy of antibiotic treatment. The connection between antibiotic susceptibility and biofilm development was examined in this study in *P. mirabilis* isolates from UTI patients to identify the profiles of antibiotic susceptibility of *P. mirabilis*, assess biofilm formation ability among these isolates, and explore potential links between antibiotic resistance and biofilm production. The urine samples of 300 UTI patients from August to November 2023 were taken in Hilla city, Iraq. *P. mirabilis* isolates were identified and subjected to disc diffusion testing for antibiotic susceptibility and microtiter plate assays for biofilm formation. The results showed that all 67 *P. mirabilis* isolates harbored the urease gene and exhibited the highest resistance rates to trimethoprim (43.28%), chloramphenicol (31.34%), vancomycin (41.79%), and nitrofurantoin (29.85%), while all isolates were imipenem susceptible. Resistance to other antibiotics ranged from 2.9% to 13.43%. Importantly, 21 (31.34%) isolates were multidrug-resistant (MDR). All isolates formed biofilms with 23 (34.3%) demonstrating strong biofilm production, significantly associated with MDR isolates. This study revealed a substantial proportion of *P. mirabilis* isolates from UTI patients displaying the development of biofilms and carrying antibiotic resistance, especially among MDR isolates. These findings underscored the crucial need for continuous antibiotic resistance surveillance and novel strategies for preventing and treating UTIs caused by *P. mirabilis*.**

Keywords: *Proteus mirabilis*; URer gene; antibiotic susceptibility; urinary tract infections (UTIs); biofilm formation.

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Introduction

Proteus mirabilis is a Gram-negative bacterium and responsible for a significant proportion of human urinary tract infections (UTIs), particularly affecting patients with indwelling catheters [1]. *P. mirabilis* is thought to be the cause of between 10% and 20% of hospital acquired UTIs [2]. This bacterium can cause UTIs due to a number of virulent factors. One key factor is its production of urease, an enzyme that hydrolyzes urea to ammonia, increasing urinary pH and promoting tissue damage and stone formation [3]. Additionally, *P. mirabilis* is equipped with

numerous fimbriae, hair-like structures, that facilitate adhesion to host tissues and biofilm formation [4].

Biofilms are well-organized populations of bacteria enclosed in an extracellular matrix made by the bacteria themselves that provide defense against the host immune system and antibiotics [5]. Imagine *P. mirabilis* bacteria teaming up to build tiny fortresses (biofilms) inside the urinary tract, biofilms are slimy shields made of sugar, proteins, and even their own DNA. They protect the bacteria from the host body's natural defenses and even make it tougher to fight with antibiotics. This ability to form biofilms is a key reason why *P. mirabilis* infections can be so persistent and difficult to treat [6]. Biofilm formation facilitates bacterial colonization on host tissues, particularly catheters in hospitalized patients, promoting persistent infections and increasing the risk of recurrent UTIs [6]. Furthermore, the urease activity of *P. mirabilis* within biofilms elevates urinary pH, creating an environment conducive to the formation of struvite stones, which can obstruct urinary flow and exacerbate UTI symptoms [7]. Additionally, biofilms impede the effective diffusion of antibiotics, contributing to the emergence of antibiotic-resistant *P. mirabilis* strains, jeopardizing treatment options, and potentially leading to severe complications [8]. Therefore, understanding the mechanisms of biofilm formation in *P. mirabilis* and its impact on UTI severity remains crucial for developing effective preventive and therapeutic strategies against this challenging pathogen [9].

While *Proteus mirabilis* can develop resistance to various antibiotics, wild-type strains often remain susceptible to a range of antibiotics including ampicillin, amoxicillin-clavulanate, cefazolin, nitrofurantoin, and fluoroquinolones [10]. Biofilm formation in *Proteus mirabilis* is a complex process involving several factors and mechanisms. Adhesion is that *P. mirabilis* possesses fimbriae, a hair-like appendage, to facilitate attachment to host uroepithelial cells. Additionally, curli fibers and extracellular polymeric substances (EPS) contribute to bacterial adhesion and biofilm development [7, 11]. Quorum sensing is a cell-to-cell communication system that allows *P. mirabilis* to sense its population density and regulate genes involved in biofilm formation. The signaling molecules such as acyl-homoserine lactones (AHLs) coordinate biofilm development and expression of virulence factors [10]. The extracellular polymeric substances (EPS) is made up by a complex combination of polysaccharides, proteins, and DNA, which surrounds biofilms. In addition to facilitating nutrient uptake and offering structural support, EPS shields bacteria from environmental stresses such antibiotics [4]. *Proteus mirabilis* can develop resistance to various antibiotics through several mechanisms including efflux pumps that antibiotics are aggressively pumped by these membrane proteins out of the bacterial cell, which lowers antibiotic intracellular concentration and potency [11]; enzymatic modification that certain strains of *P. mirabilis* are capable of generating enzymes that can alter or break down antibiotics, making them ineffective [8]; target modification that mutates the target sites of antibiotics to decrease their binding affinity, leading to resistance [6, 12].

Treatment for UTIs is made more difficult by the advent of *P. mirabilis* strains resistant to antibiotics. A rising public health issue is the rise of multidrug-resistant (MDR) *P. mirabilis* strains, especially those that show robust biofilm formation [10-12]. Many studies have shown that understanding the relationship between biofilm formation in *P. mirabilis* and its antibiotic resistance helps to develop effective treatment strategies. This study investigated the association between antibiotic resistance and biofilm formation in *P. mirabilis* isolates from UTI patients in Hilla city, Iraq. Through antibiotic susceptibility testing and biofilm formation assays, the study aimed to evaluate the prevalence of antibiotic resistance among *P. mirabilis* isolates, determine the ability of these isolates to form biofilms, and explore the potential link between antibiotic resistance and

biofilm formation in *P. mirabilis*.

Materials and methods

Sample collection

Between August and November 2023, 300 urine samples were taken from patients diagnosed with urinary tract infections (UTIs) at hospitals in Hilla City, Iraq including 180 female patients aged from 18–65 years old and 120 male patients aged from 20–70 years old. The UTI diagnosis was made based on the patient's symptoms including dysuria, urgency, frequency, hematuria and the results of the urinalysis including the presence of leukocytosis, nitrites, pyuria.

Proteus mirabilis isolation and identification

Proteus mirabilis bacteria were isolated from urine samples using a standard culture-based method. Briefly, 10 µL of each well-mixed urine sample was inoculated onto MacConkey Agar plate and Blood Agar plate (BD Difco™, Franklin Lakes, New Jersey, USA) and incubated aerobically at 37°C for 24 hours [9]. The MacConkey Agar plate was for the differentiation of lactose-fermenting and lactose-non-fermenting bacteria including *P. mirabilis* that typically appeared as pale or colorless colonies. The blood Agar plate was for the observation of bacterial growth morphology. *P. mirabilis* often exhibited a characteristic swarming motility pattern on blood agar plates. Colonies exhibiting characteristic morphologies on both media plates were subjected to further presumptive identification tests including indole test (Sigma-Aldrich, St. Louis, Missouri, USA) to differentiate bacteria that produce indole from tryptophan and *P. mirabilis* that is typically indole-negative, urease test (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to detect the presence of the urease enzyme produced by *P. mirabilis* to hydrolyze urea into ammonia, motility test (BD Difco™, Franklin Lakes, New Jersey, USA) to assess bacterial motility patterns, citrate utilization test (Sigma-Aldrich, St. Louis, Missouri, USA) to ascertain if a bacteria can use citrate as its only carbon source, while *P. mirabilis* is typically citrate-negative, phenylalanine deamination test (Himedia Laboratories, Mumbai, Maharashtra, India) to detect the ability of *P. mirabilis* to deaminate phenylalanine [2, 10, 11]. Following presumptive identification based on colony morphology and biochemical tests, bacterial isolates were further confirmed using VITEK 2 automatic identification system (BioMérieux, Marcy-l'Étoile, France) following manufacturer's instruction [10].

DNA extraction and UreR gene detection

The bacterial DNA was extracted using DNA Purification Kit (Promega, Madison, WI, USA) following kit's instructions [11]. Conventional polymerase chain reaction (PCR) was applied to confirm that *P. mirabilis* isolates had the UreR gene. The PCR primers were designed based on the sequence of UreR gene (GenBank ID: HI4320) with the forward primer of 5'-GGT GAG ATT TGT ATT AAT GG-3' and reverse primer of 5'-ATA ATC TGG AAG ATG ACG AG-3'. The PCR reaction consisted of 5 µL of 10 ng/µL template DNA, 1 µL of each 10 µM forward and reverse primers, 12.5 µL of Taq PCR master mix ((NEB, Ipswich, MA, USA), and 5.5 µL of nuclease-free water. The PCR was performed using a Biometra TOne Series thermal cycler (Analytik, GmbH. Co, Göttingen, Germany) with the program of 94°C for 4 minutes followed by 40 cycles of 94°C for 40 s, 58°C for 60 s, 72°C for 20 s. Then, 72°C for 4 minutes before stored at 4°C [12]. The PCR products were checked using 1.5% agarose gel electrophoresis.

Antibiotic susceptibility test

The Kirby-Bauer technique was applied in accordance with the recommendations set out by the Clinical and Laboratory Standards Institute (CLSI) (https://clsi.org/) to evaluate the resistance of *P. mirabilis* bacteria to various antibiotics. After *P. mirabilis* isolates were cultured overnight, the bacterial concentration was adjusted to obtain a turbidity standard corresponding to a 0.5 McFarland standard. Commercially pre-made Mueller-Hinton agar (MHA) plates were used for antibiotic susceptibility testing with commercially prepared antibiotic discs including 10 µg of topramycin, 30

µg of amoxicillin/clavulanic acid (AUG), 30 µg of ceftriaxone, 5 µg of ciprofloxacin, 10 µg of gentamicin, 30 µg of nitrofurantoin, 10 µg of imipenem, 10 µg of meropenem, 30 µg of trimethoprim, 30 µg of chloramphenicol, and 30 µg of vancomycin [13]. Following inoculation of the MHA plates with the standardized bacterial suspensions and application of antibiotic discs, the plates were incubated at 37°C for 18-24 hours. The diameters of the inhibition zones surrounding different antibiotic discs were then measured and interpreted using CLSI breakpoints to categorize the *P. mirabilis* isolates as susceptible, intermediate, or resistant to each corresponding antibiotic [14]. The multidrug-resistant (MDR) index for every isolate was calculated as follows to evaluate the extent of MDR.

$$\text{MDR index} = \frac{\text{Number of resistant antibiotics}}{\text{Total number of tested antibiotics}}$$

A MDR phenotype was indicated when the MDR index was greater than 0.2 [14].

Biofilm formation

This study used a modified microtiter plate approach to examine the production of biofilms by *P. mirabilis* [15]. The *P. mirabilis* overnight culture was diluted at 1:100 ratio using freshly prepared Tryptic Soy Broth (TSB) medium supplemented with 1% sucrose (w/v). 100 µL of the diluted bacterial sample was cultured on a sterile, flat bottom 96-well plate at 37°C for 24 h. The media were then aspirated followed by gently wash with sterile phosphate-buffered saline (PBS) to eliminate non-adherent bacteria. The adherent biofilms were then stained with 100 µL of 0.1% crystal violet solution (w/v) (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. The excess crystal violet was removed by rinsing plate with distilled water before drying the plate completely. 100 µL of 95% ethanol was then added to each well to solubilize the bound crystal violet followed by shaking the plate for 15 minutes to guarantee the completely dissolving of the crystal violet. The absorbance of the solubilized dye in each well was determined at 570 nm using SpectraMax i3x multi-Mode microplate reader (Molecular Devices, San Jose, CA, USA).

Statistical analysis

By using SPSS statistical analysis program (IBM, Armonk, New York, USA), the data were analyzed to find relationship between *P. mirabilis* samples according to the resistant of antibiotics and classified as susceptible, intermediate, or resistant to each antibiotic. One-way (ANOVA) and Chi-square tests were used to compare the different absorbance values from the crystal violet staining experiment between groups. The *P* value less than 0.05 was defined as significant difference [16].

Results and discussion

Identification of *P. mirabilis* isolates

This study examined various aspects of 67 *P. mirabilis* isolates obtained from UTI patients' urine samples. Among 300 UTI patients' urine samples, 67 isolates were presumed to be *P. mirabilis* using standard microbiological techniques [16, 17]. The confirmation of *P. mirabilis* was achieved using both VITEK 2 automated identification system and UreR gene, a well-established *P. mirabilis* marker, PCR amplification. The results showed that all presumed *P. mirabilis* isolates displayed characteristic biochemical profiles in VITEK 2 system and produced the expected UreR gene fragment (225 bp), confirming their identity as *P. mirabilis* (Figure 1).

Antibiotic susceptibility and MDR assessment

By compliance with CLSI recommendations and using the Kirby-Bauer disk diffusion assay, the antibiotic susceptibility of *P. mirabilis* isolates was assessed. The overall resistance rates showed the highest for chloramphenicol (41.79%) and trimethoprim/sulfamethoxazole (43.28%), while susceptibility rates were high for ciprofloxacin (88.05%), gentamicin (91.04%), and

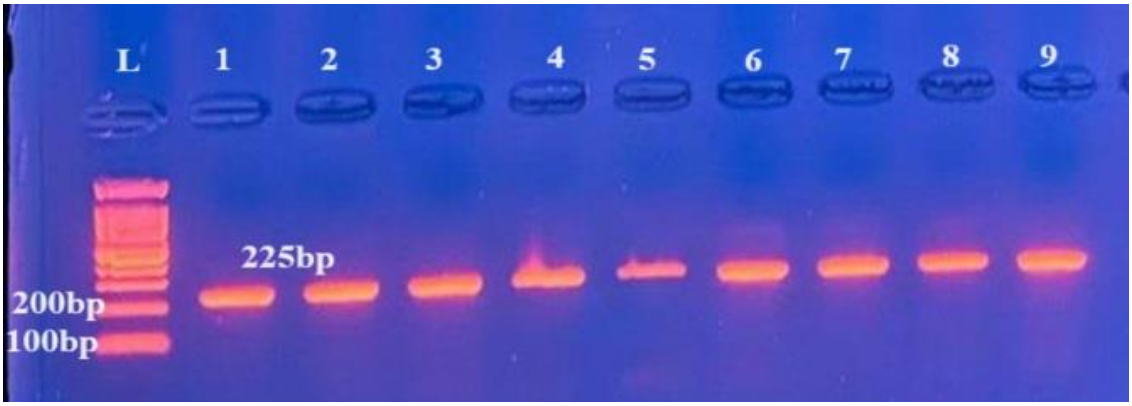

Figure 1. PCR products of *P. mirabilis* isolates using UreR gene-specific primers. Lane L: 100 bp DNA ladder. Lanes 1-9: individual *P. mirabilis* isolates' PCR products showing a single amplicon of the expected size (225 bp).

Table 1. Antibiotic resistance of *Proteus mirabilis* isolates by disc diffusion test.

Antibiotic class	Antibiotic	Case No. and sensitive (%)	Case No. and resistant (%)
Carbapenems	Meropenem (MRP 10 µg)	65 (97.01%)	2 (2.9%)
	Imipenem (IMI 10 µg)	67 (100%)	0 (0%)
Nitrofurans	Nitrofurantoin (F 30 µg)	47 (70.15%)	20 (29.85%)
Cephalosporins	Ceftriaxone (CRO 30 µg)	62 (92.53%)	5 (7.46%)
Chloramphenicol	Chloramphenicol (30 µg)	39 (58.2%)	28 (41.79%)
Beta-Lactams	Amoxicillin/Clavulanic acid (AUG 30 µg)	58 (86.56%)	9 (13.43%)
Sulfonamides	Trimethoprim (TM 30 µg)	38 (56.71%)	29 (43.28%)
Glycopeptides	Vancomycin (VA 30 µg)	46 (68.65%)	21 (31.34%)
Quinolones	Ciprofloxacin (CIP 5 µg)	59 (88.05%)	8 (11.94%)
Aminoglycosides	Gentamicin (GN 10 µg)	61 (91.04%)	6 (8.95%)
	Tobramycin (TOB 10 µg)	58 (86.56%)	9 (13.4%)

tobramycin (86.56%). However, the resistance to carbapenems including meropenem and imipenem remained low as 2.9% and 0%, respectively (Table 1). The multidrug resistance (MDR) index is a commonly used tool to evaluate the extent of antibiotic resistance in an isolate. In this study, a cut-off value of 0.2 for the MDR index was employed and the isolates with an MDR index greater than 0.2 were classified as MDR (Table 2). By applying this criterion, 21 (31.34%) out of 67 isolates were identified as MDR, which aligned with the 34% prevalence reported by Mirzaei *et al*. [18] but differed from the 14.5% lower prevalence observed by Dalia [19].

Table 2. MDR indices of *Proteus mirabilis* (n = 67).

MDR index	Case No. and percentage (%)
0.1	8 (11.94%)
0.2	21 (31.34%)
0.3	11 (16.41%)
0.4	7 (10.44%)
0.5	1 (1.49%)
0.6	1 (1.49%)
0.7	1 (1.49%)
0.8	0.00 (0%)
0.9	0,00 (0%)
1.0	0.00 (0%)

Biofilm formation

67 samples of *P. mirabilis* were investigated for biofilm formation using the established microtiter plate method. All isolates demonstrated biofilm formation, albeit with varying intensities. 27 isolates (34.3%) exhibited strong biofilm formation, while 24 isolates (35.8%) displayed moderate biofilm formation and 20 isolates (29.8%) showed the weakest biofilm formation. The results aligned with previous research on the inherent biofilm-forming propensity of *P. mirabilis* but also revealed significant intraspecific variability in biofilm formation intensity [18, 20, 21]. This observed heterogeneity warranted further investigation into the factors that might influence biofilm formation degree and potentially lead to the development of more targeted strategies for preventing and treating *P. mirabilis* infections [22].

Association between biofilm formation and MDR

The observed antibiotic susceptibility patterns exhibited variations compared to previous studies. The results showed that carbapenem antibiotic appeared low resistance level (< 3%) in this study, which aligned with the findings of Dalia *et al*. for imipenem [20]. However, discrepancies were observed for meropenem compared to the study of Poore *et al*. who reported a higher resistance rate of 4.5% [20]. Similar differences were observed for other antibiotic classes, highlighting the dynamic nature of bacterial resistance. These findings emphasized the importance of continuous monitoring and responsible antibiotic use to prevent resistance emergence. Further investigation into the factors contributing to these variations, including local antibiotic usage patterns and potential clonal dissemination, would be valuable. Further research is needed to identify if there is a relationship between the *P. mirabilis* biofilm development and MDR. The fundamental mechanisms exploration may help create new treatment approaches that address the issue of development of biofilms and antibiotic resistance [8, 10]. This study investigated the antibiotic susceptibility, biofilm formation potential, and MDR profiles of the *P. mirabilis* isolates from urinary tract infections. While carbapenems exhibited promising activity, other antibiotic classes demonstrated variable effectiveness, highlighting the challenges associated with MDR infections treatment [21]. The 100% biofilm formation prevalence combining with varying intensities further complicated treatment strategies. The results suggested the importances of the antibiotic resistant patterns monitoring and *P. mirabilis* ability to form biofilm. This information could be used to guide empirical therapy selection and inform public health interventions [20]. Further research should delve into the genetic determinants and environmental factors contributing to MDR and biofilm formation. Exploring alternative therapeutic approaches, such as targeting virulent factors or disrupting biofilm formation, holds potential for preventing and combating these complex challenges [22].

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