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Article

Study the Association of ureA Gene with Biofilm Production and Multidrug Resistance in Proteus Mirabilis Uropathogenic Isolates

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Abstract: Introduction: Proteus mirabilis is a clinically relevant uropathogen due to its urease production, biofilm forming ability, and developing antimicrobial resistance that contribute to poor treatment outcomes of urinary tract infections (UTIs). Objectives: This study investigates the association of ureA gene, biofilm formation and multi-resistance among uropathogenic P. mirabilis isolates. Materials and Methods: 215 Urine samples were obtained from female patients who were diagnosed later with urinary tract infections between September 2025 and February 2025 who attended to Al-Nasr Hospital for Obstetrics and Gynecology, Kirkuk, Iraq. Isolate, Biochemical identification, VITEK 2 identification, antimicrobial susceptibility testing, Biofilm measurement, PCR detection of ureA gene. Results: Out of 215 samples, 17 isolates (7.9%) were confirmed as P. mirabilis. Antibiotic resistance was highest against piperacillin (100%), amoxicillin (88.2%), and colistin (64.7%), while highest susceptibility was observed for meropenem (94.1%) and imipenem (88.2%). MDR accounted for 58.8% (10/17), XDR for 5.9% (1/17), and non-MDR for 35.3% (6/17). Biofilm analysis showed weak (41.2%), moderate (23.5%), strong (5.9%), and non-producers (29.4%). The ureA gene was detected in 76.5% (13/17) of isolates. A clear association was observed between MDR and biofilm formation, particularly in weak and moderate biofilm producers. Conclusions: P. mirabilis isolates were highly resistant to antimicrobials, formed a considerable amount of biofilm and possessed a high frequency of ureA gene, suggesting a close association between virulence and resistance traits.

Keywords: Proteus Mirabilis, ureA Gene, Biofilm, MDR, Urinary Tract Infection.

Introduction

UTIs due to the presence of uropathogenic strains of Proteus mirabilis continue to be important clinical challenges, especially in catheterized patients, because Proteus mirabilis can persist and cause recurrent infections involving multiple virulence factors [1]. Pathogenicity of this particular organism is directly related to the hierarchical expression of virulence genes that promote survival in ESBL conditions and biofilm development, along with swarming motility [1], [2]. The formation of biofilm is a key step in the development of chronic infection, providing structural protection and increasing

resistance to antibiotics and host immune responses. Recent evidence supports the idea that the genotypic repertoire of P. mirabilis is strongly associated with the regulation of pathogenic behavior as biofilm formation and swarming motility genome are associated with virulence genes [2]. In addition, biofilm gene expression has been associated with antimicrobial resistance, suggesting a direct association between resistance and virulence pathways [3]. There is an increasing concern regarding antimicrobial resistance in P. mirabilis from a clinical perspective. Higher resistance rates in catheter associated urinary tract infection isolates have been observed when compared to non-catheter associated urinary tract infection in studies designed to determine the role of medical devices in selecting for resistant strains [4]. Furthermore, the phenotypic and molecular characterization of clinical isolates has shown extensive heterogeneity in virulence gene profiles, patterns of antimicrobial susceptibility, and methods of strain typing [5]. Molecularly, urease is one of the key virulence factors of P. mirabilis. This enzyme, also indirectly promotes urinary alkalinization, stone formation and also enhance the biofilm stability. In addition to its enzyme activity, urease has also been shown to have non-enzymatic functions important for bacterial pathogenicity in the host and with immune modulation [6], [7]. Mutations in urease regulatory genes such as ureR have been identified in clinical isolates, confirming the genetic basis of urease-mediated virulence [8]. It has been widely acknowledged that the virulence and antimicrobial resistance are related. An essential association had been observed between multidrug resistance and virulence factor expression in P. mirabilis, and strong virulent strains were likely to be more resistant to antibiotics [9]. Additionally, the detection of extended-spectrum β-lactamase (ESBL), AmpC, and carbapenemase-producing isolates makes the therapeutic difficulty more profound [10]. Recent surveillance studies have shown that multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains are increasingly isolated in clinical samples [11]. The pathogenic power of P. mirabilis is also increased by polymicrobial interactions. Since enteric infection is commonly a complex microbial community rather than a pure culture, Enterococcus faecalis synergies with uropathogenic Escherichia coli to enhance biofilm development and subsequent invasion of the urinary tract [12]. Recent investigations in the treatment of virulence, instead of bacterial survival. Studies have reported that protocatechuic acid inhibits key biological processes relevant for biofilm formation and bacterial persistence [13]. Likewise, there are also anti-inflammatory agents like diclofenac and ketorolac that have a stronger inhibitory effect on urease activity and P. mirabilis biofilm formation [14]. Furthermore, green-synthesized nanoparticles such as zinc, selenium, and zinc-selenium composites have exhibited excellent antibacterial and antibiofilm effects [15]. Lastly, bacteriophage therapy has been repurposed as a potential candidate, as certain phages have been shown to be capable of breaking established biofilms [16]. Proteus mirabilis is a remarkably adaptable uropathogen, with strong biofilm-forming ability, redundant virulence determinants, and growing antimicrobial resistance. It is an important public health problem with limited effective control despite greater understanding of its pathogenic mechanisms. Hence, additional molecular studies are necessary to define the genetic basis of virulence and resistance. Thus, this study was aimed to determining the relationship of the ureA gene with biofilm formation and multidrug resistance of uropathogenic P. mirabilis isolates.

Materials and Methods

Study Design and Sample Collection

The data used were obtained from a prospective cross-sectional study of 215 urine specimens collected from female patients clinically diagnosed with UTIs. The samples were taken from the patients attending Al-Nasr Hospital for Obstetrics and Gynecology, Kirkuk–Iraq, from September of 2025 to February of 2026. Aseptic collection of mid-stream urine specimens in sterile and leak proof containers following standard microbiological methods.

Exclusion Criteria

Urine specimens were excluded if patients had been treated with antimicrobial therapy in the previous 72 h before sample collection, if specimens were contaminated by a mixture of bacteria (so

that an accurate identification could not be made) or if the sample was unsuitable for microbiological analysis.

Isolation and Identification of *Proteus mirabilis*

urine samples were cultured on MacConkey agar plates, with calibrated loops; these plates were incubated aerobically at 37°C for 18–24 h, and after incubation, colonies were investigated concerning their cultural and morphological characteristics. Conventional microbiological and biochemical tests were performed for the initial identification of bacterial isolates including Gram staining, catalase, oxidase, urease production, motility test, indole production, methylene red, VP, and citrate utilization. Pure cultures were preserved in nutrient agar slants at 4 °C for subsequent experiments.

Identification Using the VITEK 2 Compact System

VITEK 2 Compact System (bioMérieux, France) with GN identification cards was used to confirm presumptively identified Proteus mirabilis isolates following the manufacturer's instructions. Therefore, only isolates confirmed as Proteus mirabilis were included in the following analyses.

Antimicrobial Susceptibility Testing

Based on the recommendations of Clinical and Laboratory Standards Institute, antimicrobial susceptibility testing was carried out using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar [17]. In short, 0.5 McFarland turbidity standard-equivalent bacterial suspensions were prepared for uniform inoculation on Mueller–Hinton agar plates. Agar disk diffusion susceptibility testing was performed on Mueller–Hinton agar plates incubated aerobically at 37 °C for 18–24 h after placing antibiotic discs on the agar surface, and interpreted as susceptible, intermediate, or resistant based on CLSI guidelines.

Biofilm Formation Assay

Biofilm production was determined using the microtiter plate biofilm assay. Bacterial inoculumFresh cultures were adjusted to 0.5 McFarland standard and further diluted in 1:100 in tryptic soy broth with 1% glucose. Each were inoculated with 200 µLaliquots of the bacterial suspension into sterile 96-well flat-bottom microtiter plates and incubated at 37°C for 24 h, after which, they were washed three times with phosphate-buffered saline (PBS) to remove unattached cells and were left to air dry. Adhesion biofilm was stained with 0.1% crystal violet for 15 min. Unbound dye was washed away with distilled water and bound dye was solubilized with 95% ethanol. Microplate reader was utilized to determine the optical density (OD) at 570 nm. According to the OD values obtained, isolates could be classified as non-biofilm producer, weak, moderate or strong biofilm producers using the cut-off value method [18][19].

Genomic DNA Extraction

Genomic DNA from confirmed Proteus mirabilis isolates was extracted using Geneaid Genomic DNA Extraction Kit (Geneaid Biotech. Ltd., Taiwan) following the manufacturer's protocol. Bacterial cells were lysed with a lysis buffer and proteinase K and the DNA was adsorbed onto a silica membrane column. DNA was purified by sequential washes to remove contaminants and cellular debris followed by elution in nuclease-free elution buffer. The concentration and purity of isolated DNA were determined on a NanoDrop spectrophotometer according to the A260/A280 ratio. DNA samples were preserved in −20°C till molecular analysis was performed.

PCR Amplification of the *ureA* Gene

The presence of the *ureA* gene was detected by conventional polymerase chain reaction (PCR) using specific primers targeting the *ureA* gene of *Proteus mirabilis* (Table 1). PCR amplification was performed in a total reaction volume of 25 µL containing 12.5 µL of 2× PCR Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 2 µL of template DNA, and 8.5 µL of nuclease-free water (Table 2). The amplification program consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 46.5°C for 45 s, and extension at 72°C for 45 s. A final extension step was carried out at 72°C for 7 min to ensure complete amplification of PCR products

(Table 3). The amplified products were analyzed by agarose gel electrophoresis. The presence of a DNA fragment of 400 bp was considered positive for the *ureA* gene.

Table 1. Primer sequences used for amplification of the *ureA* gene.

Primer	Sequence (5′–3′)	Amplicon size (bp)	Ref.
ureA-F	GTTGCAGAAAGACGTTTAG	400	19
ureA-R	AGGTGAGTGAATTGAAACC		

Table 2. PCR reaction mixture (25 μL).

Component	Volume (μL)
2× Master Mix	12.5
ureA Forward Primer	1.0
ureA Reverse Primer	1.0
Template DNA	2.0
Nuclease-free Water	8.5
Total	25

Table 3. PCR thermal cycling conditions for amplification of the *ureA* gene.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 s	30
Annealing	46.5	45 s	30
Extension	72	45 s	30
Final extension	72	7 min	1

Agarose Gel Electrophoresis

PCR products were resolved on 1.5% agarose gel, casting in 1× TBE buffer and subjected to electrophoresed at 90–100 V for ~60 min. The gel was stained with a nucleic acid staining dye and visualized under ultraviolet illumination. Molecular size markers were a 100 bp DNA ladder. A unique 400 bp band suggested that the *ureA* gene was successfully amplified.

Results

Isolation and Identification of *Proteus mirabilis*

A total of 215 urine samples were collected from female patients diagnosed with urinary tract infections during the study period. After culture and identification procedures, 17 isolates were confirmed as *Proteus mirabilis*. The overall isolation rate of *P. mirabilis* was 7.9% (17/215), while 198 samples (92.1%) were negative for *P. mirabilis*, Table 4

Table 4. Distribution of *Proteus mirabilis* among urine samples.

Result	No. of Samples	Percentage (%)
Positive (*P. mirabilis*)	17	7.9
Negative	198	92.1
Total	215	100

Following primary isolation on MacConkey agar, Proteus mirabilis isolates exhibited characteristic pale, non-lactose-fermenting colonies. These colonies are typically smooth to slightly irregular in appearance and may show early swarming behavior under suitable conditions, Figure 1.

Figure 1. Colony of *P. mirabilis* on MacConkey agar after incubation at 37°C for 24 hours.

The biochemical profile of the isolates showed Gram-negative rods with positive catalase, urease, motility, methyl red, and citrate utilization tests, while oxidase, indole, and Voges–Proskauer tests were negative, table 5. Confirmation of the preliminary identification was subsequently performed using the VITEK 2 Compact system (bioMérieux, France), which verified all tested isolates as Proteus mirabilis with high confidence according to the manufacturer's identification criteria.

Table 5. Results of biochemical and phenotypic tests.

Test	Result
Gram staining	−
Catalase	+
Oxidase	−
Urease	+
Motility	+
Indole	−
Methyl Red (MR)	+
Voges–Proskauer (VP)	−
Citrate utilization	+

Antibiotic Susceptibility

Antibiotic susceptibility testing of the 17 Proteus mirabilis isolates revealed variable resistance and sensitivity patterns across the tested antimicrobial agents. Amoxicillin showed a high resistance rate of 15 isolates (88.2%), with only 2 isolates (11.8%) being susceptible. Ceftazidime demonstrated moderate activity with 6 isolates (35.3%) susceptible, 1 isolate (5.9%) intermediate, and 10 isolates (58.8%) resistant. Tobramycin exhibited relatively high effectiveness, with 13 isolates (76.5%) susceptible and only 3 isolates (17.6%) resistant. Gentamicin showed reduced activity with 6 isolates (35.3%) susceptible and 9 isolates (52.9%) resistant. Piperacillin demonstrated complete resistance, with all isolates (100%) showing resistance. Aztreonam showed moderate resistance with 10 isolates (58.8%) resistant and 7 isolates (41.2%) susceptible. Cefoperazone showed intermediate susceptibility patterns with 5 isolates (29.4%) susceptible, 3 isolates (17.6%) intermediate, and 9 isolates (52.9%) resistant. Amikacin demonstrated high effectiveness with 14 isolates (82.4%) susceptible and only 1 isolate (5.9%) resistant. Carbapenems showed the highest antimicrobial activity. Imipenem was effective against 15 isolates (88.2%), while Meropenem showed the highest susceptibility rate with 16 isolates (94.1%) susceptible and only 1 isolate (5.9%) resistant. Ciprofloxacin showed moderate susceptibility with 7 isolates (41.2%) susceptible and 9 isolates (52.9%) resistant. Colistin exhibited relatively low activity, with 4 isolates (23.5%) susceptible and 11 isolates (64.7%) resistant, Table 6.

Table 6. Antibiotic susceptibility patterns of *Proteus mirabilis* isolates (n=17).

Antibiotic	Susceptible S	Intermediate I	Resistant R
Amoxicillin	2 (11.8%)	0 (0%)	15 (88.2%)
Ceftazidime	6 (35.3%)	1 (5.9%)	10 (58.8%)
Tobramycin	13 (76.5%)	1 (5.9%)	3 (17.6%)
Gentamicin	6 (35.3%)	2 (11.8%)	9 (52.9%)
Piperacillin	0 (0%)	0 (0%)	17 (100%)
Aztreonam	7 (41.2%)	0 (0%)	10 (58.8%)
Cefoperazone	5 (29.4%)	3 (17.6%)	9 (52.9%)
Amikacin	14 (82.4%)	2 (11.8%)	1 (5.9%)
Imipenem	15 (88.2%)	1 (5.9%)	1 (5.9%)
Meropenem	16 (94.1%)	0 (0%)	1 (5.9%)
Ciprofloxacin	7 (41.2%)	1 (5.9%)	9 (52.9%)
Colistin	4 (23.5%)	2 (11.8%)	11 (64.7%)

The antimicrobial resistance profiling of the 17 *Proteus mirabilis* isolates revealed a variable distribution of resistance patterns. Multidrug-resistant (MDR) isolates represented the majority, accounting for 10 isolates (58.8%). Extensively drug-resistant (XDR) isolates were observed in 1 isolate (5.9%), while no pandrug-resistant (PDR) isolates (0%) were detected. On the other hand, 6 isolates (35.3%) were classified as susceptible or non-MDR, Table 8.

Table 7. Resistance profile of *Proteus mirabilis* isolates.

Resistance profile	Number of isolates	Percentage (%)
MDR	10	58.8%
XDR	1	5.9%
PDR	0	0%
Susceptible / non-MDR	6	35.3%

Biofilm Formation

The biofilm formation assay revealed that the 17 *Proteus mirabilis* isolates exhibited different levels of biofilm production. The majority of isolates were weak biofilm producers, accounting for 7 isolates (41.2%). Moderate biofilm production was observed in 4 isolates (23.5%), while strong biofilm formation was detected in only 1 isolate (5.9%). In contrast, 5 isolates (29.4%) showed no biofilm-forming ability, Table 8

Table 8. Biofilm formation profile of *Proteus mirabilis* isolates.

Biofilm category	**Number of isolates**	**Percentage (%)**
Strong	1	
Moderate	4	
Weak	7	
Non-producer	5	

The relationship between biofilm formation and antimicrobial resistance profiles of the 17 Proteus mirabilis isolates is summarized in Table 9. Among the strong biofilm producers (n = 1, 5.9%), all showed MDR (1, 5.9%). In the moderate biofilm group (n = 4, 23.5%), 3 isolates (17.6%) were MDR and 1 isolate (5.9%) was XDR. In weak biofilm producers (n = 7, 41.2%), 5 isolates (29.4%) were MDR and 2 isolates (11.8%) were susceptible/non-MDR. Non-biofilm producers (n = 5, 29.4%) included 1 MDR isolate (5.9%) and 4 susceptible/non-MDR isolates (23.5%). Overall, MDR accounted for 10 isolates

(58.8%), XDR for 1 isolate (5.9%), while no PDR isolates (0%) were detected, and 6 isolates (35.3%) were susceptible/non-MDR, Table 9.

Table 9. Relationship between biofilm formation and antimicrobial resistance profiles of *Proteus mirabilis* isolates.

Category	MDR	XDR	PDR	Susceptible	Total
Strong biofilm	1	0	0	0	1 (5.9%)
Moderate biofilm	3	1	0	0	4 (23.5%)
Weak biofilm	5	0	0	2	7 (41.2%)
Non-producer	1	0	0	4	5 (29.4%)
Total	10 (58.8%)	1 (5.9%)	0 (0%)	6 (35.3%)	17 (100%)

Molecular Detection of ureA Gene

Molecular analysis using PCR demonstrated that the ureA gene was detected in 13 out of 17 Proteus mirabilis isolates (76.5%), whereas 4 isolates (23.5%) showed no amplification of the target gene, Figure 2. This indicates a high prevalence of the ureA gene among the clinical urinary isolates included in the present study.

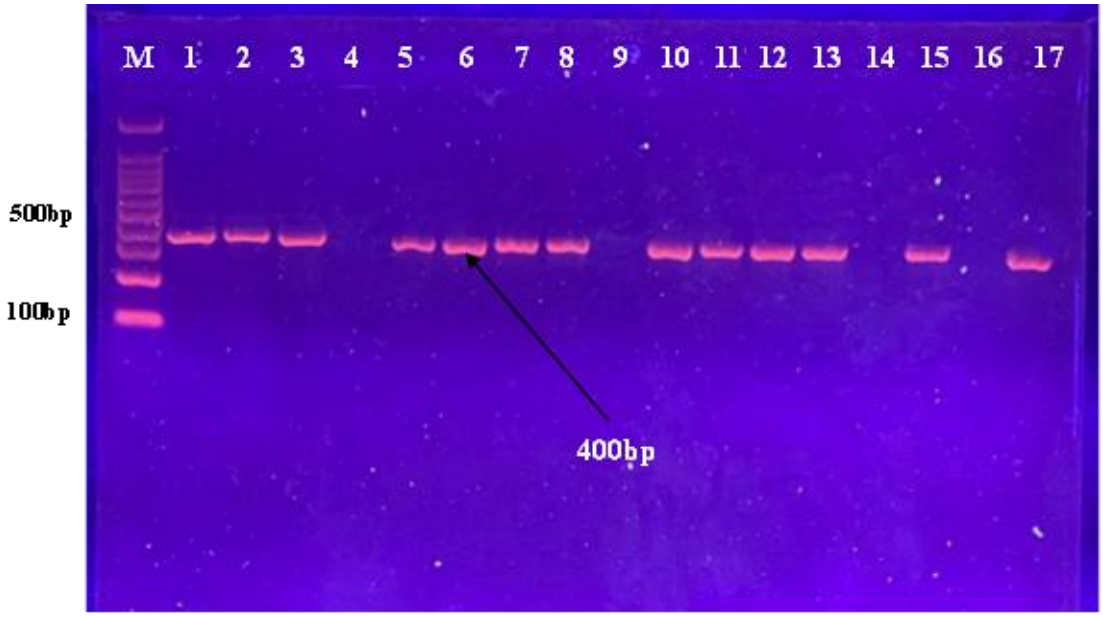

Figure 2. PCR amplification of the *UreA* gene using 1.5% agarose gel electrophoresis at 75 V, showing a 504 bp product, M: 100bp Ladder, 1-17: bacterial isolates.

Discussion

This study showed that Proteus mirabilis were isolated from 17 of 215 urine samples with a total prevalence of 7.9%. Yaseen et al. [20] reported a similar but higher prevalence of P. mirabilis isolates from urinary tract infections in Iraq, isolation rate was ~12.5%, which is higher than the prevalence found in the current study (7.9%). The effect may be due to differences in case selection because they included both complicated and uncomplicated cases and the current one only included female patients. In a similar manner, Al-khalidy and Aburesha [21] reported close to 15.8% isolation rate of P. mirabilis among urinary tract infection isolates in Iraqi clinical settings, which is almost two times higher than that found in the present work. In contrast, Badi et al. [22] described low and variable rates of P. mirabilis detection (5% and 10%) depending on the type of clinical sample and co-infection status.

These values closely mirror the results of the current study (7.9%) and also support the notion that the prevalence of P. mirabilis varies dramatically between populations and clinical conditions. Furthermore, AL-Ezzy et al. [23] referred that P. mirabilis was responsible for about 10.3% of urinary tract infection cases, with the rates varying significantly depending on the population demographics and the clinical presentation. The current study showed high antimicrobial resistance of the Proteus mirabilis isolates were found to piperacillin (100%) and amoxicillin (88.2%). Also, a significant part exhibited increased levels of calve ceftazidime(hand samples41(58/8%), imipenem50%and colistin 64.7identical hand). In comparison, the lowest susceptibility rates were found for meropenem (94.1%), imipenem (88.2%) and amikacin(82.4%). Trivedi et al. [24] showed lower resistance rates in P. mirabilis, and also reported low β-lactam resistances from 40–70%, however the aminoglycosides including amikacin over exceed 80% of susceptibility rate values. These findings are partially in line with the present study concerning aminoglycoside effectiveness but, conversely, yielded a more pronounced resistance to some β-lactams being observed here showing an increasing trend of microbial resistance. Al-Azawy et al. [25] demonstrated resistance rates exceeding 75% for amoxicillin and greater than or equal to60%for cephalosporins among clinical P. mirabilis isolates. These values are comparable to present results (amoxicillin 88.2% and ceftazidime 58.8%) indicating an agreement in general patterns of very high β-lactam antibiotic resistance rate. Pal et al. [26] In a cohort analysis, they observed 50% to 70% prevalence of MDR and up to 5–10% XDR in specific patient settings out of Proteus species. This finding is in accordance with the present study which demonstrated that 58.8% of strains were MDR and 5.9% XDR, supporting an increase worldwide of resistant Proteus. A study by Al-Bassam et al. [27] showed P. mirabilis isolates could be resistant to ampicillin-class antibiotics (70%) with moderate susceptibility against aminoglycosides that is 60-85% susceptible. These findings are consistent with the current study, especially for high aminoglycoside activity (amikacin 82.4% & tobramycin 76.5%). Biofilm formation and antibiotic resistance was found to be highly correlated with nearly 55–65% of biofilm producers being shown as MDR isolates [28]. This is in pretty close agreement with present study, which again had MDR as predominant (58.8%) among biofilm forming isolates confirming its role of biofilm per se as a factor to contribute for antimicrobial resistance. Abebe [29] highlighted that biofilm forming bacteria are 10–1000 times more resistant to antibiotics than planktonic cells, which explains the higher resistance of all isolates and specifically those from biofilms in our study. Sun et al. [30] showed that MDR rates in biofilm-forming P. mirabilis isolates were as high as 60% with a higher intensity of adherence associated with increasing resistance levels. This corresponds with the current results, where MDR was most frequently detected amongst weak to moderate biofilm producers. Bitar et al. [31] referred that Carbapenem susceptibility of P. mirabilis was reported to be 85–95% which closely corresponds with the results from the present study (meropenem 94.1% and imipenem 88.2%) indicating continued activity of carbapanems against this organism during our surveillance period. Stepanović et al. [32], referred that the most clinical isolates was classified as weak and moderate defined biofilm producers, with the predominant being weak producer (~35–45%) which consistent with our findings. In this study, the ureA gene was identified in 13 of the 17 isolates of Proteus mirabilis, accounting for 76.5%. D'Orazio et al. In a recent study, [33] showed that urease gene expression was thoroughly regulated by a transcriptional activator (UreR) in P. mirabilis, supporting the notion that regulation of UreR activity and expression is a main genetic control point for urea-dependent promoters that is important for both urease activity and virulence. This consensus reflects the prominent presence of ureA that was found in the present work since urease-related genes are critical for survival of bacteria in urinary tract environments. The current results (76.5%) are fairly comparable to these estimates, with previous results conducted by Al-Hamdani and Al-Hashimy [34] detected urease-associated genes in urinary isolates of P. mirabilis with a detection rate of nearly 70–80%. This resemblance indicates that genetic determinants related to urease are probably distributed stably among the clinical isolates in Iraq. Concordant with this study, Al-Obaidi and Al-Hashimy [35] have shown that the ureA gene and other virulence genes like hpmA were detected in 75% of urinary P. mirabilis isolates, which corresponds with our finding (76.5%). Finally, these data draw increasing support for ureA as a conserved virulence factor in clinical isolates. The prevalence of urease regulatory genes (ureR) is much lower in P. mirabilis isolates obtained from urinary tract infections, as shown by

Al-Fahham and Kareem (8) with detection rates of about 65–70%. The greater incidence in the present study may be an indication of: differences in target genes (either ureA or ureR); differences in study sample size; and/or differences in geographical variables. Additionally, Shameel and Alkhafaji [19] detected ureA gene among clinical P. mirabilis isolates (around 78%) which is close to the present findings (76.5%). This confirms the role of ureA as a common virulence trait of uropathogenic isolates.

Conclusion

The findings of the current work affirm the role of P. mirabilis as an important uropathogen with a high level of antimicrobial resistance and biofilm formation capacity. The elevated frequency of the ureA gene (76.5%) indicates its essential role in urinary colonization and pathogenesis. The association of biofilm formation with multidrug resistance justifies the need to use improved therapeutic strategies and molecular surveillance in clinical settings.

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