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Evaluation of Biofilm Formation and Distribution of ureC, LuxS, flaA, and aac(6')-Ib Genes in Proteus Mirabilis Isolated from Urinary Tract Infections

Conflict of interest: nothing to declare.
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Abstract

Introduction. Proteus mirabilis is an opportunistic pathogen associated with a variety of infections in humans, especially those in the urinary tract.
Purpose. To detect a number of Proteus mirabilis virulence genes and evaluating their ability to form a biofilm.
Materials and methods. A total of 250 samples were collected from urinary tract infections. Samples were obtained for patients of different ages were involved of both sexes; All of the isolates were diagnosed by different laboratory methods. Biofilm formation assay by Crystal violet was performed to evaluate the ability of proteus mirabilis to form a biofilm.
Results. All proteus mirabilis isolates were biofilms producers, 8 (32%) isolates produced weak biofilm, 15 (60%) of isolates produced moderate biofilm and 2 (8%) isolates produced strong biofilm. The findings of virulence genes showed the highest gene detected in P. mirabillis isolated from UTI patients were LuxS gene 25 (100%), followed by both UreC gene and aac(6)Ib gene 23 (92%). Whereas the lowest isolated gene was Fla A gene 8, 32%.
Conclusion. Proteus mirabilis have the ability to cause urinary tract infections and CAUTIs because it possesses many virulence factors that help it to cause infection, increase their resistance to antibiotics as well as the ability to form biofilms on biotic & abiotic materials. Thus increasing the pathogenicity and making it difficult to treat.
Keywords: P. mirabilis, UTI, Biofilm, virulence factors gene ureC, flaA, luxS, aac(6')Ib

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Оценка распределения генов ureC, LuxS, flaA и aac(6′)-Ib и их способности к образованию биопленок у Proteus mirabilis, выделенных из очагов инфекции мочевыводящих путей

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Резюме

Введение. Proteus mirabilis – условно-патогенный микроорганизм, являющийся возбудителем различных инфекций у человека, среди которых наиболее часто встречаются инфекции мочевыводящих путей.
Цель. Выявление ряда генов вирулентности Proteus mirabilis и оценка их способности к образованию биопленки.
Материалы и методы. Всего было получено 250 образцов, выделенных из очагов инфекции мочевыводящих путей. Образцы отбирали у пациентов различного возраста, обоих полов; все изоляты идентифицировали с использованием различных лабораторных методов. Для оценки пленкообразующей способности Proteus mirabilis был проведен тест на формирование биопленки методом окрашивания генцианом фиолетовым.
Результаты. Все изоляты Proteus mirabilis продуцировали биопленки, причем 8 (32%) изолятов были слабыми продуцентами биопленки, 15 (60%) изолятов – умеренными и 2 (8%) изолята – сильными продуцентами биопленки. Результаты исследования генов вирулентности показали, что самым распространенным геном, выявленным у P. mirabillis, выделенных от пациентов с ИМП, был ген LuxS – 25 (100%), за которым следовали ген UreC и ген aac(6′)Ib – 23 (92%). Наименее распространенным геном был ген flaA – 8 (32%).
Заключение. Proteus mirabilis вызывают инфекции мочевыводящих путей и катетер-ассоциированные инфекции мочевыводящих путей (CAUTI), поскольку они экспрессируют ряд факторов вирулентности, способствующих возникновению инфекции, повышению резистентности к антибиотикам и усилению продуцирования биопленок на биотических и абиотических материалах. Как следствие, повышается их патогенность и затрудняется процесс лечения.
Ключевые слова: P. mirabilis, ИМП, биопленка, гены вирулентности, ureC, flaA, luxS, aac(6′)Ib

■ INTRODUCTION

Proteus spp. is a motile, dimorphic, Gram-negative bacterium of the order Enterobacterales [1]. Proteus mirabilis is a well-known rod-shaped Gram-negative bacterium that swarms across agar plates to generate distinctive bull's eye-shaped motility. It is a member of the class Gammaproteobacteria and family Enterobacteriaceae [2]. Its ability to transform from short rods into long, multinucleate swarmer cells expressing thousands of flagella has intrigued scientists for many years [3] Proteus mirabilis is linked to a number of human illnesses, including infections of the gastrointestinal tract, wounds, eyes, and UTIs, particularly catheter-associated urinary tract infections (CAUTI) [4]. The severity of any infection caused by the members of the genus Proteus depends mainly on the availability of virulence factors that may include β- Lactamase, extended-spectrum β-lactamases, Protease, Urease and hemolysin production other factors like swarming motility, adhesion and biofilm formation are also important. All these factors collectively or separately play important roles in the pathogenicity and pathogenesis of disease accordingly [5]. UTI is primarily caused by the presence and growth of bacteria in the urinary system, which represents the most prevalent infection in all age groups [6]. P. mirabilis infection of the upper urinary tract can result in sepsis, bacteremia, urolithiasis, and permanent kidney damage [7]. It predominantly forms apatite and struvite (CaPO4 and MgNH3PO4) crystals, which inhibit urine flow [8]. The symptoms of P. mirabilis infections, including bacteriuria, acute pyelonephritis, catheter occlusion, and fever, may progress to bacteremia and sepsis, resulting in a greater fatality rate compared to other infections [9]. P. mirabilis possesses a high biofilm-forming capability [10]. Proteus mirabilis biofilms have recently become a source of growing concern [11]. P. mirabilis coordinates an increase in the production of numerous virulence factors, including Urease, the most significant enzyme in the creation of kidney and bladder stones. The (ureRDABCEFG) genes on the ure operon are responsible for the synthesis of the urease enzyme, while ureC is the main gene responsible for urease synthesis [12]. The motility of Protues mirabilis is a result of its flagella, which not only aid in colonization but have also been linked to its ability to form biofilms and resistance to host defenses and specific medications. Flagella is encoded by flaA gene [13]. LuxS enzyme synthesis autoinducers (AI-2) are the most prevalent and common kind of Quorum sensing (QS) in Gram-negative bacteria. LuxS enzyme encoded by luxS gene [14]. In the past few decades, clinical strains of P. mirabilis have developed greater resistance to antimicrobial medicines [15] aminoglycosides resistance AME (aminoglycoside modifying enzymes), and 16S rRNA methylase genes. Enzymatic inactivation by producing AMEs is the most prevalent mechanism against aminoglycosides, followed by methylation of 16S rRNA, which confers a high level of resistance to tobramycin, amikacin, and gentamicin [16].

■ PURPOSE OF THE STUDY

To detect a number of Proteus mirabilis virulence genes and evaluating their ability to form a biofilm.

■ MATERIALS AND METHODS

Samples collection

A total of 250 samples were collected from patients with UTI infections attendance at hospitals and private clinics in Thi-Qar province. The samples were immediately cultured on bacteriological media and incubated at 37 °C for 24 hrs.

Biofilm formation assay by Crystal violet

A 96-well microtiter plates with Brain heart infusion broth (HiMedia) were employed to detect the development of biofilms. Semi-quantitative evaluations of biofilm development were made according to [17] with some modifications Briefly, the overnight culture of the isolates was diluted 1:100 in BHI broth medium and cultured in polystyrene microtiter 96-well plates. Then, the plates were incubated for 1 day at 37°C. Following this, the plates were washed three times with distilled water (D.W.) and dyed with 0.1% (w/v) crystal violet for 20 mins at room temperature (RT). Then, 200 µL of absolute ethanol was added to the wells to measure their absorbance with an Enzyme-linked immunosorbent assay (ELISA) reader at wavelength 630 nm. The results were classified as follows: non-biofilm, weak biofilm, moderate Biofilm, and strong biofilm according to optical density of microorganisms' cells. The results were interpreted as follows: if OD ≤ODc had no biofilm development, ODc < OD ≤2 ×ODc had weak biofilm, 2 ×ODc < OD ≤4 ×ODc had moderate biofilm and OD > 4 × ODc had high biofilm.

Extraction of the bacterial DNA

Genomic DNA was extracted from p.mirabilis Culture isolates by using Geneaid Genomic DNA extraction Kit (Taiwan) and after this process, to ensure the DNA was extracted, and its quality was tested, the samples were run on a 1.5% gel agarose.

Statistical Analysis

The data of the present study were statistically analysis by using SPSS (Statistical Package of Social Science version 26) based in using both descriptive and non-parametric Chi-Square at p. value <0.05.

■ RESULTS

A total of 250 samples, 25 (13.89%) were P. mirabilis. The results demonstrated that all isolates of Proteus mirabilis (n=25 100%) were biofilm forming. Biofilm production is 8 (32%) isolates of Proteus mirabilis produce weak biofilm, 15 (60%) of isolates moderate biofilm and 2 (8%) isolates strong biofilm (Fig. 1). The present study showed the highest gene detected in P. mirabillis isolated from UTI patients were LuxS gene (Fig. 3) and gyrA

Table 1
Primers and the molecular size of the PCR product in the study

No.	Gene	Primer Sequence[3] to[5]	Product size Annealing	Reference
1	ureC	F-GTTATTCGTGATGGTATGGG	316 bp 52 C	[18] [19]
		R-GTAAAGGTGGTTACGCCAGA		
2	LuxS	F-GTATGTCTGCACCTGCGGTA	464 bp 55 C	[20] [21]
		R-TTTGAGTTTGTCTTCTGGTAGTGC		
3	flaA	F-AGGATAAATGGCCACATTG	417 bp 55 C	[22]
		R-CGGCATTGTTAATCGCTTTT		
4	aac(6')-Ib	F-CCCGCTTTCTCGTAGCA	500 bp 55 C	[16] [19]
		R-TATGAGTGGCTAAATCGAT		

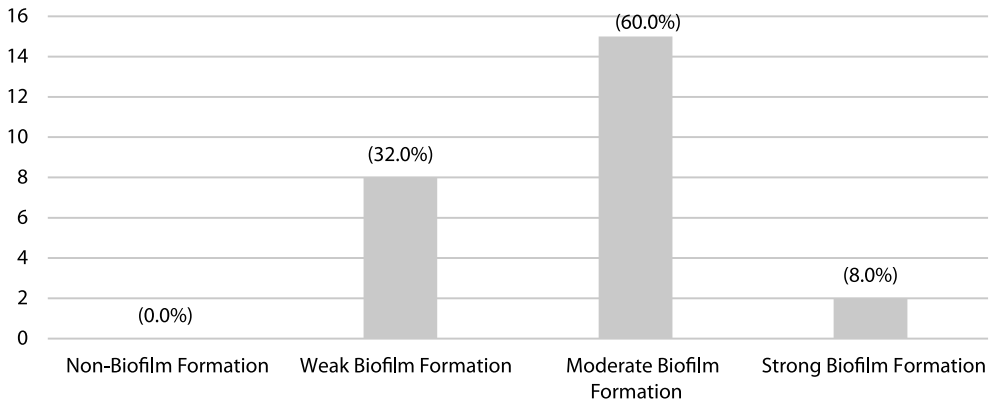
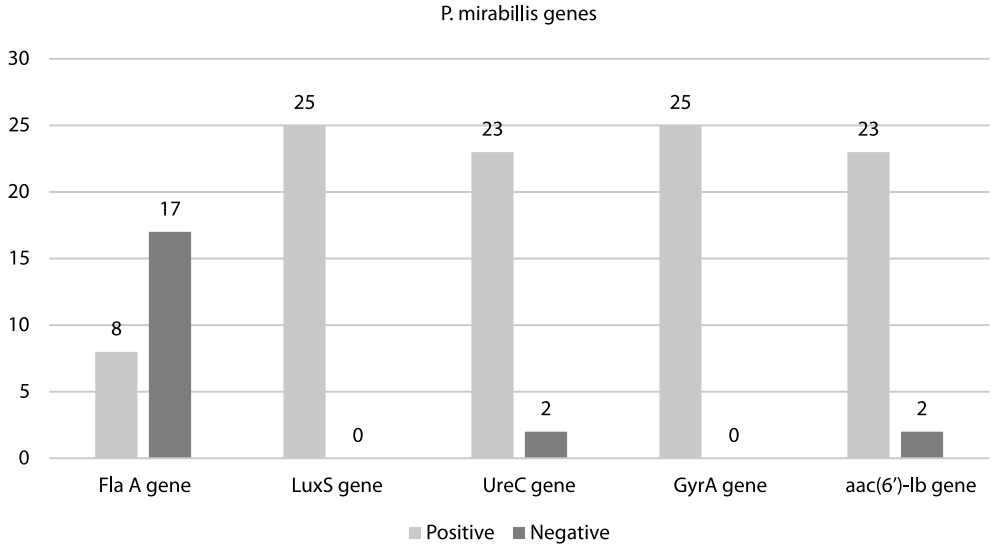


Fig. 1. The biofilm formation among the isolated Proteus mirabilis



CalX^2=55.494	TabX^2=9.49	DF=4	p. value <0.001 ^{sig}

Fig. 2. Molecular detection P. mirabillis isolates genes

gene (Fig. 5) 25, 100%, followed by both UreC gene (Fig. 4), and aac(6)Ib gene (Fig. 5) 23, 92%. Whereas the lowest isolated gene was Fla A gene (Fig. 6) 8, 32%. The study also noted a significant difference at p. value <0.05 between gene prevalence in P. mirabillis bacteria as in (Fig. 2).

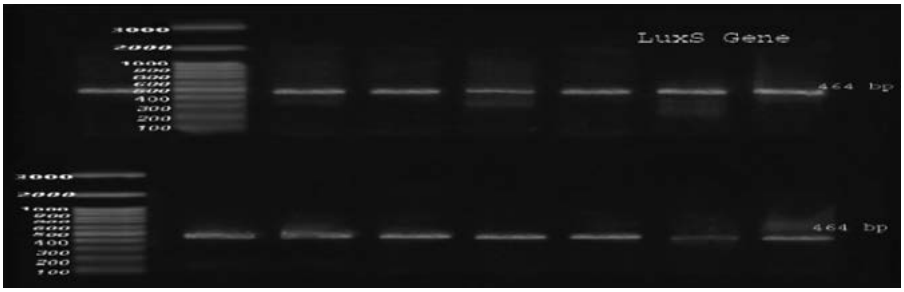


Fig. 3. Agarose gel electrophoresis of amplified LuxS gene PCR product (464bp) (L: DNA Ladder 100–3000 bp, Agarose: 1.5%, Volt: 60 v, Lanes showing (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25) represent bands of P. mirabilis isolates

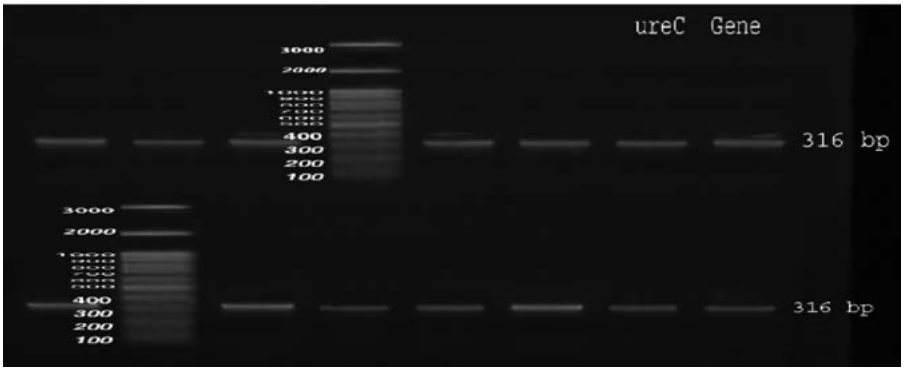


Fig. 4. Agarose gel electrophoresis of amplified ureC gene PCR product (316bp) (L: DNA Ladder 100–3000 bp, Agarose:1.5%, Volt: 60 v, Lanes showing (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 24 and 25) represent bands of P. mirabilis isolates

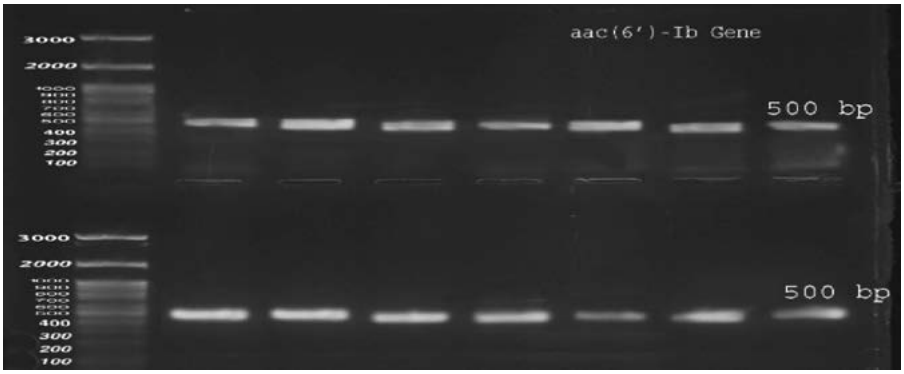


Fig. 5. Agarose gel electrophoresis of amplified aac(6′)-Ib gene PCR product (500bp) (L: DNA Ladder 100–3000 bp, Agarose: 1.5%, Volt: 60 v, Lanes showing (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 22, 23, 24 and 25) represent bands of P. mirabilis isolates

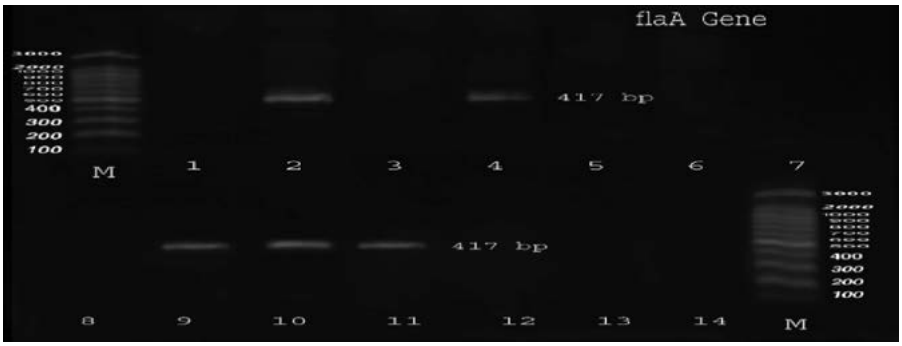


Fig. 6. Agarose gel electrophoresis of amplified flaA gene PCR product (417bp) (L: DNA Ladder 100–3000 bp, Agarose: 1.5%, Volt: 60 v, Lanes showing (2, 4, 9, 10, 11, 18, 19 and 20) represent bands of P. mirabilis isolates

■ DISCUSSION

In the present study, the prevalence of P. mirabilis was (13.88%) that slightly more when compared with findings of [23] who reported a prevalence rate of 10.7% in human urine samples but, differ with [24] who reported a prevalence rate (19.3%). Variation in the prevalence rate can be attributable to numerous factors, including geographic location and antibiotic use [23] or it may be related to changes in the size or number of samples, and the number of hospitals surveyed [25]. Proteus mirabilis had several virulence factors responsible for the pathogenicity of the organism, including urease, flagella and biofilm formation beside to antibiotic resistance such as aminoglycoside resistance.

According to [26] A biofilm is a group of adherent microbial cells that are protected by an extracellular matrix. Biofilms unwittingly aid bacterial survival by promoting enhanced environmental adaption and enhanced nutrient use. Our results resemble to study reported by [17, 27, 28] that all P. mirabilis isolates had the capacity to form biofilm on polystyrene that certainly is important to establish P. mirabilis in the urinary tract, especially among catheterized patients [26] found the P. mirabilis was able to form a weak biofilm at rate 12 (24.0%), moderate by 13 (26.0%), and strong by 25 (50.0%), [29] found the P. mirabilis was able to form ,a strong biofilm at rate 29 (58%), moderate by 21 (42%), while [30] the percentage were 9 (15%) strong, 8 (13.33%) moderate, 10 (16.66%) weak, and 33 (55%) was non-producer to biofilm and [31] found the strong rate (40%), the Moderate rate (24.7%), the weak rate (21.1%) and Non biofilm forming were (14.1%) which are not completely comparable to our study results. Numerous environmental conditions, including as oxygen, temperature, and others, may influence the formation of the slime layer, with varying effects. According to the findings of this study, the existence of a strong or moderate biofilm enables bacteria to strongly adhere to the urinary tract. However, a weak positive may reveal that the bacteria are under stress or that their development is insufficient, so rendering the biofilm in effective or impossible to form [28].

The molecular results showed the prevalence of the luxS gene in Proteus mirabilis isolates was completely agree with other studies for [32], and [33]. Another studies by [34, 35] and [21] reported the luxS gene prevalence rates was 70%, 55% and 47% respectively.

The luxS gene contributes to the creation of autoinducer 2, which is produced by bacteria and used to communicate information about the metabolic capability and cell density of the surrounding environment [34]. Urease production is an important characteristic of the genus Proteus, it is a main virulence factor in the pathogenicity of Proteus [36]. Urease is produced by the vast majority of clinical strains of the bacteria and catalyzes the hydrolysis of urea into ammonia and carbon dioxide. Ammonia, a highly alkaline agent, is a major cause of tissue injury [37] The molecular results indicated the prevalence of the ureC gene in Proteus mirabilis isolates agree with [38] that show (96.66%) positive for ureC gene and, converged with [11] study which found that the gene frequency was (100%) However, it disagreed with [35] and [23] who found the presence of ureC gene was (60%, 80.5%) respectively.

The flagella that are encoded by flaA gene are recognized as one of the major virulence factors that assist these bacteria to move aggressively [39]. The study finding agree with [23] that found the distribution of flaA gene was only (28.5%), but disagree with [40], and [41] who reported the prevalence rates of flaA gene were 100% and 96.15% respectively. The prevalence rates of luxS, ureC and flaA genes most likely attribute to the differences in the distribution of virulence genes among different populations and geographic locations [33].

Aminoglycoside – modifying enzymes (AMEs) catalyze the covalent modification of amino or hydroxyl groups on aminoglycoside molecules, resulting in poor binding to the ribosome target [43]. Our finding for aac(6)Ib gene was much higher than [16, 35, 42] and [19] that found the distribution of aac(6)Ib gene was 43%, 40%, 33.3% and 22.2% respectively, which are differ from our results.

The prevalence of these genes may change according to the clinical condition of the host and the genetic composition of the isolates producing UTIs [33]. Future research should investigate more virulence and antibiotic-resistant genes, one of the study's limitations being that it evaluated only a subset of virulence and antibiotic-resistant genes.

■ CONCLUSION

Proteus mirabilis have the ability to cause urinary tract infections and CAUTIs because it possesses many virulence factors that help it to cause infection, increase their resistance to antibiotics as well as the ability to form biofilms on biotic & abiotic materials. Thus increasing the pathogenicity and making it difficult to treat.

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