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Original Article

Evaluation of virulence genes in *Proteus* strains isolated from diabetic foot infections and urinary tract infections

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Abstract

Introduction: *Proteus* species are frequently isolated from urinary tract infections (UTIs) and diabetic foot infections (DFIs). We aimed to evaluate the presence of virulence genes in *P. mirabilis* and *P. vulgaris* strains isolated from DFI and UTI.

Methodology: A total of 78 *Proteus* isolates (57 *P. mirabilis* and 21 *P. vulgaris*) collected from patients were studied. The isolates were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The presence of virulence-associated genes (*hlyA*, *mrpA*, *atfA*, *pmfA*, *hmpA*, *ptaA*, *ureA*, *ureC*, *zapA*, *ireA*, *rsbA*, *flaA*, and *ucaA*) was evaluated by polymerase chain reaction (PCR).

Results: 46 isolates were obtained from wound cultures, and 32 were obtained from the midstream urine cultures. All virulence genes, except *hlyA,* were detected in the study. *ureA* was the most detected gene in both UTI (100%) and DFI isolates (84.8%). The distributions of *ureC*, *flaA*, *hpmA*, *ireA*, *rsbA*, *pmfA*, *zapA*, *ucaA*, *ptaA*, *atfA*, and *mrpA* genes in DFI and UTI isolates were as follows: 82.6% and 96.9%, 71.7% and 93.8%, 69.6% and 93.8%, 69.6% and 96.9%, 69.6% and 96.9, 67.4% and 81.3%, 65.2% and 43.8%, 54.3 and 71.9%, 34.8% and 96.9%, 26.1 and 93.8%, and 23.9% and 46.9%, respectively.

Conclusions: We demonstrated that *P. mirabilis* and *P. vulgaris* express a variety of virulence genes related to pathogenicity. All virulence genes were found to be more frequent in UTI isolates except *zapA.* There is limited data on the virulence factors of *Proteus* species in DFIs. Further studies are needed to investigate virulence genes in wound isolates.

Key words: *Proteus* spp., urinary, diabetic foot, infection, virulence.

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Introduction

Proteus species, which belong to the order Enterobacterales, are Gram-negative bacteria commonly found in the environment. They are also members of the normal human intestinal microbiota. *P. mirabilis*, the most common species associated with human infections, along with *P. vulgaris*, and *P. penneri*, are the predominant species responsible for opportunistic infections in humans [1,2]. *P. mirabilis* is noted for causing complications in patients undergoing care for indwelling bladder catheterization [3]. *P. mirabilis* causes not only urinary tract infections (UTIs), but also respiratory tract infections, food poisoning, ear infections, and wound infections, particularly in people with diabetes [4].

Proteus species have swimmer cells that differentiate into swarmer cells, which are capable of movement on surfaces. This is supported by virulence factors such as the mannose-resistant *Proteus* (MR/P) fimbriae, and *P. mirabilis* P-like fimbriae normally found in *Proteus* to generate biofilm-forming factors mediated by hyper-flagella, which contribute to swarming [5]. Another virulence factor is the production of the urease enzyme, which promotes the hydrolysis of urea into carbon dioxide and ammonia, thus increasing the pH of urine. This process initiates the formation of bladder and kidney stones (struvite and apatite), which is a condition known as urolithiasis, and may even cause the complication of renal failure [6,7].

The World Health Organization (WHO) has categorized diabetes as one of the epidemics of the 20th century, and about 10% of the adult population worldwide are at high risk of being diagnosed with this endocrine disease [8]. One of the most important

complications of diabetes is diabetic foot infection (DFI). Patients with DFI are at risk of amputation even with appropriate treatment [9]. Mortality from DFI is higher in patients with chronic osteomyelitis, those who have acute necrotizing soft tissue infections, and those with other complications that result in a poor immune system [10].

UTIs affect the urinary tract and usually occur endogenously when bacterial colonies, grow, and disseminate upstream to the urinary tract. About 150 million people worldwide are affected by UTIs each year [5]. DFIs and UTIs are common nosocomial and community-acquired infections caused by pathogenic agents such as *P. mirabilis*, which carries many virulence factors that contribute to its pathogenesis. In the present study, we aimed to investigate the presence of the virulence genes related to the pathogenesis of clinical *P. mirabilis* and *P. vulgaris* strains isolated from DFIs and UTIs at two centers and to compare their distribution among the respective isolates.

Methodology

Bacterial strains and identification

A total of 78 *Proteus* (57 *P. mirabilis* and 21 *P. vulgaris*) isolates obtained from patients at two different centers between January and December 2020 were included in this study. Of these, 46 were isolated from wound cultures collected from patients with DFIs at the Aydın Adnan Menderes University Hospital, and 32 were isolated from the midstream urine cultures of patients at the University of Health Sciences Gülhane Training and Research Hospital. The isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker, Germany).

DNA isolation

A single bacterial colony grown on trypto-casein soy agar was selected and dissolved in 1 mL of Tris-EDTA buffer. The suspension was centrifuged at 11,000 g, and the pellet was used for DNA isolation. DNA extraction was performed using the DNA4PCR kit (R-Tech, Aydın, Türkiye) in accordance with the manufacturer's recommendations. Briefly, the pellet was dissolved in the extraction kit solution and incubated at 56 °C for 30 minutes. After incubation, the suspension was vortexed and incubated at 100 °C for 10 minutes. It was then centrifuged at 11,000 g, and the supernatant was used for the polymerase chain reaction (PCR). The DNA was stored at −20 °C until the time of use.

Detection of virulence genes using PCR

The virulence genes evaluated in *P. vulgaris* and *P. mirabilis* in this study were *mrpA*, *atfA*, *pmfA*, *ucaA*, *hmpA*, *hlyA*, *ptaA*, *ureC*, *ureA*, *zapA*, *ireA*, *rsbA*, and *flaA*. The oligonucleotide primers that were used to determine the species-specific region are presented in Table 1 [2,11,12]. PCR amplification was performed separately in a 50 µL reaction mixture for each gene.

Table 1. Primers used in the study.

Genes	Primer sequences (5'→3')	Base pair	Annealing temperature	Reference
ireA	F: ACTACGATAACGAGCGCCAG R: GCCCTAACTGCGGGAATACG	681	60 °C	[11]
mrpA	F: GAGCCATTCAATTAGGAATAATCCA R: AGCTCTGTACTTCCTTGTACAGA	648	58 °C	[11]
ucaA	F: GCTTTTACATCCCAGCGGT R: GCTGCATTTGCTGGCTCATC	476	60 °C	[11]
pmfA	F: CAAATTAATCTAGAACCACTA R: ATTATAGAGGATCCCTTGAAGGTA	617	54 °C	[11]
atfA	F: CATAATTTCTAGACCTGCCCTAGCA R: CTGCTTGGATCCGTAATTTTTAACG	382	50 °C	[11]
ptaA	F: CCACTGCGATTATCCGCTCT R: ATCGGCAGAAGTGACAAGCA	688	60 °C	[11]
hpmA	F: GTTGAGGGGCGTTATCAAGAGTC R: GATACTGTTTTGCCCTTTTGTGC	709	55 °C	[11]
hlyA	F: AACAAGGATAAGCATGTTCTGGCT R: ACCATATAAGCGGTCATTCCCGTA	1177	63 °C	[11]
rsbA	F: TTGAAGGACGCGATCAGACC R: ACTCTGCTGTCCTGTGGGTA	467	58 °C	[12]
ureC	F: GTTATTCGTGATGGTATGGG R: ATAAAGGTGGTTACGCCAGA	317	56 °C	[12]
zapA	F: ACCGCAGGAAAACATATAGCCC R: GCGACTATCTTTCCGCATAATCA	540	59 °C	[12]
ureA	F: GATCTGGGCGACATAATCGT R: TCACCGGGGATCATGTTATT	362	54 °C	[2]
flaA	F: AGGATAAATGGCCACATTG R: CGGCATTGTTAATCGCTTT	417	54 °C	[2]

The PCR program was as follows: an initial denaturation step at 95 °C for 4 minutes; followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at the temperatures shown in Table 1, and an extension at 72 °C for 1 minute; with a final extension at 72 °C for 7 minutes. The amplicons were electrophoresed in 1% agarose gel at 100 V for 30 minutes and visualized under ultraviolet light. One amplicon was selected for each positive gene detected by PCR and then processed for sequencing. The sequencing results were analyzed and compared using the Basic Local Alignment Search Tool (BLAST) database program provided by the National Center for Biotechnology Information (NCBI). The confirmed amplicons were used in the study as positive controls.

Statistical analysis

Chi-squared (χ^2) and Fisher's exact tests were used to compare the prevalence of the virulence-related genes among the study isolates using SPSS statistical software (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). A *p* value less than 0.05 was considered statistically significant.

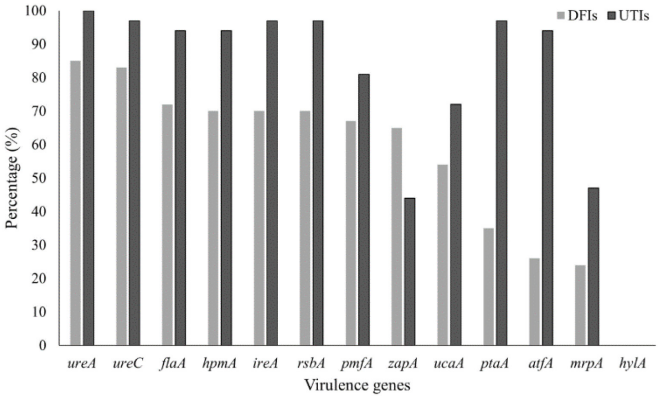
Results

In this study, a total of 78 *Proteus* species (21 *P. vulgaris* and 57 *P. mirabilis*) isolated from two groups of patients with DFI and UTI were investigated for the presence of pathogenesis-related virulence genes. Of the 78 *Proteus* isolates, 46 (59%) were obtained from the wound cultures of the patients with DFI, and 32 (41%) were obtained from the midstream urine cultures of the patients with UTI.

We conducted tests to determine the presence of 13 virulence genes among the isolates. Except for *hlyA*, all the virulence genes were detected in at least one of the isolates. We determined up to 12 virulence genes in two *P. mirabilis* isolates, one from DFI culture and the other from UTI culture. The percentages of the virulence genes were predominantly higher among *P. mirabilis* than *P. vulgaris* in both the DFI and UTI isolates. Except for *zapA*, all the virulence genes investigated in the study were detected at higher percentages in the UTI *P. mirabilis* isolates than in the DFI *P. mirabilis* isolates (Figure 1). Similarly, with the exception of *zapA*, the virulence genes were determined to be present at higher rates in the UTI *P. vulgaris* isolates compared to the DFI *P. vulgaris* isolates.

The distributions of *mrpA* ($p = 0.034$), *atfA* ($p < 0.001$), *hpmA* ($p = 0.009$), *ptaA* ($p < 0.001$), *ureA* ($p = 0.037$), *rsbA* ($p = 0.003$), *flaA* ($p = 0.015$), and *ireA* ($p = 0.003$) were significantly higher in the UTI isolates compared to the DFI isolates. The presence of *ureC* was found to be close to statistical significance ($p = 0.052$) in the UTI isolates. *ureA* was the most detected gene for both the DFI (84.8%) and UTI (100%) isolates. The percentages of *ureC*, *flaA*, *hpmA*, *ireA*, *rsbA*, *pmfA*, *zapA*, *ucaA*, *ptaA*, *atfA*, and *mrpA* virulence genes detected in the DFI and UTI isolates were as follows: 82.6% and 96.9%, 71.7% and 93.8%, 69.6% and

Figure 1. Presence of virulence genes in the isolates.



DFI: diabetic foot infection; UTI: urinary tract infection.

Table 2. Distributions of virulence genes in the isolates.

Virulence genes	Characteristics	*P. vulgaris* (n = 21) n (%)	*P. mirabilis* (n = 57) n (%)	Total (n = 78) n (%)	DFI			UTI			*p* value*
					P. vulgaris (n = 14) n (%)	*P. mirabilis* (n = 32) n (%)	Total (n = 46) n (%)	*P. vulgaris* (n = 7) n (%)	*P. mirabilis* (n = 25) n (%)	Total (n = 32) n (%)	
mrpA	Adhesion, biofilm and fimbriae formation	7 (33.3)	19 (33.3)	26 (33.3)	2 (14.3)	9 (28.1)	11 (23.9)	5 (71.4)	10 (40.0)	15 (46.9)	0.034⁺
atfA		9 (42.9)	33 (57.9)	42 (53.8)	3 (21.4)	9 (28.1)	12 (26.1)	6 (85.7)	24 (96.0)	30 (93.8)	< 0.001⁺
pmfA		9 (42.9)	48 (84.2)	57 (73.1)	5 (35.7)	26 (81.3)	31 (67.4)	4 (57.1)	22 (88.0)	26 (81.3)	0.174
ucaA		8 (38.1)	40 (70.2)	48 (61.5)	5 (35.7)	20 (62.5)	25 (54.3)	3 (42.9)	20 (80.0)	23 (71.9)	0.117
hpmA	Cytotoxins	12 (57.1)	50 (87.7)	62 (79.5)	6 (42.9)	26 (81.3)	32 (69.6)	6 (85.7)	24 (96.0)	30 (93.8)	0.009⁺
hlyA		-	-	-	-	-	-	-	-	-	
ptaA		9 (42.9)	38 (66.7)	47 (60.3)	3 (21.4)	13 (40.6)	16 (34.8)	6 (85.7)	25 (100)	31 (96.9)	< 0.001⁺
ureC	Urease production	15 (71.4)	54 (94.7)	69 (88.5)	9 (64.3)	29 (90.6)	38 (82.6)	6 (85.7)	25 (100)	31 (96.9)	0.052
ureA		16 (76.2)	55 (96.5)	71 (91.0)	9 (64.3)	30 (93.8)	39 (84.8)	7 (100)	25 (100)	32 (100)	0.037⁺
zapA	Protease expression	5 (23.8)	39 (68.4)	44 (56.4)	5 (35.7)	25 (78.1)	30 (65.2)	0	14 (56.0)	14 (43.8)	0.060
rsbA	Swarming regulation	12 (57.1)	51 (89.5)	63 (80.8)	6 (42.0)	26 (81.3)	32 (69.6)	6 (85.7)	25 (100)	31 (96.9)	0.003⁺
flaA	Flagella moderation	13 (61.9)	50 (87.7)	63 (80.8)	8 (57.1)	25 (78.1)	33 (71.7)	5 (71.4)	25 (100)	30 (93.8)	0.015⁺
ireA	Siderophore receptor	12 (57.1)	51 (89.5)	63 (80.8)	6 (42.9)	26 (81.3)	32 (69.6)	6 (85.7)	25 (100)	31 (96.9)	0.003⁺

DFIs: diabetic foot infections; UTIs: urinary tract infections; * denotes comparison variables between DFIs and UTIs isolates. ⁺ indicates statistically significant.

93.8%, 69.6% and 96.9%, 69.6% and 96.9, 67.4% and 81.3%, 65.2% and 43.8%, 54.3 and 71.9%, 34.8% and 96.9%, 26.1 and 93.8%, and 23.9% and 46.9%, respectively. The distribution of the virulence genes detected in the isolates are summarized in Table 2.

Discussion

The most studied and well-known species of the genus *Proteus* is *P. mirabilis*. It is mainly problematic for catheterized patients due to the virulence factors and is the third most common bacterial agent that causes UTIs [1]. Wound infection is a major concern for individuals with diabetes and increases the three-year mortality rate of people with diabetes from 13% to 28% [10]. DFIs not only increase mortality, but also impair quality of life and increase treatment costs. Diabetes-related complications further pose a major problem due to the global implications (e.g., economic and social burden), given that the worldwide incidence of diabetes is expected to increase by 55% over the next 20 years [10,13].

The most defining characteristic of *Proteus* spp. is its swarming ability, which plays a role in its pathogenesis, and is regulated by genes including *rsbA*. In our study, although *rsbA* was found in all the UTI *P. mirabilis* isolates, we detected *rsbA* in 81.3% of the DFI *P. mirabilis* isolates. Among all study isolates, *rsbA* was found to be present at a higher rate in *P. mirabilis* isolates (89.5%) than in *P. vulgaris* isolates (57.1%). This result is consistent with the data reported from studies conducted by Hussein *et al*. [7] and Pathirana *et al*. [12], who reported that *rsbA* gene was present in 100% of the *P. mirabilis* isolates recovered from UTIs. However, several factors contribute to the swarming ability of *P. mirabilis*, such as flagella and chemotaxis, thus swarming regulating genes may not be strictly necessary to enhance its swarming ability [14].

Urease activity is a significant characteristic of the pathogenesis of *Proteus*. Urease hydrolyzes urea to release ammonia and accelerates the formation of kidney stones through the precipitation of compounds such as calcium and magnesium, which make the urine pH alkaline [15,16]. Moreover, only *P. mirabilis* has a positive association with catheter occlusion, although other strains of urease-positive bacteria can cause catheter-associated UTIs [17]. In our study, the genes that encode the urease enzyme, namely, *ureA* and *ureC*, were present in 91% and 88.5%, of all the isolates, respectively. On the other hand, both enzymes were detected in all the *P. mirabilis* isolates obtained from the UTI cultures. Moreover, *ureA* was detected in all the urinary isolates of *P. vulgaris*. These results are consistent with the data from a study by Alatrash *et al.* [18], who found a 100% *ureA* ratio in 36 *P. mirabilis* isolates collected from urine. The prevalence of the *ureC* gene, which was determined to be 96.9% and 82.6% in the UTI and DFI isolates in our study, was higher than that in the study by Alsherees *et al.*, who reported 33.3% in urinary isolates and 50% in wound isolates [19]. Urease activity, which is considered one of the most important virulence factors of *P. mirabilis* and *P. vulgaris* strains, contributes to biofilm formation and provides the ability of bacteria to cluster on living or non-living surfaces. The presence of at least one urease gene in all UTIs isolates in our study supports the view that this gene is one of the most important virulence factors in the development of infection.

Biofilm formation, adhesion ability, and fimbriae are important factors that contribute to bacterial dissemination and invasion in infections. *Proteus* species encodes viable fimbriae such as MR/P fimbriae, ambient temperature fimbriae, uroepithelial cell adhesion fimbriae, *P. mirabilis* fimbriae, and *P. mirabilis* P-like fimbriae [15,20]. In this study we assessed the biofilm and fimbriae-related virulence genes in the *P. mirabilis* and *P. vulgaris* isolates. The prevalence rates of *mrpA*, *atfA*, *pmfA*, and *ucaA* in the DFI isolates were 23.9%, 26.1%, 67.4% and 54.3%, respectively; whereas the rates in the UTI isolates were 46.9%, 93.8%, 81.3%, and 71.9%, respectively. These results are inconsistent with the findings of Oliveira *et al.,* who reported the prevalence of *mrpA*, *pmfA*, and *atfA* genes as 100%; and *ucaA* as 81.4% in 183 strains isolated from the urine of catheter-associated UTI patients [21]. Mirzaei *et al.* detected an even higher prevalence of 100% for the *mrpH*, *mrpA*, *pmfA*, *ureG*, and *hpmA* genes among UTI isolates from patients in Iran [17]. On the other hand, we found that the *mrpA*, *atfA*, *pmfA*, and *ucaA* distributions among the *P. mirabilis* urinary isolates had considerably higher rates at 40%, 96%, 88%, and 80%, respectively.

We also checked for the virulence genes that are responsible for hemolytic and flagellar activity (i.e., *hmpA*, *ptaA*, and *flaA*). The *flaA* gene is responsible for the flagella moderations that facilitate swarming and swimming motility and thereby cause the spread of infection. Among the urinary isolates in our study, the prevalence rates of *hmpA*, *ptaA*, and *flaA* were 93.8%, 96.9%, and 93,8%, respectively, while those for the DFI isolates were 69.6%, 34.8%, and 71.7%, respectively. These results are in accordance with the results reported by Hind *et al.* [2], who reported that the prevalence of the *flaA* gene was 86.7% among UTI isolates; and Al-Hamdani *et al.* [22], who reported that the prevalence

of the *hmpA* gene was 90%. The hemolytic properties and cytotoxins of uropathogenic *Proteus* species rely on *hpmA* and *hlyA* hemolysins, and *ptaA* cytotoxic agglutinins [7,17]. In general, a high prevalence of *hpmA* and *hpmB* hemolysins has been reported in previous studies, while the range of *hlyA* has been found to be variable [23]. In our study, *hlyA* was not detected in the urinary and wound isolates. The results of our study are inconsistent with those of Abd El-Baky *et al.* [24], who reported that the *hlyA* gene was found in 81.8% and 55.5% of uropathogenic and bloodstream isolates, respectively. However, our results are consistent with a study conducted using a large study population that included 211 urinary *P. mirabilis* isolates; the researchers reported the presence of *hpmA* and *hpmB* in 97.15% of the isolates, whereas none of the investigated isolates expressed *hlyA* [23]. The remarkable features of uropathogenic strains are that they can be more resistant to antibiotics and the host's immune system, and their ability to form biofilms plays a role in this resistance. The relationship between biofilm formation, biofilm density, urease activity, and cytotoxins with resistance to various antibiotics has been shown in various studies [7,17]. The high rates of genes associated with biofilm formation, flagellar activity and urease activity found in our study constitute a warning, since these structures may block urinary catheters, especially in hospitalized patients, and complicate the antibiotic effect and the immune response of the patients. This may lead to the need to develop one or more new forms of UTIs treatment, such as intravesical antibiotic administration, vaccines, receptor analogs, or phage therapy.

Another gene examined in our study was *ireA*, which encodes a siderophore receptor, and is involved in urinary tract colonization as well as bacterial iron uptake [11,25]. Iron uptake is essential for bacterial growth, and its level is restricted by the host to overcome bacterial infection. To highlight the importance of this system, recent studies have revealed that *P. mirabilis* has 21 iron-regulated systems [6]. We detected the presence of the *ireA* gene in 69.9% of the DFI isolates and 96.9% of the UTI isolates in our study. Notably, *ireA* was encountered in all the *P. mirabilis* urinary isolates. Similarly, Oliveira *et al.* found the expression of *ireA* in all 183 *P. mirabilis* strains that they isolated from urine samples [21].

The *zapA* gene encodes for an extracellular metalloprotease, also known as IgA protease, which belongs to the serralysin family of the zinc metalloproteases. ZapA protease not only digests peptides such as complement components, cellular matrix (i.e., collagen, fibronectin, and laminin), and the protein components of microtubules and microfilaments (i.e., actin and tubulin), but also disrupts the activity of host immunoglobulins such as IgA and IgG and causes tissue damage [26–28]. This tissue damage worsens the condition of the wound by disrupting the cell matrix and cytoskeletal proteins [27]. The presence of the *zapA* gene improves bacterial survival in the urinary tract through ZapA-related degradation of the host proteins and antimicrobial peptides [16]. In a recent study with *P. aeruginosa* isolates obtained from diabetic foot wounds, bacterial biofilm formation at the wound sites decreased because of in vitro inhibition of the protease gene *lasB* [29]. In another study conducted with *P. aeruginosa* obtained from diabetic foot wounds, the importance of bacterial proteases in chronic wound development was emphasized [13]. The prevalence of *zapA* among the diabetic foot isolates in our study was higher (65.2%) than among the UTI isolates (43.8%). Studies have also reported a higher *zapA* gene prevalence in urinary tract isolates than in wound isolates, which is unlike the observations made in our study [19].

Conclusions

A limitation of our study is that we examined a limited number of virulence genes and isolates. Notwithstanding, the fact that the focus of the study was on isolates obtained from DFIs, it contributes valuable data to the literature.

We evaluated and compared the prevalence of virulence genes in the *P. mirabilis* and *P. vulgaris* isolates obtained from UTIs and DFIs. Urease activity, biofilm formation, adhesion ability, fimbriae formation, cytotoxins, iron uptake systems, and flagellar activity are the most important factors contributing to bacterial spread and invasion in *Proteus* infections. Our data indicate that the *Proteus* species possess several virulence genes related to pathogenicity, such as motility, cytotoxicity, tissue and protein degradation, and iron uptake systems. It was found that the isolates from UTIs had a higher prevalence of virulence-related genes (i.e., *mrpA*, *atfA*, *hpmA*, *ptaA*, *ureA*, *rsbA*, *flaA*, *ireA*) compared to those from DFIs, with the exception of *zapA*. The strong capacity of strains to possess these virulence-associated genes can complicate antibiotic therapy. ZapA is a broad-spectrum protease that is responsible for complement and cell components, cytoskeletal proteins, and immunoglobulins degradation. The *zapA* gene may play a crucial role in tissue damage and may contribute to treatment complications. Further studies are needed to investigate

virulence-related genes in *Proteus* species isolated from UTIs and DFIs.

Authors' contributions

BB and RMP: study design, methodology; TH, RMP, and MBE: data collection and data integrity; TH and BB: manuscript draft with input from all authors. All authors verified the analysis and interpretation of data. All authors have read and approved the final version of the manuscript.

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