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VIRULENCE FACTORS OF *Proteus mirabilis* ISOLATED FROM CLINICAL URINE IN BRAZIL

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Abstract: Urinary tract infection (UTI) is among the most prevalent human infections and is defined as microbial colonization and tissue invasion of the genitourinary tract. *Proteus mirabilis* is one of the main causative agents of UTI. Some of its virulence factors have been characterized, such as flagella, enzymes, cytolysins, siderophores and adhesins. Due to its virulence factors, individuals with UTI caused by *P. mirabilis* often develop bacteriuria, kidney and bladder stones, acute pyelonephritis, catheter obstruction and fever. In this study, 47 isolates of uropathogenic *P. mirabilis* were analyzed and characterized genotypically (PCR) and phenotypically (biofilm formation and cytotoxicity assay). Diverse virulence genes were found, such as *hpmA* (present in 93.61% of the isolates), *ptA* (68.75%), *ureA* (100%), *zapA* (6.25%), *atfA* (100%), *mrpA* (91.48%), *pmfA* (77.08%), *ucaA* (85.41%) and *ireA* (54.16%). All isolates were able to form biofilm and presented cytotoxic activity. In general, the antimicrobial resistance rate was lower than that demonstrated in other studies. Therefore, *P. mirabilis* can presented several virulence factors, resulting in various infections in human urinary tract.
Keywords: *Proteus mirabilis*, Virulence factors, Urinary Tract Infections.

INTRODUCTION

Proteus mirabilis is na opportunistic pathogen causing complicated urinary tract infections (UTIs) in patients with anatomical or functional problems (JAMIL; FORIS; SNOWDEN, 2020). UTIs are caused mainly by Gram-negative bacteria, with P. *mirabilis* being the third most common of them, responsible for about 5% of UTI cases, after *Escherichia coli* (70-95%) and *Klebsiella pneumoniae* (about 7%). The infections caused by *P. mirabilis* are serious because of its virulence factors, resulting in some clinical problems such as catheter obstruction and stone formation in the bladder and kidneys (BUNYAN; ALBAKERY, 2021; KOT et al., 2021).

P. mirabilis uses a diverse set of virulence factors to establish and promote UTI, including urease, fimbriae, flagella, toxins, iron acquisition system and biofilm formation. The production of urease is clearly associated with urolithiasis and catheter encrustation. During infection, the generated ammonia by the hydrolysis of urea, alkalinizes the urinary pH, followed by the precipitation of polyvalent ions (Mg^{2+} and Ca^{2+}) and subsequent formation of urinary stones composed by struvite or apatite crystals (GRAHL et al., 2021; JIANG et al., 2020; YUAN et al., 2021).

Fimbriae are also important virulence factors, which mediate attachment to uroepithelial cells and contribute to biofilm formation. Particularly, sequencing of the genome of a *P. mirabilis* isolate revealed the existence of at least 17 fimbrial operons, and among them, the most widely researched are mannose-resistant Proteus-like (MR/P) fimbriae, *P. mirabilis* fimbriae (PMF), and uroepithelial cell adhesin (UCA) (FILIPIAK et al., 2020; JIANG et al., 2020).

The ability of *P. mirabilis* to form crystalline biofilms is particularly related to the encrustation and blockage of urethral catheters (WHITE et al., 2021). Catheter blockage prevent the free flow of urine and promote the ascending spread of infection, which can result in bacteriuria, pyelonephritis, bacteremia and, some cases, sepsis (MILO et al., 2021; RICE et al., 2021). In addition, the formation of crystalline biofilms is a key factor that contributes to the persistence of this microorganism in the urinary tract (WASFI et al., 2020; WHITE et al., 2021). The biofilms provided a physical barrier that hinders the penetration of antibiotics and antibodies, therefore, pathogens become highly resistant

to conventional antimicrobials and the host immune response (WASFI et al., 2020; YUAN et al., 2021).

Regarding to antimicrobial susceptibility, *P. mirabilis* is intrinsically resistant to tetracyclines, nitrofurans and polymyxins, including colistin (OLIVEIRA et al., 2021; SHELENKOV et al., 2020). Besides, the acquired resistance by this pathogen to β-lactams, fluoroquinolones and aminoglycosides has been reported worldwide, compromising the effectiveness of an empirical treatment (GIRLICH et al., 2020).

Therefore, further studies on the virulence factors of *P. mirabilis* are essential to determine its uropathogenesis. The objective of this study was to evaluate the presence of virulence factors in *P. mirabilis* isolated from urine of patients with UTI, characterizing them phenotypically and genotypically.

MATERIALS AND METHODS

BACTERIAL ISOLATES

A total of 47 isolates of uropathogenic *P. mirabilis* were studied. All strains were isolated from the urine of patients with UTI admitted to the University Hospital in the city of Londrina, Brazil, from January to December 2009. The bacterial isolates were identified by the Phoenix® automated method. The wild HI4320 *P. mirabilis* strain was used as the positive control in all experiments (MOBLEY; WARREN, 1987). The isolates were maintained in Broth Heart Infusion (BHI) with glycerol (30%) and stored at -80°C.

DETECTION OF VIRULENCE GENES

Virulence genes were detected by PCR. The bacterial DNA was extracted by boiling in ultrapure sterilized water. The reaction was performed in a thermocycler, containing a final volume of 25 µL, composed of 0.5 µL of bacterial DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 10X buffer, 20 pmol of each primer and 1.5 U of Taq DNA polymerase. For *hlyA*, the positive control was *E. coli* O157:H7 (EDL 933) (WELLS et al., 1983). Primers sequencing and conditions used for PCR amplification according to: hpmA (CESTARI et al., 2013); atfA (MASSAD, BAHRANI and MOBLEY., 1994); hlyA (JOHNSON and STELL., 2000); mrpA (ROCHA et al., 2007); pmfA (ZUNINO et al., (2003); ureA, zapA, ucaA, IreA and ptA (SANCHES et al., 2021).

BIOFILM FORMATION

Bacterial biofilm formation test using violet crystal was performed on polystyrene plates according to the methodology described by Kwiecinska-Piróg et al. (2014). The standard strain Enteroaggregative *E. coli* 042 (O44:H18) was used as the very strong biofilm formation control (CZECZULIN et al., 1997). The strain *E. coli* DH5α was used as the weakly biofilm formation control and TSB sterile was used as negative control (SAMBROOK; FRITSCH; MANIATIS, 1989).

VERO CELL CYTOTOXICITY ASSAY

All strains were evaluated for ability to produce cytotoxic effect on Vero cells as described by Lascowski et al. (2013). The cytotoxicity of isolates was quantified after analysis of the metabolic activity of Vero cells by the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. An isolate was considered highly cytotoxic when it presented 50% or more of Vero cells death when compared to the control.

ANTIMICROBIAL SUSCEPTIBILITY

This assay was performed by the disk diffusion technique, following the Clinical and Laboratory Standards Institute (COCKERILL et al., 2012). All the antimicrobials used were obtained from Oxoid™. The drugs tested were nalidixic acid (NAL), 30 µg; amoxycillin +

clavulanic acid (AMC), 30 µg; cephalothin (KF), 30 µg; cefoxitin (FOX), 30 µg; cefotaxime (CTX), 30 µg; ceftazidime (CAZ), 30 µg; aztreonam (ATM), 30 µg; cefepime (FEP), 30 µg; ertapenem (ETP), 10 µg; meropenem (MER), 10 µg; gentamicin (GEN), 10 µg; amicacin (AMI), 30 µg; ciprofloxacin (CIP), 5 µg; levofloxacin (LEV), 5 µg; sulfamethoxazole + trimethoprim (SXT), 25 µg; and norfloxacin (NOR), 10 µg.

STATISTICAL ANALYSIS

Statistical analysis was performed using the R Core Team software (2015). The mean values and statistical analysis were calculated based on the p value, where $p \leq 0.05$ was considered statistically significant.

RESULTS

The results of the analysis of virulence genes from the 47 isolates are shown in Table 1. The most prevalent virulence gene was *ureA*, present in all (100%) of the isolates. Most adhesin genes also showed a high prevalence: *atfA* (100%), *mrpA* (91.48%), *ucaA* (85.41%) and *pmfA* (77.08%). The toxin genes were found in the following percentages *ptA* (68.75%) and *hpmA* (93.61%) and *hlyA* (0%). The siderophore receptor gene *ireA* was found in 54.16% of the isolates. The metalloprotease *zapA* gene was found only in 6.25% of the isolates.

The phenotypic characteristics of all isolates analyzed are shown in Figures 1 and 2. All strains exhibited cytotoxic effect in the test performed with Vero cells. Most of the isolates (82.97%) presented higher OD than the control (Fig. 1).

The results of current study were categorized as strong, moderate, and weak biofilm formation by *P. mirabilis* isolates. From the total microorganisms studied, 100% presented ability for very strong biofilm formation on polystyrene plates (Fig. 2).

ISOLATES	VIRULENCE GENES										ANTIBIOTICS RESISTANCE
	hpmA	atfA	*ptA*	*hlyA*	ureA	*zapA*	*mrpA*	*pmfA*	*ucaA*	*ireA*	
PM-1	+	+	+	-	+	-	+	+	+	-	-
PM-2	+	+	-	-	+	-	+	-	+	+	-
PM-3	+	+	-	-	+	-	+	+	-	-	-
PM-4	+	+	+	-	+	-	+	+	-	+	-
PM-5	+	+	-	-	+	-	+	-	+	+	-
PM-6	+	+	+	-	+	-	+	+	+	-	STX
PM-7	+	+	-	-	+	-	+	-	+	+	NAL/ CIP
PM-8	+	+	-	-	+	-	+	-	+	-	-
PM-9	+	+	+	-	+	-	+	+	+	+	-
PM-10	+	+	+	-	+	-	+	+	+	+	-
PM-11	+	+	-	-	+	-	+	+	+	-	-
PM-12	+	+	-	-	+	-	+	+	+	-	-
PM-13	+	+	+	-	+	-	+	-	+	-	-
PM-14	+	+	+	-	+	-	+	+	+	-	NOR/ FOX/ KF/ FEP

PM-15	+	+	+	-	+	-	+	-	+	+	-
PM-16	+	+	-	-	+	-	+	-	+	-	-
PM-17	+	+	+	-	+	-	+	+	+	-	KF
PM-18	+	+	+	-	+	-	+	+	-	+	-
PM-19	+	+	-	-	+	-	+	+	+	+	CTX
PM-20	+	+	+	-	+	-	+	+	+	+	-
PM-21	+	+	+	-	+	-	+	-	+	+	NOR/ NAL/ LEV/ CIP
PM-22	+	+	+	-	+	-	+	+	+	+	-
PM-23	+	+	+	-	+	-	+	+	+	+	NAL
PM-24	+	+	+	-	+	-	+	+	+	+	-
PM-25	+	+	+	-	+	-	+	+	+	+	-
PM-26	+	+	-	-	+	-	+	+	+	-	-
PM-27	+	+	+	-	+	-	+	+	+	+	-
PM-28	+	+	+	-	+	-	+	+	+	+	-
PM-29	+	+	+	-	+	-	+	+	+	-	STX/ NAL
PM-30	+	+	+	-	+	-	+	+	+	-	-
PM-31	+	+	-	-	+	-	+	+	+	+	-
PM-32	+	+	+	-	+	-	+	+	-	+	NAL
PM-33	+	+	-	-	+	+	+	+	-	+	-
PM-34	+	+	+	-	+	+	+	+	+	-	-
PM-35	+	+	+	-	+	+	+	+	+	-	STX/ LVX
PM-36	-	+	+	-	+	-	-	+	+	+	-
PM-37	+	+	+	-	+	-	-	-	+	+	-
PM-38	-	+	+	-	+	-	-	+	+	-	-
PM-39	+	+	+	-	+	-	+	+	-	+	STX
PM-40	+	+	+	-	+	-	+	-	+	+	-
PM-41	+	+	+	-	+	-	+	+	+	-	NAL
PM-42	+	+	-	-	+	-	+	+	+	+	NAL
PM-43	-	+	+	-	+	-	+	+	+	-	FOX/ NAL/ ATM/ CAZ
PM-44	+	+	-	-	+	-	+	-	+	+	AMC
PM-45	+	+	+	-	+	-	-	+	+	-	-
PM-46	+	+	-	-	+	-	+	+	+	+	-
PM-47	+	+	+	-	+	-	+	+	+	-	NAL

- negative; + positive

Table 1. Virulence genes and antibiotics resistance characteristics of *P. mirabilis* isolates from clinical urine of patients with UTI

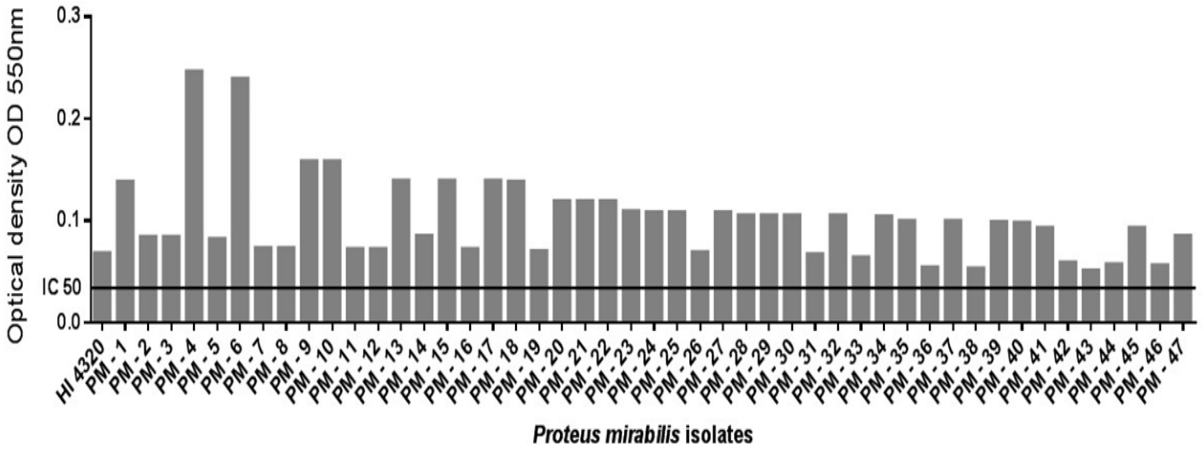

Figure 1. Vero cells cytotoxity of *P. mirabilis* isolates. *P. mirabilis* HI 4320 was used as control. The $p \leq 0.05$ was considered statistically significant. IC_{50}: Inhibitory concentration of the bacteria that can kill 50% of the cells.

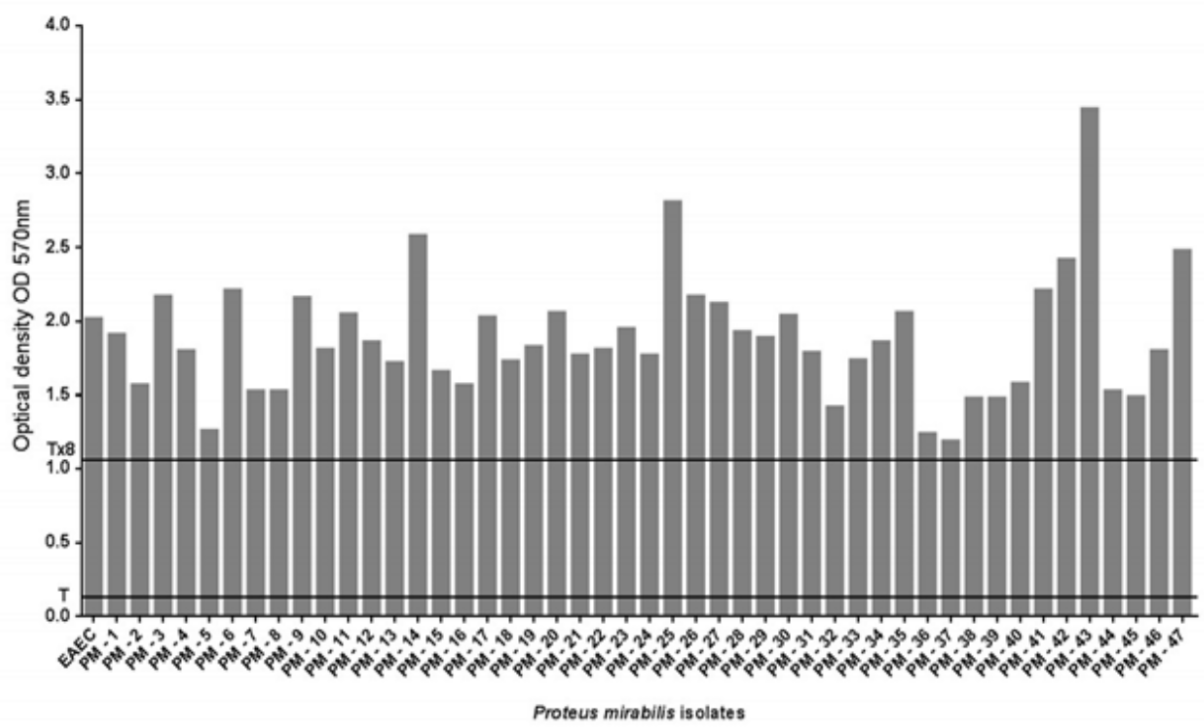

Figure 2. Biofilm of *P. mirabilis* isolates. Control *Escherichia coli* (EAEC). The $p \leq 0.05$ was considered statistically significant. (T): Threshold; (Tx8): The value of threshold multiplied by 8. Values above 1.063nm (Tx8), means that isolates are very strong.

Antimicrobial susceptibility test showed that most isolates were sensitive to the drugs examined. Regarding the resistance to β-lactams, none of the 47 microorganisms studied showed an extended spectrum β-lactamases (ESBL) production profile. In total, 91.4% of the isolates were sensitive to STX, 91.4% to CAZ, 95.7% to FOX, 95.7% to KF, 95.7% to NOR, 80.8% to NAL, 97.8% to LEV, 97.8% to AMC, and 95.7% to CIP. All isolates were sensitive to MER, AMI, GEN, and ETP.

DISCUSSION

In this study, phenotypic and genotypic tests were performed with the aim of contributing toward the knowledge of the main virulence factors of the important uropathogen *P. mirabilis*. To our knowledge this is the first work that comprehensively characterizes virulence factors of *P. mirabilis* isolated from hospital clinical urine in Brazil.

Urease is one of the main virulence factors in uropathogenesis. The gene *ureA* were found in all the 47 microorganisms examined by us. This enzyme promotes alteration of the urine pH, causes ion precipitation, and favors struvite and apatite crystal formation. These crystals can protect bacteria against antibiotics, antibodies, and urease inhibitors in addition to causing obstruction of the catheters. Our results are in conformance to other studies, showing that *P. mirabilis* is the most related bacterium with urinary stones formation and urease is one of the reasons for the recurrence of UTI (RAMPURE et al., 2013; TORZEWSKA et al., 2014).

As described in this study and confirmed by several other authors, *P. mirabilis* presents biofilm formation capacity (JANSEN et al., 2004; ROCHA et al., 2007; SABBUBA; HUGHES; STICKLER, 2002). These robust structures can block drainage of urine from ureters, resulting in reflux and promoting progression to pyelonephritis, sepsis, and shock as well as urinary catheter blockade (ARMBRUSTER; MOBLEY, 2012). All isolates in this study formed a strong biofilm in polystyrene plate. These results were different from the results of Kwiecinska-Piróg et al. (2014), that examined 50 *P. mirabilis* isolates for biofilm formation and found that weak biofilm was formed by 12 (24%), moderate by 13 (26%), and strong by 25 (50%).

The adhesins favor bacterial adhesion to host cells and biofilm formation, being one of the main mechanisms of pathogenicity and establishment of infection. In a study performed with isogenic mutants of MR/P, ATF, UCA, and PMF fimbriae, it was demonstrated that MR/P and ATF are essential in the initial steps of biofilm formation, and UCA and PMF probably has a role once the biofilm is structured.[34] In our study, MR/P, UCA, and PMF genes were found in more than 77% of isolates and ATF was found in 100% of isolates (Table 1). However, these isolates exhibited high level of biofilm formation, corroborating with the study of Scavone et al. (2016).

The MR/P fimbria is one of the major virulence factors related to biofilm formation and consequent colonization of the bladder and kidneys (ROCHA et al., 2007). The gene coding for this fimbria was found in 43 isolates (Table 1). Furthermore, these isolates exhibited high levels of biofilm formation, corroborating with the results found by Rocha et al. (2007).

In a study, Bijlsma et al. (1995) found that all *P. mirabilis* isolates tested presented the *ucaA* gene. Pellegrino et al. (2013) reported that mutations in this gene lead to a decrease in the uroepithelial cell adhesion and the ability to colonize rat kidneys. In our study, 41 isolates presented the *ucaA* gene. Another fimbria related to urinary tract colonization is PMF. Mutation in the *pmfA* gene leads to

a decrease in colonization of the urinary tract (MASSAD et al., 1994; ZUNINO et al., 2003). We found 41 isolates presented with the *pmfA* gene.

Proteus spp. produce two types of cytolysins, HpmA and HlyA hemolysins and Pta cytotoxic agglutinin. Hemolysin in *P. mirabilis* facilitates the propagation of bacteria in the kidney and development of pyelonephritis during UTI (MOBLEY; CHIPPENDALE, 1990). Several studies corroborate with our results regarding the presence of hemolysin and its effect on Vero cells. Senior and Hughes (1988) found that 94% of the *P. mirabilis* isolates produced the hemolytic factor HpmA associated with the cells. Swihart and Welch (1990) reported that HpmA is the most common hemolysin found in *P. mirabilis*. Cestari et al. (2013) verified that 211 isolates (97.15%) presented the *hpmA* gene. Independent *E. coli* alpha-hemolysin (HlyA) is found in the supernatant of some *Proteus spp.*, primarily in *P. vulgaris* (SWIHART; WELCH, 1990). None of the 47 isolates examined in this study presented the *hlyA* gene, which was also verified by Cestari et al. (2013). Therefore, these data indicate that this toxin is not prevalent in *P. mirabilis*.

The PtA toxin mediates cell-cell aggregation and is also capable of lysing renal and bladder cells (ALAMURI; MOBLEY, 2008). In this study, 32 isolates presented with the *ptA* gene, indicating a high prevalence of this gene in the studied microorganisms. In a study by Alamuri et al. (2009), the inactivation of *pta* but not *hpmA* resulted in significant decreases in bacterial infection in kidneys and spleen in a murine model, indicating that the activities of HpmA and Pta are independent of each other but additive as they contribute to cytotoxicity. These data are in conformance with the present study, suggesting that not all isolates that present the *hpmA* gene present the *ptA* gene (Table 1). Regarding the cytotoxicity, the isolates with the highest OD value (PM-04, PM-06, PM-09, PM-10, PM-13, PM-14, PM-15, PM-17, PM-18, PM-20, PM-21, PM-22, PM-23, PM-24, PM-25, PM-27, PM-28, PM-29, PM-30, PM-32, PM-34, PM-35, PM-37, PM-39, PM-40, M-41, PM-45 and PM-47 isolates) presented both genes and those with the lowest OD value (PM-36, PM-38 and PM-42 isolates) presented only the *hpmA* gene (Table 1 and Fig. 1).

The ZapA enzyme is a metalloprotease that cleaves immunoglobulins, cytoskeletal proteins and antimicrobial peptides (BELAS; MANOS; SUVANASUTHI, 2004; SENIOR; ALBRECHTSEN; KERR, 1987). In the present study, only three isolates presented the *zapA* gene. On the contrary, Carson et al. (2011) studied 160 *P. mirabilis* strains and 100% were positive for this gene. The results of these studies demonstrate that the presence of *zapA* gene is variable in *P. mirabilis*.

Iron is an extremely important element for the metabolism of cells. Furthermore, iron is required for the pathogenicity of *P. mirabilis*, and a lack of iron results in attenuation of virulence. Siderophore appears to be the primary mechanism for iron chelation in *P. mirabilis*, being more prevalent in UTI isolates than in non-UTI isolates (HIMPSL et al., 2010). In the present study, 54.16% of the isolates were positive for the *ireA* gene.

Regarding to the resistance to antibiotics, in general, *P. mirabilis* exhibits a high level of resistance to first-generation cephalosporins as well as quinolones and fluoquinolones (NABER et al., 2008; RZECZKOWSKA; PIEKARSKA; GIERCZYŃSKI, 2012). Overall, the antimicrobial resistance rate of the isolates examined in this study was lower than that pointed in other studies (ABREU et al., 2013; WAGENLEHNER et al., 2008). But, as *P. mirabilis* isolates examined in the present study exhibited a high ability to form urinary stones and biofilm, we believe that these abilities prevented the interaction of the

bacterium with the antimicrobials, and thus, there was no selective pressure.

CONCLUSION

In conclusion, the results of this study showed that the uropathogenic *P. mirabilis* presents several virulence factors that are cytotoxic and capable of forming urinary stones and biofilm. Furthermore, we observed the sensitivity of this bacterium toward certain antibiotics, which can be studied further for clinical use. Because of the expression of potent urease and biofilm by *P. mirabilis*, antibiotics should be selected carefully to treat UTI because the sensitivity observed *in vitro* is not always similar to the sensitivity observed *in vivo* owing to crystal production. Therefore, the uropathogenic *P. mirabilis* presents with several virulence factors, contributing toward its pathogenicity, and can constitute a human health problem, primarily for people with urinary catheters.

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