TAYNARA DE LACQUA WALDRICH

CARACTERIZAÇÃO GENOTÍPICA E FENOTÍPICA DE ISOLADOS CLÍNICOS DE *PROTEUS MIRABILIS* DE PACIENTES DO HOSPITAL UNIVERSITÁRIO DE LONDRINA

Londrina
2022

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Tese Apresentada ao Programa de Pós Graduação em Microbiologia, da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutora em Microbiologia.

Orientador: Prof. Dr. Sérgio Paulo Dejato da Rocha

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Londrina, 28 de janeiro de 2022

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“Tende coragem, que tudo irá bem com a graça de Deus”
(Santa Paulina)

RESUMO

Waldrich, Taynara de Lacqua. **Caracterização genotípica e fenotípica de isolados clínicos de *Proteus mirabilis* de pacientes do Hospital Universitário de Londrina** 2022. 60 f. Tese (Doutorado em Microbiologia) – Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, 2022.

O gênero *Proteus* é causador de diferentes infecções hospitalares, como: infecções no trato respiratório, feridas, olhos, nariz, garganta, pele e gastroenterite. Dentre as espécies deste gênero, *Proteus mirabilis* é a mais prevalente em infecções em humanos, principalmente as do trato urinário. Este trabalho objetivou investigar a ocorrência e a diversidade de *P. mirabilis* isolados de diversas infecções em humanos, já que há pouco conhecimento sobre a epidemiologia e o genoma deste patógeno em isolados que não são de fonte urinária. Deste modo, foram realizados 3 estudos, sendo o primeiro com análise genômica de um isolado de secreção traqueal – Este é o primeiro estudo de isolado de secreção traqueal que foi sequenciado. A cepa *Proteus mirabilis* LBUELH-11 tem 3.973.423 bp e 99.13% de similaridade com *Proteus mirabilis* PM52260 e 99.06% com *Proteus mirabilis* AR0155, sugerindo pela análise filogenética que LBUELH-11 é *P. mirabilis*. O segundo estudo, com base em dados epidemiológicos da resistência de *Proteus mirabilis* e no terceiro estudo, foram analisadas 68 cepas de *P. mirabilis* atendidos no Hospital Universitário de Londrina. Quanto ao perfil genotípico e fenotípico, constatou-se a presença do gene *hpmA* em 97.05% dos isolados de origem urinária e em 100% dos isolados de outras fontes de infecção, *pmfA* em 97.05% dos isolados de origem urinária e 100% dos isolados de outras fontes de infecção, *ucaA* em 82.35% dos isolados de origem urinária e em 85.29% dos isolados de outras fontes de infecção, *zapA* está presente em 94.11% dos isolados de origem urinária e em 88.23% dos isolados de outras fontes de infecção, *mrpA* está presente em 100% dos isolados de origem urinária e em 97.05% dos isolados de outras fontes de infecção. Os genes *hlyA* e *fimH* não foram encontrados em nenhum dos isolados e os genes *atfA* e *ureA* estão presentes em 100% deles. Todos os microrganismos analisados apresentaram forte capacidade em formar biofilme. No teste de adesão, 100% dos isolados de fonte urinária foram capazes de formar adesão agregativa (AA); já nos isolados de outras fontes de infecção, 17.65% não foram aderentes (NA) e 82.35% foram capazes de formar adesão agregativa (AA). O patógeno apresentou resistência à maioria dos fármacos testados, principalmente à ceftriaxona. Portanto, pode-se inferir que houve diferenças genotípicas e fenotípicas entre os isolados de *Proteus mirabilis*, sejam ele de fonte urinária ou não urinária

Palavras-chave: Infecção do trato urinário; agentes antimicrobianos; genes de virulência; *Proteus;* biofilme; cristais.

ABSTRACT

Waldrich, Taynara de Lacqua. **Genotypic and phenotypic characterization of clinical isolates of *Proteus mirabilis* from patients at the University Hospital of Londrina** 2022. 60 p. Thesis (Doctorate Degree in Microbiology) – Center of Biological Sciences, Universidade Estadual de Londrina, Londrina, 2022.

The *Proteus* genus causes many different hospital infections, such as: respiratory tract infections, wounds, eyes, nose, throat, skin and gastroenteritis. Among the species of this genus, *Proteus mirabilis* is the most prevalent in human infections, mainly those of the urinary tract. This work aimed at investigating the occurrence and diversity of *P. mirabilis* isolates of many infections in humans, as little is known about the epidemiology and genome of this pathogen in isolates which are not from a urinary source. Thus, three studies were carried out, the first with the genomic analysis of an isolate of tracheal secretion – This study is the first Brazilian isolate sequenced. *Proteus mirabilis* strain LBUELH-11 has 3.973.423 bp and 99.13% of similarity with *Proteus mirabilis* PM52260 and 99.06% with *Proteus mirabilis* AR0155, suggesting by phylogenomic analysis that the LBUELH-11 is a *P. mirabilis*. The second study was based on epidemiologic data on the resistance of *Proteus mirabilis* and, in the third study, 68 strains of *P. mirabilis* observed at Hospital Universitário de Londrina regarding the phenotypic and genotypic profiles. The presence of the gene *hpmA* was noted in 97.05% of the isolates of urinary origin and in 100% of the isolates from other sources of infection, *pmfA* in 97.05% of the isolates of urinary origin and 100% of isolates from other sources of infection, *ucaA* in 82.35% of the isolates of urinary origin and 85.29%% of isolates from other sources of infection, *zapA* being present in 94.11% of the isolates of urinary origin and in 88.23%%% of isolates from other sources of infection, *mrpA* being present in 100% of the isolates of urinary origin and 97.05% of isolates from other sources of infection. The genes *hlyA* and *fimH* were not found in any of the isolates and the genes *atfA* and *ureA* are present in 100% of them. All microorganisms analyzed showed a strong ability to form biofilm. In the adherence test, 100% of the isolates of urinary origin were able to form aggregative adherence (AA); among the isolates from other sources of infection, however, 17.65% were non-adherent (NA) and 82.35% were able to form aggregative adherence (AA). The pathogen showed resistance to most of the drugs tested, mainly ceftriaxone. Therefore, it may be inferred that there were genotypic and phenotypic differences among the isolates of *Proteus mirabilis*, both from urinary and non-urinary sources.

Key-words: Urinary tract infection; antimicrobial agents; virulence genes; Proteus; biofilm; cyrstals.

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LISTA DE ABREVIATURAS E SIGLAS

ATF	*Ambient-temperaure fimbriae*
ITU	Infecção do trato urinário
MR/P	*Mannose-resistant/proteus-like*
PMF	*Proteus mirabilis fimbriae*
PTA	*Proteus toxic agglutinin*
NAF	*Non-agglutinating fimbriae*
UCA	*Uroepithelial cell adhesion*

SUMÁRIO

1 INTRODUÇÃO

O gênero *Proteus* compreende bactérias Gram-negativas pertencentes à família *Morganelacea*, que são frequentemente encontradas no solo, na água, e no trato gastrointestinal de humanos e de animais, representando aproximadamente 0,05% da composição da microbiota intestinal humana (WASFI *et al*., 2020). Este gênero compreende as espécies *P. cibarius*, *P. hauseri, P. mirabilis*, *P. penneri, P. terrae* e *P. vulgaris* (HAMILTON *et al.*, 2018; FUSCO *et al*., 2017). Em 1885, *Proteus mirabilis* foi descoberto pelo patologista alemão Gustav Hause, caracterizando-se como um microrganismo oportunista, sendo o agente causador de infecções humanas envolvendo olhos, ouvidos, pele, trato respiratório, infecção do trato urinário (ITU) (WASFI *et al*., 2020), principalmente nas complicadas e nas ocasionadas por uso de cateter e, no estabelecimento da infecção costuma expressar fatores de virulência, que garantem sua sobrevivência e fixação nas células do hospedeiro (JAMIL, FLORES, SNOWDEN, 2017; MIRZAEI *et al.*, 2019; YUAN *et al.*, 2021).

Comumente, a antibioticoterapia é a estratégia terapêutica mais utilizada para o tratamento das ITU; porém a resistência bacteriana aos agentes antimicrobianos e o risco de recidiva tornam-se fatores limitantes à eficácia do tratamento (BARBER *et al*., 2013*;* FLORES-MEIRELES *et al.*, 2015; KO, CHOI, SONG, 2019).

ITU causadas por *P. mirabilis* encontram-se amplamente descritas na literatura; entretanto, infecções por ele causadas em sítios de infecção que não os do trato urinário não estão totalmente elucidadas, o que justifica a busca de informações a respeito do referido patógeno na fisiopatologia das mesmas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Caracterizar por testes fenotípicos, genotípicos e perfil de sensibilidade a agentes antimicrobianos de *P. mirabilis* visando avaliar o risco de infecções causadas por este microrganismo em vários sítios de infecção.

2.2 OBJETIVOS ESPECÍFICOS

- Detectar os genes associados aos fatores de virulência de *P. mirabilis*;
- Caracterizar o padrão de adesão em células HEp-2;
- Comparar a capacidade de produzir Biofilme;
- Detectar a formação de cristais de estruvita e apatita;
- Avaliar o perfil de sensibilidade a antimicrobianos de uso clínico.

3 REVISÃO DE LITERATURA

3.1 INFECÇÕES CAUSADAS POR *Proteus mirabilis*

Infecções do trato urinário (ITU) consistem em desordens infecciosas relacionadas à invasão de um patógeno no trato urinário e acometem órgãos como rins, bexiga, ureteres e uretra, causando complicações como pielonefrite, cistite, ureterite e uretrite (BARBER *et al*., 2013; FUSCO *et al*., 2017).

ITU representam um dos tipos mais comuns de infecção e mundialmente, estima-se que mais de 150 mil pessoas sejam acometidas anualmente por elas (FUSCO et al., 2017). Podem ser causadas tanto por bactérias Gram-positivas e Gram-negativas quanto por fungos (minoria), tendo como principais agentes etiológicos *Escherichia coli* (75 a 90% dos casos), *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus* e *Proteus mirabilis* (FLORES-MEIRELES *et al.*, 2015; FUSCO et al., 2017; MOHIUDDIN, NASIRULLAH, 2019).

As ITU podem acometem ambos os sexos e sua incidência varia conforme a faixa etária e o sexo do indivíduo, costumando ser mais frequente entre os 20 e 50 anos e em mulheres (FUSCO *et al*., 2017; JAMIL, FORIS, SNOWDEN, 2017). Em homens, a incidência costuma ser maior em idade neonatal e na velhice, principalmente quando o paciente apresenta doenças prostáticas; já em mulheres, é mais comum da infância até os 30 anos (FUSCO *et al*., 2017).

Tradicionalmente, as ITU acometem majoritariamente mulheres por conta do tamanho de sua uretra (mais curta que a dos homens), o que facilita a migração do patógeno dela para a bexiga (BARBER *et al*., 2013). Entre as mulheres, estima-se que 50% serão afetadas durante algum momento da vida, sendo muito comum a recidiva da doença (McLELLAN, HUNSTAD, 2016).

Além do sexo feminino, outros fatores predisponentes à doença incluem presença de comorbidades (diabetes, hipertensão, artrite, entre outras), atividade sexual desprotegida, imunodeficiência, longos períodos de cateterização, cuidados incorretos e má higienização do cateter, histórico de ITUs anteriores, obstrução do trato urinário, baixo nível de estrogênio, micção incompleta e obesidade (BARBER *et al*., 2013; JAMIL, FORIS, SNOWDEN, 2017; MOHIUDDIN, NASIRULLAH, 2019).

Clinicamente, as ITUs são classificadas de acordo com a presença de anormalidades que comprometam o funcionamento adequado do trato urinário do paciente, sendo divididas em complicadas e não complicadas. UTIs não complicadas normalmente acometem indivíduos saudáveis, cujo trato urinário não apresenta anormalidades funcionais ou estruturais, podendo ser divididas em inferior (cistite) e superior (pielonefrite). Nestes casos, *Escherichia coli* é o principal agente etiológico envolvido (mais de 80% dos casos) (BONO, REYGAERT, 2017; FLORES-MEIRELES *et al.*, 2015; KO, CHOI, SONG, 2019). Sintomas associados a UTIs não complicadas incluem disúria (dor ao urinar), presença de sangue na urina (hematúria), micção frequente (poliúria) e vontade repentina de urinar (BONO, REYGAERT, 2017).

Já as ITUs complicadas geralmente afetam indivíduos imunocomprometidos, com comorbidades, grávidas ou que apresentem anormalidades no trato urinário como: insuficiência renal, urolitíase, obstrução urinária, implantação de equipamentos de drenagem (como cateter) e longo tempo de cateterização (FLORES-MEIRELES *et al.*, 2015). Este tipo de ITU costuma ser causada por uma associação de patógenos, a saber, *Proteus mirabilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Providencia stuartii* e *Morganella morganii* (KO, CHOI, SONG, 2019).

O diagnóstico costuma ser feito de acordo com o histórico médico do paciente, sua atividade sexual e os sintomas por ele relatados, e a confirmação ocorre por meio de urinálise e cultura bacteriológica da urina para correta identificação do agente etiológico. No caso de ITUs não complicadas, o diagnóstico é confirmado quando há uma quantidade maior ou igual a 10^5 unidades formadoras de colônia/mL de urina. A partir do diagnóstico adequado, pode-se estabelecer uma estratégia terapêutica adequada às necessidades do paciente (BARBER *et al*., 2013; JAMIL, FORIS, SNOWDEN, 2017; MOHIUDDIN, NASIRULLAH, 2019).

Normalmente, o tratamento de UTIs costuma ser de curta duração (entre 3 dias a 6 semanas) e conta com o uso de antibióticos sistêmicos como nitrofurantoína, cefalosporinas (como a cefalexina), trimetoprima+sulfametoxazol, fluoroquinolonas (como ciprofloxacino e levofloxaxino) e fosfomicina trometamol (Tabela 1) (BARBER *et al*., 2013; CHU, LOWDER, 2018). Geralmente, a maioria dos pacientes consegue observar os primeiros indícios de melhora entre 24 e 48h após o início do tratamento (SABIH, LESLIE, 2017).

O sucesso terapêutico pode ser comprometido por conta da resistência bacteriana ao antibiótico, da não adesão do paciente ao tratamento, bem como em casos de infecção polimicrobiana, hidronefrose ou abscessos perinéfricos, por exemplo (BARBER *et al*., 2013; CHU, LOWDER, 2018; SABIH, LESLIE, 2017). Aliada à resistência bacteriana, o grande risco de recidiva e possível alteração da microbiota intestinal a longo prazo sugerem a busca por alternativas terapêuticas que contornem estes obstáculos, através de medicações que, por exemplo, atuem de forma a neutralizar os fatores de virulência do patógeno, contudo, sem alterar a microbiota intestinal do hospedeiro (FLORES-MEIRELES *et al.*, 2015; KO, CHOI, SONG, 2019).

3.2 Proteus mirabilis

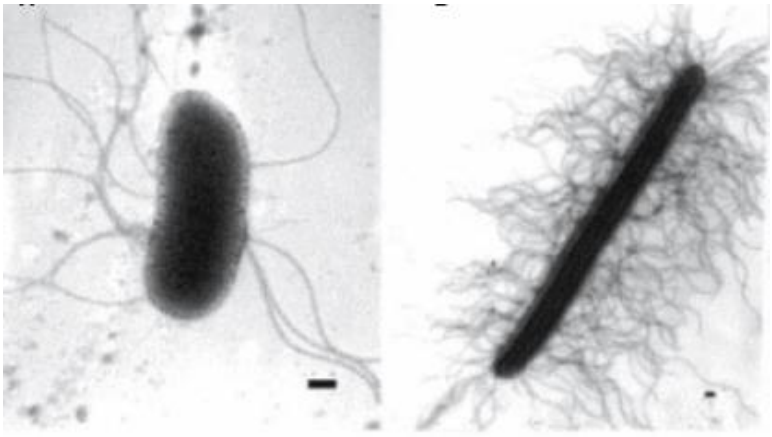
Proteus mirabilis é um bactéria Gram-negativa, anaeróbia facultativa, pertencente à família *Morganelacea*, e pode ser encontrada em diversos ambientes como: fontes de água, solo, esgoto e no trato gastrointestinal de animais e humanos. Este é um patógeno oportunista e responsável por várias doenças humanas envolvendo os tratos gastrointestinal e respiratório, olhos, ouvidos e principalmente o trato urinário (ARMBRUSTER, MOBLEY, PEARSON, 2018; JAMIL, FORIS, SNOWDEN, 2017; WASFI *et al.*, 2020). Complicações associadas à infecção por *P. mirabilis* incluem esplenomegalia, pneumonia, peritonite, meningite, tromboflebite, prostatite, cistite e pielonefrite (JAMIL, FORIS, SNOWDEN, 2017; MOTTA *et al.*, 2012).

As células desta bactéria têm a capacidade de sofrer diferenciação em resposta ao contato com um meio sólido, passando de uma célula vegetativa simples, em forma de bastão, para uma forma mais alongada e com milhares de flagelos, mais conhecida como célula *swarmer* (Figura 1). Este processo de diferenciação é denominado de *swarming* e permite uma locomoção coordenada das células microbianas e, consequentemente, seu crescimento neste meio (MANOS, BELAS, 2006; RATHER, 2005). *P. mirabilis* também secreta um polissacarídeo conhecido como Cmf (*colony migration fator*) que facilita sua locomoção e fixação em superfícies (JAMIL, FORIS, SNOWDEN, 2017; RATHER, 2005).

Em infecções do trato urinário complicadas, como aquelas envolvendo pacientes com cálculos renais ou com uso prolongado de cateter urinário, *P. mirabilis* é frequentemente encontrado *(*CHEN *et al.*, 2012). Pacientes com histórico de infecções

recorrentes, instrumentação uretral, anormalidades no trato urinário ou infecção hospitalar apresentam maior susceptibilidade a infecções por *Proteus spp* (JAMIL, FORIS, SNOWDEN, 2017).

Figura 1 – Diferenciação celular de *Proteus mirabilis*: da forma *swimming* (à esquerda) para a forma *swarmer* (à direita)



Fonte: Adaptado de Manos, Belas (2006)

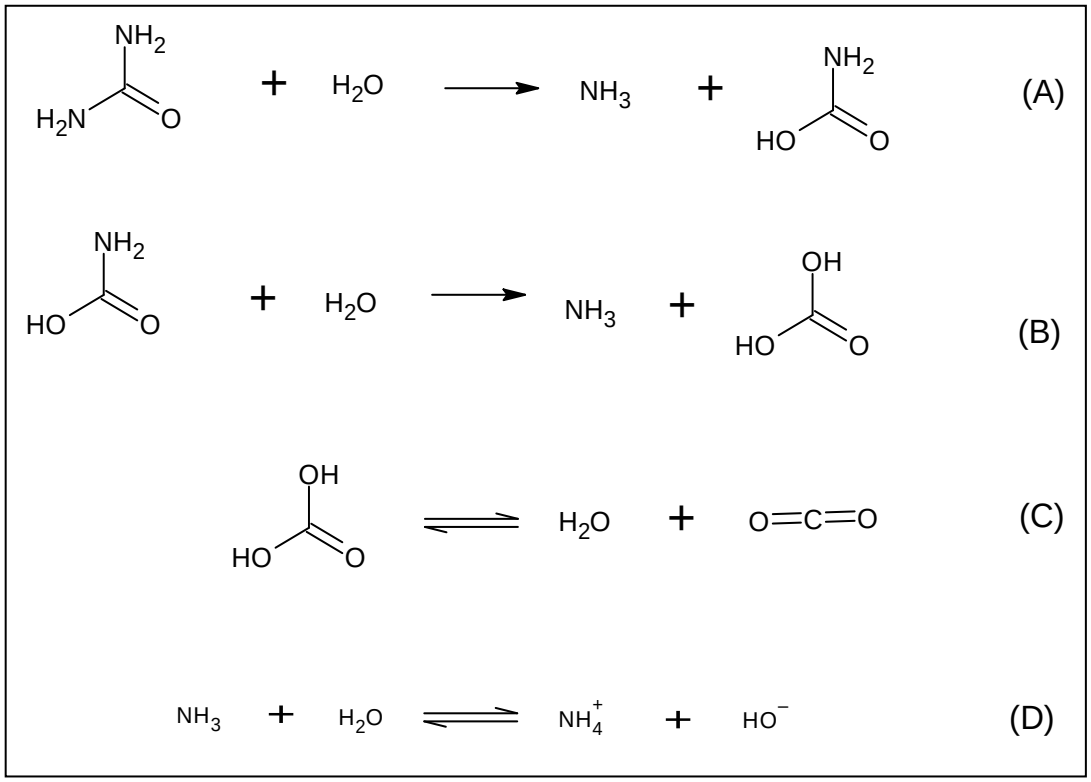
3.3 FATORES DE VIRULÊNCIA DE *Proteus mirabilis*

Para estabelecer uma infecção no trato urinário do hospedeiro, *P. mirabilis* utiliza-se de diversos fatores de virulência, expressos no curso da infecção, como: flagelos, urease, adesinas, toxinas, proteases, sistemas para captação de metais (ferro e zinco) e formação de biofilme (PELLEGRINO *et al.*, 2013; YUAN *et al.*, 2021).

3.3.1 Urease

A urease é uma metaloenzima dependente de níquel responsável por catalisar a hidrólise de ureia em dióxido de carbono e amônia (Figura 2), que serve como fonte de nitrogênio para o microrganismo, possibilitando sua sobrevivência (GRAHL *et al.*, 2021; YUAN *et al.*, 2021).

Figura 2 – Reação de hidrólise da ureia catalisada pela urease de *P. mirabilis*: cada molécula de ureia (reação A) origina duas moléculas de amônia (reações A e B) e uma de gás carbônico (reação C). A amônia em meio aquoso é protonada, gerando os íons amônio e hidroxila, tornando o meio alcalino (reação D).



A amônia é toxica e causa elevação acentuada do pH da urina (para aproximadamente 9,0), o que ocasiona a precipitação de íons solúveis (como Mg^{2+} e Ca^{2+}) na forma de sais insolúveis como carbonato apatita [$Ca_{10}(PO_4)_6{\cdot}CO_3$] e estruvita ($MgNH_4PO_4{\cdot}6H_2O$), que promovem a formação de cálculos renais (urolitíase) em órgãos do sistema urinário e/ou depósitos cristalinos em cateteres (ARMBRUSTER, MOBLEY, PEARSON, 2018; BROLL, 2013; NORSWORTHY, PEARSON, 2017; YUAN *et al.*, 2021).

A urolitíase pode causar obstrução do sistema urinário e impedimento do fluxo de urina, resultando em complicações como a distensão dolorosa da bexiga (devido à retenção urinária), refluxo vesicoureteral, pielonefrite, infecção ascendente, bacteriúria e até mesmo sepse (ARMBRUSTER, MOBLEY, 2012; GRAHL *et al.*, 2021). Já para o patógeno pode ser benéfica, pois o cálculo o protege da ação de antibióticos e do sistema imunológico do hospedeiro e além disso, a amônia produzida pode danificar as células epiteliais do trato urinário do hospedeiro, disponibilizando seus nutrientes para o

microrganismo, o que favorece sua sobrevivência (NORSWORTHY, PEARSON, 2017; YUAN *et al.*, 2021).

3.3.2 Adesinas

A adesão do patógeno às células uroepiteliais é uma etapa fundamental à colonização bacteriana e estabelecimento da infecção no trato urinário e para isso, *P. mirabilis* conta com apêndices extracelulares, denominados de fímbrias (HASAN, ALASEDI, JALOOB, 2021, YUAN *et al.*, 2021). De natureza proteica, as fímbrias se estendem pela superfície da bactéria na forma de filamentos, geralmente com 1 a 2 µm de comprimento e 2 a 8 nm de diâmetro, e contam com proteínas adesivas (adesinas) em suas extremidades para melhor fixação no tecido-alvo (JUGE, 2012).

P. mirabilis possui elevada capacidade adesiva devido à alta quantidade de fímbrias que é capaz de codificar; entretanto, as mais comumente estudadas são: *mannose-resistant/Proteus-like* (MR/P), *uroepithelial cell adhesion* (UCA) – ou *non-agglutinating fimbriae* (NAF), *ambient-temperature fimbriae* (ATF) e *Proteus mirabilis fimbriae* (PMF), (PELLEGRINO *et al.*, 2013, YUAN *et al.*, 2021).

A fímbria mais importante e mais estudada é a MR/P por conta do papel que desenvolve na adesão ao epitélio do hospedeiro (HAMILTON *et al*., 2018). Logo após a ligação inicial à superfície, a bactéria produz a referida fímbria, que viabiliza a colonização da bexiga e dos rins, bem como contribui para a formação de biofilme. A expressão de MR/P é favorecida sob baixo suprimento de oxigênio e desencadeia uma forte resposta imunológica no hospedeiro (FLORES-MEIRELES *et al.*, 2015; RÓŻALSKI *et al*., 2012).

Em 1986, Wray e colaboradores identificaram UCA em *P. mirab*ilis, sendo a primeira fímbria a ser descoberta. Também conhecida como *non-agglutinating fimbriae* (NAF), UCA tem um papel fundamental à patogênese de ITU em seu estágio inicial, podendo ser expressa sob diversas condições ambientais e aderir a células uroepiteliais descamadas, HEp-2 e EJ28 por exemplo (PELLEGRINO et al., 2013). Possivelmente, esta fímbria seja mais importante na colonização dos rins do que da bexiga (PELLEGRINO et al., 2013; SCHAFFER, PEARSON, 2017).

Já ATF é uma fímbria cuja temperatura ótima de expressão é de 23°C (YUAN *et al.*, 2021). Inicialmente, pensava-se que esta fímbria não exercia qualquer papel na

patogênese da infecção; entretanto, o estudo de Scavone et al. (2016) aponta que sua atividade esteja relacionada à adesão em superfícies abióticas e formação de biofilme.

Por fim, a função da fímbria PMF foi verificada principalmente por dois estudos: no primeiro, Massad et al. (1994) sugeriram que esta fímbria era importante na colonização da bexiga e não dos rins; já Zunino et al. (2003) contrariaram esta declaração, sugerindo sua importância também na colonização bacteriana dos rins. Segundo Scavone et al. (2016), PMF contribui para a formação de biofilmes sobre superfícies abióticas.

3.3.3 Proteases

Para sobrevivência no trato urinário, *P. mirabilis* produz uma variedade de proteases com o objetivo de combater os mecanismos de defesa do hospedeiro, incluindo peptídeos antimicrobianos e anticorpos (SCHAFFER, PEARSON, 2017). As proteases são produzidas por muitas bactérias Gram-negativas e constituem-se um importante fator de virulência na patogênese de ITU, atuando em processos essenciais à colonização bacteriana e estabelecimento da infecção no hospedeiro (CARSON *et al.*, 2011).

P. mirabilis produz uma metaloprotease dependente de zinco conhecida como ZaPa (ou mirabilisina). Esta é expressa em células *swarmer* e capaz de degradar imunoglobulinas, como IgA e IgG, além de proteínas do citoesqueleto (tubulina e actina), componentes da matriz celular (laminina, fibronectina e colágeno) e peptídeos antimicrobianos (como catelicidina e β-defensina-1). Assim, a bactéria consegue combater o sistema de defesa do hospedeiro durante o processo infeccioso (SCHAFFER, PEARSON, 2017; YUAN *et al.*, 2021).

Tal fator de virulência constitui-se um possível alvo terapêutico e por isso, inibidores desta metaloprotease são uma alternativa terapêutica para tratamento de ITUs (CARSON *et al.*, 2011; SCHAFFER, PEARSON, 2017).

3.3.4 Toxinas

Hemolisina e *Proteus toxic agglutinin* (Pta) são duas toxinas produzidas por *P. mirabilis* e ocasionam danos no tecido do trato urinário, além de estar associadas ao surgimento de pielonefrite e cistite (YUAN *et al.*, 2021).

3.3.4.1 Hemolisina

O gênero *Proteus* pode sintetizar dois tipos de hemolisinas: HlyA e HpmA; porém, somente a última é expressa por *P. mirabilis* (HAMILTON *et al*., 2018). A hemolisina HpmA é uma toxina dependente de cálcio (Ca^{2+}) capaz de invadir a membrana celular do hospedeiro, formar poros e causar o extravasamento de íons (como Na^{+}). Também causa lise celular, não só de células epiteliais, mas também de eritrócitos e células do sistema imunológico, como monócitos e células B, além de estar associada à pielonefrite em casos de infecções ascendentes (HAMILTON *et al*., 2018; HASAN, ALASEDI, JALOOB, 2021; YUAN *et al.*, 2021).

Os genes *hpmA* e *hpmB* são responsáveis por codificar a hemolisina HpmA, sendo que *hpmB* transporta e ativa *hpmA* e este, por sua vez, codifica a secreção da toxina nas células do hospedeiro (HASAN, ALASEDI, JALOOB, 2021). Possivelmente, as hemolisinas contribuem para infecções gastrointestinais através da indução do inflanossoma NLRP3 (do inglês *NOD-like receptor protein 3*), da liberação de interleucina-1 β e da lise de células do sistema imunológico (HAMILTON *et al*., 2018).

3.3.4.2 *Proteus toxic agglutinin* (Pta)

A toxina Pta é uma protease citotóxica capaz de perfurar a membrana celular do hospedeiro, causando desregulação da pressão osmótica, extravasamento do conteúdo citoplasmático e despolimerização dos filamentos de actina, com consequente comprometimento da integridade da estrutura celular (KO, CHOI, SONG, 2018; YUAN *et al.*, 2021). Além da ação citotóxica contra células epiteliais do trato urinário, esta toxina ainda possui função aglutinadora, ocasionando a autoagregação das células bacterianas (KO, CHOI, SONG, 2018; RÓŻALSKI *et al*., 2012).

Pta é codificada pelo ICEPm1, que é um autotransportador presente na parte externa da membrana celular do patógeno. Este transportador atua no processo de autoagregação das células de *P. mirabilis* e graças a um domínio α-catalítico ativo presente em sua estrutura, consegue causar a disruptura das células do fígado e dos rins, resultando na danificação destes órgãos. E tal como o pH gerado pela atividade da enzima urease, o funcionamento desta toxina também ocorre somente em meio alcalino (ARMBRUSTER, MOBLEY, 2012; HASAN, ALASEDI, JALOOB, 2021; KO, CHOI, SONG, 2018).

3.3.5 Biofilmes

Biofilmes consistem em conglomerados de células microbianas, de uma ou várias espécies, que se encontram fortemente ligadas entre si e a um substrato, sendo esta ligação irreversível. Em seu interior, tais células são envoltas por uma matriz extracelular constituída por lipídeos, proteínas, polissacarídeos e DNA extracelular (WASFI *et al.*, 2020).

A formação de um biofilme é um processo que ocorre em múltiplas etapas e se inicia com a adesão bacteriana a uma superfície, seja ela biótica ou abiótica, através das adesinas. Após a adesão, as células bacterianas ligam-se irreversivelmente, formando uma microcolônia e posteriormente, o biofilme maduro, que representa uma fonte de contaminação, uma vez que de sua superfície são liberadas células do patógeno que se disseminam em condições ambientais favoráveis (ALI, 2012; WASFI *et al.*, 2020).

Algumas espécies de microrganismos, como *P. mirabilis*, se utilizam deste fator de virulência como estratégia para sobrevivência em condições ambientais severas, pois o biofilme constitui uma barreira física que impede a penetração de agentes antimicrobianos em seu interior, bem como a ação do sistema imunológico do hospedeiro sobre o patógeno (WASFI *et al.*, 2020).

Uma característica peculiar de *P. mirabilis* é sua capacidade de formar biofilmes cristalinos, que podem obstruir cateteres e agravar as ITUs associadas a cateterização, além de aumentar o risco de urolitíase (ITU-AC) (ALI, 2012; ARMBRUSTER, MOBLEY, PEARSON, 2018; WASFI *et al.*, 2020). Fatores de virulência que contribuem à formação deste tipo de biofilme incluem a enzima urease, motilidade *swarming*, fímbrias e polissacarídeos capsulares (WASFI *et al.*, 2020).

P. mirabilis é capaz de se fixar em superfícies de materiais clínicos feitos de vidro, sílica gel, látex e poliestireno, alterando suas propriedades (ALI, 2012; ARMBRUSTER, MOBLEY, PEARSON, 2018). Na formação de biofilme no cateter, inicialmente, esta bactéria adere à sua superfície através das adesinas. Então, a partir da urina alcalinizada pela ureia (gerada pela atividade da urease) ocorre a precipitação de cristais de estruvita e hidroxiapatita e estes se incorporam ao aglomerado de células bacterianas, dando origem ao biofilme cristalino (ARMBRUSTER, MOBLEY, PEARSON, 2018; WASFI *et al.*, 2020).

O desenvolvimento de biofilmes em cateteres pode bloquear completamente o fluxo urinário através dele, levando a complicações como distensão dolorosa da bexiga (provocada pela urina retida na bexiga), que leva a complicações como pielonefrite, refluxo urinário, pielonefrite e septicemia. Ademais, a formação de biofilme pode aumentar o risco de urolitíase (ARMBRUSTER, MOBLEY, PEARSON, 2018; YUAN et al., 2021).

Estratégias comuns para prevenir a formação de biofilme em cateteres consistem na substituição do cateter incrustado, além da limitação do tempo de cateterização. Novas estratégias incluem a confecção de cateteres com materiais alternativos, emprego de revestimentos antimicrobianos, controle do pH urinário e dispersão elétrica ou mecânica do biofilme (ARMBRUSTER, MOBLEY, PEARSON, 2018).

3.3.6 Captação de metais

Durante o processo infecioso, o hospedeiro e o patógeno costumam competir pela captação de micronutrientes (como ferro, zinco e níquel), essenciais à sua sobrevivência. Enquanto que o primeiro tenta isolar os nutrientes para si e privar o microrganismo de absorvê-los, este, por sua vez, também possui a habilidade de captá-los através de sistemas especializados (YUAN *et al.*, 2021).

Como o ferro é um elemento fundamental ao funcionamento adequado de enzimas e proteínas do patógeno e o trato urinário é um ambiente com baixa disponibilidade deste elemento, *P. mirabilis* tem a capacidade de codificar sistemas de captação deste nutriente, que incluem transportadores ABC, transporte de íons ferrosos (Fe^{2+}) e sistemas baseados em sideróforos (ARMBRUSTER, MOBLEY, 2012; SCHAFFER, PEARSON, 2017).

Sideróforos são agentes quelantes com alta afinidade pelo íon férrico (Fe^{3+}), afinidade esta maior do que com os sistemas de captação do hospedeiro. O íon liga-se ao sideróforo formando-se um complexo sideróforo-ferro, que é absorvido para o meio intracelular por um receptor especifico da membrana externa do microrganismo (ARMBRUSTER, MOBLEY, PEARSON, 2018; SCHAFFER, PEARSON, 2017). *P. mirabilis* se utiliza do sistema sideróforo de peptídeos não ribossomais (Pnr) e também de α-cetoácidos e proteobactina como sideróforos (SCHAFFER, PEARSON, 2017).

Zinco também é outro elemento essencial ao metabolismo de *P. mirabilis*, garantindo um funcionamento adequado de suas enzimas e proteínas, além da síntese de flagelos. O patógeno possui um sistema para captação do íon Zn^{2+} (sistema ZnuABC), que permite seu desenvolvimento mesmo em ambientes com restrição de zinco, como o trato urinário (ARMBRUSTER, MOBLEY, 2012; SCHAFFER, PEARSON, 2017; YUAN *et al.*, 2021). Aponta-se que o zinco esteja relacionado a dois outros fatores de virulência - os flagelos e a metaloproteinase ZaPA – e caso o sistema de captação não funcione adequadamente, a expressão destes fatores fica comprometida e, consequentemente, a colonização bacteriana no trato urinário (SCHAFFER, PEARSON, 2017).

3.4 SENSIBILIDADE A AGENTES ANTIMICROBIANOS

P. mirabilis é sensível a ativos antimicrobianos das classes dos aminoglicosídeos, fluoroquinolonas, β-lactâmicos e à trimetoprima+sulfametoxazol e apresenta resistência a tetraciclinas e nitrofurantoína. Este patógeno pode apresentar resistência a antibióticos, contribuindo para elevação nas taxas de mortalidade em pacientes hospitalizados. Esse fator incentiva o desenvolvimento de novas alternativas terapêuticas (CHEN *et al.*, 2012).

P. mirabilis apresenta diversos mecanismos de resistência a agentes microbianos e estes incluem o efluxo do fármaco, a alteração da permeabilidade da membrana celular (para evitar a entrada do antibiótico em suas células), a produção de substâncias degradadoras do fármaco (como as β-lactamases) (FURLAN *et al.*, 2021).

A susceptibilidade de *P. mirabilis* a antibióticos pode variar de acordo com a localização geográfica (WANG *et al.*, 2014): no Brasil, por exemplo, o estudo de Magalhães et al. (2008) com mulheres acima de 18 anos, em Recife, revelou que este patógeno foi resistente a ampicilina (em 24,32% dos casos), a trimetoprima+sulfametoxazol (18,92% dos casos) e a quinolonas (5,41% dos casos); já o trabalho de De Oliveira et al. (2021), em Londrina, mostrou que esta bactéria foi sensível a todos os antimicrobianos testados (100% dos casos).

Estudos em várias partes do mundo têm verificado a susceptibilidade a antimicrobianos deste microrganismo, por exemplo: o estudo de Lin e colaboradores (2019) a respeito da susceptibilidade antimicrobiana em hospitais do norte de Taiwan revelou que a maioria dos isolados de *P.mirabilis* (90%) foram susceptíveis a antibióticos como carbapenem, amicacina, cefalosporinas de terceira geração (como ceftazidima) e

piperaciclina+tazobactam e cefepima; enquanto que 47% foram susceptíveis a trimetoprima+sulfametoxazol, 58% a floroquinolonas, 4% a cefazolina e 100% a cefmetazol. Já o estudo de Sader e colaboradores (2014) com isolados de pacientes de hospitais dos Estados Unidos e da União Europeia indicou que cerca de 90% dos isolados foram susceptíveis a ampicilina, tobramicina, tigeciclina, imipenem, levofloxacino, gentamicina e ciprofloxacino.

Sokhn e colaboradores (2020), relataram altos índices de susceptibilidade (entre 72,0 e 96,0%) em pacientes hospitalizados no Líbano; Zanichelli e colaboradores (2019) conduziram um estudo entre 2009 e 2016, na Suíça, e verificaram que nitrofurantoína foi o antibiótico ao qual as cepas foram mais resistentes (mais de 98%), seguido de cotrimoxazol (30,5%), fosfomicina e quinolonas (abaixo de 20%) e cefalosporinas de 3ª e 4ª gerações (cerca de 1%). Já Smaoui e colaboradores (2015) conduziram pesquisas na Tunísia e descobriram que os isolados foram mais resistentes a amoxicilina (58,3%) e totalmente susceptíveis à gentamicina e tobramicina (100%).

Agentes antimicrobianos não são capazes de distinguir entre as bactérias patogênicas e as benéficas e por conta disso, acabam inibindo ou mesmo eliminando todas as bactérias que são susceptíveis ao fármaco escolhido. Ademais, os genes de resistência podem ser transferidos a outras bactérias do próprio hospedeiro ou de um novo hospedeiro; consequentemente, durante a aplicação do tratamento, pode haver um aumento na quantidade de bactérias resistentes, além da alteração da microbiota do hospedeiro (SCHWARZ, LOEFFLER, KADLEC, 2017).

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5 ARTIGO CIENTÍFICO I

Draft genome sequence of *Proteus mirabilis* strain LBUELH-11, isolated from human tracheal secretion of hospitalized patient

Abstract

Proteus mirabilis is an important human pathogen. This study is the first Brazilian isolate sequenced. *Proteus mirabilis* strain LBUELH-11 have 4.141.164 bp and 99.13% of similarity with *Proteus mirabilis* PM52260 and *Proteus mirabilis* AR0155 with 99.06%, suggesting by phylogenomic analysis that the LBUELH-11 is a *P. mirabilis*.

ANNOUCEMENT

The genus Proteus is responsible for several hospital infections, such as: respiratory tract infections, wounds, eyes, nose, throat, skin, gastroenteritis and urinary tract infection. They can be found in soil, water and urinary tract of different mammals [1];[2]. Proteus spp. rods are typical opportunistic pathogens, relatively infectious and contribute to the infections mostly in immunocompromised patients. Those infections are usually long-term and difficult to cure [3]. Among the species of this genus the most prevalent in infections in humans is *Proteus mirabilis*, a Gram-negative bacterium, has main virulence factors adesins, hemolysins, toxins, capsule, siderophores, urease and proteases [4]. In this study, we report the draft genome sequence of *Proteus mirabilis* LBUELH-11, isolated in 2015 of tracheal secretion of a 64-year-old male hospitalized patient in the city of Londrina, Brazil. The strain was deposited at the Bacteriology Collection of the Bacteriology Laboratory, Universidade Estadual de Londrina.

DNA was extracted with the Gentra Puregene Genomic DNA kit, Qiagen Brazil, according to the manufacturer's procedure, and then quantified. Sequencing was carried on the Illumina

MiSeq platform, with a MiSeq version 3 reagent kit (600-cycle, Illumina, Brazil) at the Soil Biotechnology Laboratory in Embrapa Soja, Londrina, Paraná, Brazil. The reaction generated 5.351.396 paired-end reads with a maximum length of 300 bp providing a coverage of around 380-fold. Quality of the reads was assessed via FastQC v0.11.9 [5], using its report the trimming parameters were selected for read trimming using Trimmomatic v0.39. Filtered reads were used for contig assembly using SPAdes v. 3.13.0 [6]. All the assemblies with different k-mers were compared using QUAST v5.0.2. Gurevich [7] and the best one was selected according to N50 values (22.713 bp), number of contigs (369), largest contig and total length of the assembly. Contigs were mapped to a reference genome for scaffolding using CONTIGuator v2.7 [8]. Genome size was estimated at 3.973.423 bp with a G+C content of 39.6%. Annotation was carried out with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) finding 3,878 CDS within the chromosome. Contigs that did not found similarity to the reference during scaffolding were analyzed using BLASTN for plasmid similarity, 2 plasmids named p1LBUEL11 and p2LBUEL11 were found and assembled respectively, with a size of 7171 bp and 160563 bp. Genomics comparisons were made with ANI Calculator by EZBioCloud [9]. LBUELH-11 showed 99.13% of similarity with *Proteus mirabilis* PM52260 and *Proteus mirabilis* AR0155 with 99.06%, suggesting by phylogenomic analysis that the LBUELH-11 is a *Proteus mirabilis*.

Using ResFinder [10] it was found that the chromosome possesses 2 genes for resistance against Phenicol and Tetracycline antibiotics. The p1LBUEL11 plasmid harbours resistance genes against: Sulphonamides; Tetracyclines; Trimethoprim; Beta-lactam and Aminoglycoside. The second plasmid p2LBUEL11 possesses one resistance gene against Aminoglycoside antibiotics. The genome of *Proteus mirabilis* strain LBUELH-11 may help to explore its pathogenic potential.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The genome sequence was deposited in the NCBI-GenBank accession number CP086377 (Bioproject PRJNA775607, BioSample SAMN22602883, Proteus_mirabilis_LBUELH11_p1, Proteus_mirabilis_LBUELH11_p2).

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7 ARTIGO CIENTÍFICO II

Review

Epidemiology of antibiotic resistance of Proteus mirabilis isolates from human and non-human sources

Abstract: *Proteus mirabilis* can cause infections in humans and animals. This bacterium has several factors that contribute to virulence and antimicrobial resistance. Antimicrobial resistance is a serious problem in infections of food, veterinary infections and infectious diseases in humans, and has been reported in several countries. *P. mirabilis* may also present multidrug-resistance to antibiotics, regardless of the isolation site. Worldwide studies have reported resistance to β-lactams, fluroquinolones, fosfomycin, aminoglycosides, and sulfonamides. Due to few studies on the susceptibility profile and antimicrobial resistance of *P. mirabilis* from non-human sources, it is difficult to compare human and non-human isolates. In this sense, the present study aimed to evaluate the worldwide epidemiology of resistance of *P. mirabilis* from human and non-human sources, because there are no studies aiming to survey the epidemiological resistance of this species.

Keywords: *P. mirabilis*; antimicrobial resistance; human and non-human isolates.

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1. Introduction

Proteus spp. are common inhabitants of the soil and part of the normal microbiota of the enteric region of man and animals. They are opportunistic pathogens responsible for causing infections at several human anatomical sites [1,2]. Among the species of this genus, *Proteus mirabilis* is responsible for the most common opportunistic nosocomial infections in humans, such as urinary tract infections (UTI), wounds, otitis, and other infections [1]. Some studies have reported the isolation of this bacterium from non-human sources, such as from UTI in companion animals [3,4]. Other studies evaluated the antimicrobial susceptibility profile of this microorganism when isolated from food sources [5,6].

P. mirabilis has several virulence factors, including antibiotic resistance genes [7,8,9,10,11,12] and has been identified in several outbreaks of nosocomial infections and community-acquired infections in different parts of the world [7,13,14,15,16].

Antimicrobial drug resistance has become a global concern recognized by the World Health Organization [7,17]. Several studies around the world have reported resistance of *P. mirabilis* to several classes of antibiotics used against infections in humans, animals, and food sources. Resistance to β-lactams, fluroquinolones, fosfomycin, aminoglycosides, and sulfonamides has been reported, as well as the natural resistance to tetracyclines, nitrofurantoin, and polymyxin [18,19,20].

For the writing of this article, we searched the databases, such as Pubmed, Google Scholar and Scielo, and selected 60 articles around the world investigating antibiotic resistance of *P. mirabilis*. These studies included human populations (community patients and hospital patients) and resistance of *P. mirabilis* in animals or food sources. The key words used were: *P. mirabilis*, antibiotic resistance, food production, animals.

2. Antibiotic resistance of *P. mirabilis* isolated from human infections

2.1. Asian continent

Extended Spectrum Beta-Lactamase (ESBL) producing bacteria have been isolated worldwide. In Japan, the increased isolation of ESBL producing bacteria has been observed since 2000. A study conducted in Japan from September to November 2012, by Shibasaki *et al*. [18] in 2016, investigated ESBL-producing bacteria in community-acquired infections. Eleven hospitals across Japan participated in the study, where the SHV, TEM, CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9 genotypes were investigated using the Polymerase Chain Reaction (PCR) technique. Community acquired infection was defined as an infection detected from

outpatient samples or submitted within 48 hours of hospitalization, while a hospital infection was defined as an infection detected in specimens submitted 48 hours or more after admission. It was observed that 17 of 107 (15.9%) strains of *P. mirabilis* isolated in this study were ESBL producers, all belonging to the CTX-M-2 group of enzymes. Nakamura *et al*. (2012) [21] found that isolation rates of *P. mirabilis* from ESBL producers increased from 0 to 12.9% in the Kinki region of Japan from 2000 to 2009.

Goltsman *et al*. (2018) [22] conducted a study in a geriatric hospital in Israel in 2017. Due to the high frequency of antimicrobial resistance by ESBL, this study indicated that *P. mirabilis* was the most common isolate from specialized wards (29.6%) and mechanical ventilation (42.1%). Of 200 isolates, 15 were collected from blood and 185 from urine. *P. mirabilis* was resistant to ceftriaxone in 54% of isolates, ciprofloxacin in 57%, trimethoprim/sulfamethoxazole in 62% and nitrofurantoin in 84%; 40% of the isolates were positive for ESBL.

In a study carried out in a hospital in Saudi Arabia, the authors found that 94% of *P. mirabilis* isolates were multiresistant. A resistance rate of 29.3% and 25.8% was observed for the antibiotics imipenem and meropenem, respectively, among the isolates. Among the species tested in this study, *P. mirabilis* exhibited the highest rate of resistance to cefepime (76.8%). The high resistance rates found in this study can be explained by the biofilm formation that occurs in this species, a phenomenon that not only helps to avoid the lethal effect of antimicrobials, but also induces bacterial changes to resist and escape the host's defenses. In addition, the free availability of antimicrobials and non-adherence to treatment, which are frequent in Saudi Arabia, may be associated with increased resistance and the emergence of multiresistant strains [23]. Studies in China show that, in hospitalized patients, *P. mirabilis* presents high resistance to fluoroquinolones, ceftriaxone, ceftazidime, cefepime, penicillins, sulfonamides and monobacchaeam, a complex resistance to multiple drugs, besides being able to produce beta-lactamases [24]. In addition, of the community acquired patient isolates, from several Chinese hospitals, 39% were ESBL positive and had sensitivity to meropenem and biapenem, but 23.4% were not sensitive to imipenem. These results indicate that there was an increase in resistant ESBL-producing strains in community-acquired infections [25]. Yang & Ji (2020) [26] conducted a study in a Chinese hospital with hospitalized patients and found the following rates for the species *P. mirabilis*: resistance greater than 90% for ampicillin; greater than 80% for sulfamethoxazole, levofloxacin and ciprofloxacin and greater than 70% for cefazolin and gentamicin; the most susceptible antibiotics were Cefoxitin, Imipenem and Amikacin (sensitivity greater than 80%); meropenem and piperacillin/tazobactam (sensitivity greater than 90%). Another study guided by Guan et al. (2021) [27], in China, found high resistance rates of *P. mirabilis* to the antimicrobials cefazolin (72.9%), ciprofloxacin (64.6%) and ampicillin (62.5%).

Wang *et al*. (2014) [28] carried out a national study between 2002 and 2012 on the antimicrobial susceptibility of *P. mirabilis* in several hospitals in Taiwan and included both hospital and community patients. The authors evaluated 1157 isolates from different sources (urine, blood, pus, sputum) and obtained the following sensitivity results: amoxicillin + clavulanate (84.6%), ampicillin (34.3%), aztreonam (99.3%), cefazolin (71.9%), cefuroxime (87.5%), cefotaxime (85.7%), ceftazidime (97.2%), cefoxitin (94.2%), cefepime (97.4%), ertapenem (99.7%), imipenem (99.8%), meropenem (100%), piperacillin (43.4%), amikacin (90%), gentamicin (57.7%), ciprofloxacin (68.7), and trimethoprim + sulfamethoxazole (34.1%). The prevalence of isolates producing ESBL and AmpC was 8.2 and 4.7%, respectively. This study revealed a significant decrease in the sensitivity of *P. mirabilis* to cefotaxime, ceftazidime and ciprofloxacin in Taiwan in the last decade. In addition, the authors compared the data with recent reports from the United States, Canada, and the United Kingdom, [29,30,31,32] which showed that *P. mirabilis* in Taiwan had lower rates of susceptibility to cefotaxime (85.7% vs. 97% in the USA, Canada, and the United Kingdom) and gentamicin (57.7% vs. 90%). Susceptibility to ciprofloxacin was also much lower than rates found in the United States and Canada (68.7% vs. 80%). The increase in β-lactamases and AmpC producers over the years that was observed in the present study may be due to clonal dissemination and horizontal gene transfer. The reduction in susceptibility to ciprofloxacin during study periods may be due to the increased consumption of fluoroquinolones in Taiwan in recent years. Although the prevalence of ESBL producers remained stable, producers of β-lactamase AmpC increased significantly during the study years. It is important to note that only one isolate showed no susceptibility to ertapenem. Therefore, carbapenemases do not currently appear to prevail in *P. mirabilis* in Taiwan.

2.2. African Continent

In Ethiopia, Engda *et al*. (2018) [19] the magnitude, distribution, and antimicrobial susceptibility of ESBL-producing Enterobacteriaceae in reference hospital settings at the University of Gondar. From a total of 384 samples, 14.8% were ESBL-producing Enterobacteriaceae, where 7.01% of the isolates were *P. mirabilis* (isolated from bed frames, floors and sinks) and were resistant to ceftriaxone, ceftazidime, cefpirome, cefpodoxime, and amoxicillin/clavulanic acid. There is also a threat from this pathogen due to the recurrent isolation of CMY-2 beta-lactamase-containing strains worldwide [33,34,35].
A study on antibiotic resistance of Enterobacteria responsible for community-acquired UTI was conducted in Tunisia. Of the 2511 isolates, 84 (3.3%) were *P. mirabilis* and had resistance to amoxicillin (58.3%), amoxicillin/clavulanic acid (11.9%), cefotaxime (4.8%), gentamicin (0%), tobramycin (0%), nalidixic acid (9.5%), ofloxacin (4.8%), ciprofloxacin (3.6%), cotrimoxazole (16.7%), and fosfomycin (13.1%). As observed, the study showed a high resistance of *P. mirabilis* to amoxicillin (58.3%) [36].
In Nigeria, the most common classes of antibiotics employed are third generation cephalosporins, fluoroquinolones, and aminoglycosides[7] since the determining genes of resistance are: ESBL (TEM, CTX-M, SHV, and OXA), AmpC Beta-Lactamases (CMY, DHA, and ACT), plasmid-mediated quinolones (*qnrA*, *qnrB*, *qnrD*, *aac(6')-Ib-cr* e *qepA*), and resistance to aminoglycosides (*aac(6')-Ib*) [34, 35]. However, several authors have reported resistance of *P. mirabilis* to these classes of antibiotics [39,40,37,41].

2.3. European continent

On the European continent, studies on antibiotic sensitivity involving *P. mirabilis* are found more frequently than other continents.
In Croatia, Bedenic *et al*. (2016) [42] carried out a survey about the sensitivity of *P. mirabilis* strains obtained from a nursing home and found that the isolates showed resistance to amoxicillin alone and combined with clavulanic acid, piperacillin, cefuroxime, cefoxitin, gentamicin, ciprofloxacin, sulfamethoxazole / trimethoprim, and norfloxacin; but were susceptible to cefepime, imipenem, and meropenem. There was a variable susceptibility to ceftazidime, cefotaxime, ceftriaxone, and the combination of piperacillin and tazobactam. Meropenem was the most potent antibiotic. Another Croatian study with hospitalized patients evaluated 288 multidrug-resistant (MDR) bacterial isolates collected from seven medical centers, which were tested from February 2014 to October 2016 for susceptibility to fosfomycin. They observed that 82.6% of the isolates were susceptible to fosfomycin: *E. coli* ESBL 97%, *K. pneumoniae* ESBL 80%, Enterobacter 85.7%, *C. freundii* 100%, *P. mirabilis* 93%, and *P. aeruginosa* 60%. Thus, the study suggests that fosfomycin may be an effective treatment option for infections caused by MDR bacteria in the hospital environment in Croatia. [20]. Rubic et al. (2020) [43] evaluated 100 strains of *P. mirabilis* multiresistant and AmpC-producing and isolated from different biological materials in a Croatian Hospital. Regarding the resistance profiles found in this study, the authors highlight the concern with the emergence of AmpC enzymes acquired among clinical isolates and that cause resistance to cephalosporins, a phenomenon that combined with other resistance mechanisms, drastically reduces the choice of therapeutic options. In addition, the authors highlight the high potential for clonal dissemination of these strains in the clinical environment.
In Italy, Mezzatesta *et al*. (2017) [44] conducted a study on the activity of fosfomycin against MDR uropathogens and compared the activity of this drug with other antibiotics commonly used in the treatment of UTI. A total of 106 clinical isolates from urinary catheters were collected in 2014. Of these, 10 were *P. mirabilis*, ESBL producers and the results showed that fosfomycin was effective against all of them. Cotrimoxazole and levofloxacin were active against 80% of strains while amoxicillin/clavulanic acid, cefuroxime and ciprofloxacin were active against 100% of *P. mirabilis* strains.
In Portugal, Costa *et al*. (2017) [45] evaluated the frequency of antimicrobial resistance of bacteria commonly causing UTI between 2011 and 2014 in community patients. The isolates of *P. mirabilis* presented the following resistance rates: ampicillin: 48.1%; amoxicillin + clavulanic acid: 17.3%; cephalothin: 23.9%; cefaclor: 19.6%; cefuroxime: 21.3%; cefoxitin: 10.7%; cefotaxime: 5.5%; cefixime: 6.8%; ceftazidime: 7.1%; ertapenem: 3.4%; imipenem: 40.9%; ciprofloxacin: 30.4%; gentamycin: 14.8%; tobramycin: 12.6%;

erythromycin: 4.2%; fosfomycin: 34.9%; and TMP-SMX: 39.3%. In this study, *P. mirabilis* was the species that presented the highest rate of resistance against ampicillin (48.1%), which is concerning, since this antibiotic is commonly used in the treatment of UTI. The high incidence of *P. mirabilis* infection in men compared to women (10.7% vs. 4.6% of women) found in this study is consistent with other studies. *P. mirabilis* is a serious medical problem in UTI associated with a catheter, due to the ability to produce a biofilm, which explains the higher incidence rate in the elderly.

In France, a study with community and hospital patients found that *P. mirabilis* was the second most frequent microorganism (5.2%) in urine of male patients who used a catheter and presented resistance rates for ampicillin (40%), cefoxitin (1%), cefotaxime (1.5%), ceftriaxone (1.5%), cefoxitin (3%), cefoxitin), ertapenem (0%), imipenem (0%), gentamicin (18%), amikacin (1.8%), nalidixic acid (26%), norfloxacin (22%), ciprofloxacin (21%), trimethoprim + sulfamethoxazole (32%), fosfomycin (19%), and nitrofurantoin (100%) [46]. After evaluating and comparing the resistance profile of two clinical isolates of P. mirabilis found in bronchial aspirate samples from a patient undergoing lung transplantation and at an interval of 3 weeks in a hospital in Paris (France), Lecuru et al. (2020) [47] observed that the first clinical isolate exhibited susceptibility to carbapenems, amikacin, amoxicillin, ticarcillin, cefotaxime, levofloxacin and cotrimoxazole, while the clinical isolate obtained after 3 weeks had MICs increased by 16x for imipenem and amikacin and 8x for gentamicin. Genetic analysis identified a mutation in the *cpxA* gene and the authors suggested that this change could be related to increased resistance (especially to imipenem and aminoglycosides) in *P. mirabilis* strains after antibiotic treatment.

In Switzerland, a study was carried out to assess the resistance of *P. mirabilis* (and other bacteria) isolated from the urinary tract of outpatients and hospitalized patients between 2009 and 2016 and the authors highlighted that, in this period, the highest rate of resistance of *P. mirabilis* was given to the antimicrobial drug cotrimoxazol (>30%), while the rate of resistance to quinolones and phosfomycin remained below 20%. However, for quinolones, there was an increase in the resistance rate during the study period (11.8% in 2009 to 17.6% in 2016). In contrast, the resistance rate to cotrimoxazole decreased from 35.5% in 2009 to 30.5% in 2016. Resistance to 3rd and 4th generation cephalosporins remained low throughout the study period (1.3% in 2009 for 0.9% in 2016) [48].

Fagan et al. (2015) [49] conducted a study in Norway that compared the differences in uropathogen resistance rates isolated from nursing homes and elderly patients living in the community to the extent that different recommendations of empirical treatment for urinary tract infection would be needed. In addition, they assessed whether specific gender-specific recommendations for treatment were needed in the elderly. Resistance rates of *P. mirabilis* to ciprofloxacin were higher in nursing home patients than in community patients. In addition, with respect to gender, bacteria isolated from male patients showed greater resistance to mecilinam. The differences between the bacterial origins and the antimicrobial resistance rates among the uropathogens isolated from the two groups were not very different. However, there was a significant difference between genders, both in terms of bacterial origin as well as resistance. Studies indicated that increased bacterial resistance in uropathogens isolated from the elderly living in nursing homes is similar to UTI acquired in the hospital environment.

In a study conducted in Germany, Belgium, and Spain, using urine samples from patients with community-acquired urinary tract infection, all cephalosporins tested were active against *P. mirabilis* and resistance to fluoroquinolones among isolates was higher in Belgium [50].

Resistance to quinolones in *P. mirabilis* has increased over the years [51] and was also reported in 40% of isolates in a recent study in Poland [52]. The increased resistance to fluoroquinolones and the need for potent broad spectrum analogs have motivated researchers to develop derivatives or hybrids of fluoroquinolones with increased activity or properties, especially those with a better pharmacokinetic profile and effectiveness against resistant strains. According to Ezelarab et al. (2018), [53] the hydrazide derivatives of ciprofloxacin and levofloxacin-hydroxamic acid showed higher activity than parental quinolones, such as anti-*Proteus* agents with urease inhibiting activities. For future studies, the researchers intend to use the quinolone skeleton in the discovery of ideal antibiotics. In a study carried out with patients hospitalized in Poland in 2020, the *P. mirabilis* isolates exhibited the following resistance rates: 83.3% to ampicillin; 60% to amoxicillin/clavulanic acid; 33% to piperacillin/tazobactam; 16.7% to cefuroxime and cefotaxime; 83.3% to ciprofloxacin; 33.3% to amikacin; 40% to gentamicin; 66.7% to trimethoprim/sulfamethoxazole; 0% to the

carbapenems ertapenem, imipenem and meropenem. This study elucidates the importance of using carbapenem antimicrobials with caution in order to prevent antimicrobial resistance to these drugs, as these drugs are characterized as one of the last therapeutic options for multiresistant pathogens [54].

2.4. American Continent

In Wisconsin (US state) the Wisconsin surveillance program for antimicrobial resistance and epidemiology was created and the authors presented the results of the first year of surveillance. The results showed the following sensitivity rates of *P. mirabilis* isolates: ampicillin/sulbactam (93.9%), piperacillin/tazobactam (100%), cefazoline (96.1%), cefoxitin (98.6%), ceftriaxone (98.6%), ceftazidime (99.6%), cefepime (99.3%), aztreonam (99.6%), meropenem (100%), ertapenem (99.6%), levofloxacin (81.0%), ciprofloxacin 6%), gentamicin (91.4%), tobramycin (92.1%), and trimethoprim/sulfamethoxazole (82.4%). Sensitivity rates for fluoroquinolones (levofloxacin and ciprofloxacin), trimethoprim/sulfamethoxazole, ampicillin, ampicillin/sulbactam, and aminoglycosides (gentamicin and tobramycin) were lower in the Northeast when compared to the state mean. In the center-south region, a lower sensitivity to aminoglycosides was observed. The results of the program in 2016 indicate geographical differences with respect to the sensitivity of the microorganisms to the antimicrobials tested [55].
Ramos *et al*. (2018) [56] characterized the first report of MDR clones of *P. mirabilis* bearing a new *bla*IMP-1 gene transmitted by integron class 1 in a hospital located in the city of Diadema in São Paulo (Brazil) between July and August 2015. A total of 10 isolates of *P. mirabilis* resistant to carbapenems originated from tracheal aspirates and inpatient rectal swabs. All isolates were identified as lactamase resistant carbapenemase producers and exhibited intermediate resistance to aminoglycosides. Ciprofloxacin was the only antimicrobial agent tested to demonstrate *in vitro* activity against the isolates. The present report highlights the potential for in-hospital dissemination of an MDR *P. mirabilis* clone among patients admitted to different hospital units, reinforcing the need for adherence to adequate infection control measures. Oliveira et al (2020) [57] conducted a study on the resistance, virulence and clonal relationship of 183 strains of *P. mirabilis* isolated from the urinary tract of patients treated at several Basic Health Units (UBS) in Londrina (Brazil). The results show that the antibiotics with the lowest sensitivity rates were trimethoprim/sulfamethoxazole and ampicillin (78.1% and 94.5%, respectively); 7.1% had multidrug resistance phenotype and one strain was ESBL positive (positive strain for the *CTX-M-9* gene). The findings found by the authors suggested that there is a circulation of *P. mirabilis* strains in the studied region, which is worrisome since this can lead to an increase in the resistance of these pathogens to antibiotics commonly used for the treatment of urinary tract infections.
A study carried out in Colombia with diabetic patients draws attention to the circulation of strains resistant to third generation cephalosporins and carbapenemase producers in UTI acquired in the community. The study warns of the need to know the additional risk factors of this type of infection, in this specific group of patients, the need for early clinical suspicion, and the development of mechanisms that contribute to its prevention [58].

2.5. Oceania continent

There are a few studies involving the resistance of *P. mirabilis* to antimicrobials in Oceania. Osthoff *et al.* [59] in 2015, performed a study describing the epidemiology and clinical characteristics of UTI due to ESBL bacteria in Australia. During the period between 2003 and 2009, *E. coli*, *P. mirabilis* and *Klebsiella* species were identified in 15,142 urine samples from 10,111 patients. *P. mirabilis* isolates (1,658, 11%) were culture positive but not ESBL positive. The authors do not detail the percentage of *P. mirabilis* positive for other antibiotics tested.

3. Antibiotic resistance of *P. mirabilis* isolated from non-human sources

A few studies have been conducted with *P. mirabilis* from non-human sources, including some that have reported isolation of *P. mirabilis* from UTI in companion animals, such as dogs and cats, and highlighted the

impact on both animal and public health, alerting to possible zoonotic risks [3,4]. One of these studies conducted by Harada *et al*. (2014) [3] in Japan evaluated the susceptibility profile of 103 *P. mirabilis* strains isolated from UTI in dogs and found that most isolates (63, 64.7%) were sensitive to all antimicrobials tested, such as ampicillin, cephalothin, cefoxitin, ceftazidime, cefotaxime, aztreonam, imipenem, streptomycin, kanamycin, gentamicin, chloramphenicol, ciprofloxacin, and enrofloxacin. The highest frequencies of resistance were for chloramphenicol (20.4%), streptomycin (15.5%), enrofloxacin (12.6%), and trimethoprim/sulfamethoxazole (10.7%). None of these isolates showed resistance to ceftazidime, aztreonam, and imipenem. The presence of resistance class 1 and 2 integrons, such as the plasmid genes qnrD, *bla*CMY-2, and *bla*DHA-1 were detected in 2.9% and 11.7% of the isolates, respectively.

Other studies, like that of Marques et al. (2018) [60] carried out in Lisbon, evaluated the resistance profile associated with the clonal relationship of *P. mirabilis* isolated from UTI in humans and companion animals, and observed that the isolates from both sources of infection were frequently resistant to ampicillin and amoxicillin/clavulanic acid, in which resistance was strongly associated with the presence of the *bla*CMY-2 gene. Isolates from human UTI exhibited a higher frequency of resistance to the quinolones nalidixic acid, enrofloxacin, and ciprofloxacin since they had the qnrD gene. In addition, the aphAI-IAB aminoglycoside resistant gene was detected in both human and non-human isolates, as well as *bla*TEM, sul1, and sul2. In contrast to the study by Harada *et al*. (2014) [3] who did not find resistance to third generation cephalosporins, it was observed that 10/107 isolates presented this resistance profile. Interestingly, the high frequency of trimethoprim/sulfamethoxazole resistance in *P. mirabilis* isolated from companion animal UTI has also been documented, as in the study of Harada *et al*. 2014 and Moyaert *et al*. (2017) [3,4].

Marques et al. (2018) [60] screened isolates from UTI of companion animals and identified an increase in MDR strains and strains containing plasmid resistance genes to the class of antimicrobials that are frequently used in the treatment of human infections. Considering the intimate clonal relationship between *P. mirabilis* causing UTI in companion animals and in humans, it is of great importance that further studies assessing the susceptibility profile of these isolates are carried out and that their zoonotic potential is evaluated in order to better understand the risk that *P. mirabilis* isolated from animals represent to human health.

Other studies evaluated the susceptibility profile to antimicrobials of *P. mirabilis* isolated from food sources, especially chicken meat. A study by Wong, Wan and Chen (2013) [5] in China genotypically and phenotypically characterized the resistance profile of 50 *P. mirabilis* isolates from chicken carcasses and showed considerable antimicrobial resistance rates to antimicrobials that are frequently used in the treatment of human infections, such as trimethoprim/sulfamethoxazole (80%), chloramphenicol (66%), nalidixic acid (66%), ampicillin (60%), streptomycin (56%), ciprofloxacin (52%), kanamycin (46%), gentamicin (38%), ceftriaxone (36%), cefotaxime (34%), ceftiofur (22%), and amoxicillin/clavulanic acid (16%). Studies evaluating the presence of β-lactamases in food-borne *P. mirabilis*, such as chicken meat, are scarce, and further research is essential to elucidate their prevalence. In the same study, Wong, Wan and Chen (2013) [5] found a high prevalence of *bla*TEM-1 (n = 21), *bla*OXA-1 (n = 18), and *bla*CTX-M-9 (n = 6), highlighting the fact that some isolates contained more than one type of *bla* gene.

Similar results were found by Sanches *et al*. (2019) [6] in Brazil, whose study had the same objective and showed a high resistance rate for the penicillins, cephalosporins, aminoglycosides, and quinolones. This study specifically highlighted the high resistance rates for quinolone antimicrobials, such as nalidixic acid, ciprofloxacin, enrofloxacin, third and fourth generation cephalosporins, as well as ceftazidime, ceftriaxone, cefotaxime, and cefepime. In the study by Wong, Wan and Chen (2013), [5] the resistance genes of the *bla*CTX-M-2 group, CIT, and a high prevalence of qnrD were also found, which contributed to the MDR phenotype in 25 (78.13%) strains. The CTX-M, ACC, CMY, DHA, qnrB and qnrD resistance genes have also been reported in *P. mirabilis* isolated from broilers [61].

The use of antimicrobials in animal production, especially in poultry, contributes directly to the increase of bacterial resistance. Broiler chickens have great commercialization worldwide, and due to the high sales demand, the intensive production of these birds uses high amounts of antimicrobial agents as prophylaxis and treatment and growth promoters [62]. The use of antimicrobials in poultry farming is a risk factor for human health. Especially as the presence of residues of these chemical compounds in the tissues of animals, and the extended use of these compounds in poultry farming may result in the selection of resistant bacteria, which are pathogenic to humans [63,64].

The high incidence of new antimicrobial resistant strains in veterinary medicine has become a major concern as resistant bacteria are more related to high morbidity and mortality when compared to susceptible strains [65,66]. Many antimicrobials were introduced into veterinary medicine after their commercialization for therapeutic purposes in humans. Some of these have been exclusively assigned to veterinary use from the outset, but belong to the classes of antimicrobials commonly used in human medicine, which are structurally similar, such as macrolides and fluoroquinolones [67]. In this sense, the resistance to antimicrobials in veterinary infections poses serious risks to human health, especially considering the reports of MDR bacteria found in food from animal origin, representing a potential pathogenic source for consumers.

In addition to being isolated from broiler chickens, *P. mirabilis* has also been reported causing mortality in fish [68]. In this study, the *P. mirabilis* strain responsible for fish deaths was MDR and expressed resistance to antimicrobials of various classes, including penicillins (amoxicillin), aminoglycosides (tobramycin), monosaccharides (aztreonam), cephalosporins (cephalothin), macrolides (erythromycin), and sulfa (sulfadiazine). Interestingly, one of the few classes of antimicrobials the isolate was not resistant to was the quinolones. The strain was susceptible to the antimicrobials amikacin, gentamycin, streptomycin, piperacillin, oxacillin, imipenem, ceftazidime, cefuroxime, cefotaxime, ciprofloxacin, levofloxacin, norfloxacin, and chloramphenicol.

The small number of studies performed with *P. mirabilis* isolated from non-human sources makes it impossible to understand the prevalence of resistance of these isolates according to the source and site of isolation. However, it is clear that isolates from chickens and companion animals may be resistant to several antimicrobials used in the human clinic and carry resistance genes that contribute to the MDR phenotype, making them difficult to treat. Therefore, it is of great importance that antimicrobial use in both veterinary and human medicine is cautious, in order to avoid selection of resistant strains that pose a threat to public health.

4. Conclusions

In conclusion, further studies need to be performed which report the profile of *P. mirabilis* susceptibility and resistance to antimicrobials isolated from both human and non-human sources, in order to monitor and better understand to which antimicrobials these isolates are resistant. The few resistance studies performed in isolates from non-human sources make it impossible to compare the resistance of human clinical isolates of community and hospital origin. However, the presence of MDR and ESBL-producing strains in several continents in both human and non-human clinical isolates, highlights the resistance to important antimicrobials frequently used for the treatment of clinical infections, as well as cephalosporins and quinolones.

Conflicts of Interest: The authors declare no conflict of interest.

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8 ARTIGO CIENTÍFICO III

Genotypic and phenotypic profiles of *Proteus mirabilis* isolates from several infection sources in humans

ABSTRACT

The genus *Proteus* is responsible for several hospital infections, such as: respiratory tract infections, wounds, eyes, nose, throat, skin and gastroenteritis. They can be found in soil, water and urinary tract of different mammals. Among the species of this genus, the most prevalent in infections in humans is *Proteus mirabilis*. It presents adhesins, toxins, biofilms, siderophores, urease and proteases as main virulence factors. This work aimed at investigating the presence of these virulence factors in 68 isolates of *P. mirabilis*, as well as biofilm and crystals formation, antimicrobial susceptibility and adherence pattern. These isolates were collected from patients of the University Hospital of State University of Londrina (HU-UEL) from January 2015 to March 2017. The virulence genes were assessed by the Polymerase Chain Reaction (PCR), as well as biofilm formation, crystals formation, adherence pattern and antimicrobial susceptibility were investigated. All of the virulence genes were found in the clinical isolates, except *hlyA* and *fimH*. Apatite and struvite formation was clearly observed after 48h of incubation of the isolates. Biofilm formation was very intense and microbial cells were resistant, in different levels, to most of the antibiotics tested. Moreover, 100% of urinary isolates and 82.35% of non urinary isolates presented an aggregative adherence pattern. Hence, we can conclude that *P. mirabilis* is resistant to many antimicrobial agents and potentially pathogenic, presenting different genotypic and phenotypic characteristics depending of the source of infection.

Keywords: Virulence factors, biofilm, antimicrobial susceptibility

INTRODUCTION

Proteus genus is comprised by Gram-negative bacteria, including *P. vulgaris*, *P. penneri*, *P. hauseri*, *P. cibarius*, *P. terrae* and *P. mirabilis*. These species are widely spread in environment and gastrointestinal tract of animals and humans and are commonly associated to urinary tract infections (UTIs) [1, 2, 3].

Proteus mirabilis is rod-shaped bacteria, also found in environment and in gastrointestinal tract of humans. It is an opportunistic microorganism able to cause infections in eyes, ears, skin, as well as in respiratory and gastrointestinal tracts, mainly in the last one. This pathogen expresses many virulence factors, such as adhesins, toxins, biofilm, proteases, siderphores, urease, in order to set the infection successfully in human host [1,4].

UTIs represent common sorts of infection that compromises suitable function of bladder, kidney, urether and urethra and affect about 150 million people wordwide, mainly women, obese and

imunossupressed patients, as well as cases of long-term catheterization [5]. Complicated UTIs and those associated to catheterization are frequently associated to *P. mirabilis* and their symptoms usually comprise dysuria, hematuria, polyuria and sudden will of peeing [6, 7].

Current treatments for UTIs include the use of antibiotics, like β-lactams, cephalosporins and fluoroquinolones, for example; however, antimicrobial resistance of *P. mirabilis* difficults the elimination of UTIs, and relapse is common [7, 8]. Hence, new more efficient therapeutic strategies are needed in order to solve this issue [9, 10].

Alternative therapeutic strategies for treating infections caused by *P. mirabilis* comprise the use of nanotechnology as well as medicinal plants, once they have shown antimicrobial activity against this pathogen. For instance, Cock and Van Vuuren [11] assessed the antimicrobial activity of plants extracts from South Africa and found out that most of them were able to inhibit microbial growth; likewise, Disaanayake and coworkers [12] assessed the same parameter using nanosilver particles and also observed microbial growth inhibition.

This work aimed at studying genotypic and phenotypic profiles of *P. mirabilis* isolates from different sources of infection by investigating their virulence factors, biofilm and crystals formation, adherence cell pattern as well as antimicrobial susceptibility profile.

METHODS

Sampling

This work was performed using 68 *P. mirabilis* strains isolated from fluids like urine, blood, secretions, as well tissues and wounds. Strains were isolated from patients of Hospital Universitário Regional Norte do Paraná (HU-UEL), from January 2015 till March 2017 (CAAE 43013315.8.0000.5231). All of the bacterial isolates were identified by Vitek 2 System (bioMérieux) and stored in Brain Heart Infusion (BHI) Broth, with 25% of glycerin, at -80°C.

Genotypic analysis

Virulence genes *hpmA*, *hlyA*, *ureA*, *zapA*, *mrpA*, *fimH*, *pmfA*, *ucaA*, *ireA, atfA* were assessed by Polymerase Chain Reaction (PCR). Bacterial DNA extraction was performed by boiling technique and its amplification was made with volumes of 25 µL, containing 1.0 µL of lysate supernatant, 200 µM of desoxirribonucleotides tri phosphate (dNTPs) (Invitrogen®), 1.5 mM of magnesium chloride (Invitrogen®), 20 pmol of each initiator primer (Invitrogen®) and 1.5U of Taq DNA polymerase (Invitrogen®). Afterwards, amplification product was undergone to agarose gel electrophoresis (1% or 2% according to product size), stained with SYBR Safe and visualized using UV light.

Phenotypic analysis

HEp-2 cell adherence pattern testing was performed according to Rocha [13] in 96-well plate. After 3h of incubation, wells were washed with phosphate-buffered saline (PBS buffer), fixed with methanol and dyed with May–Grunwald stain.

Biofilm formation testing was performed using 96-wells polystyrene plates, according to Kwiecinska-Piróg et al [14]. Quantitatively, this parametrer was assessed by crystal violet assay using spectrophotometry at a wavelength of 570 nm and ranked as following (Table 1):

Table 1 – *P. mirabilis* biofilm formation quantitative testing by spectrophotometry at 570 nm [11]

ABSORBANCE	BIOFILM INTENSITY
A ≤ T	VERY WEAK
T < A ≤ 2T	WEAK
2T < A ≤ 4T	MODERATE
4T < A ≤ 8T	STRONG

Detection of apatite and struvite crystals was performed according to Silva et al. [15]. Artificial urine was produced and then centrifuged (5000 xg; 10 min) and filtered through syringe filter with polyvinylidene fluoride membrane (Neobio®; 0,22 µm diameter). Bacteriological agar was prepared apart, autoclaved and poured into the sterilized artificial urine, reaching a final concentration of 1%. Bacterial isolates were cultivated in tryptone soya broth (TSB) at 37ºC for 18h. Afterwards, pour plate technique was performed and 100 µL of the bacterial culture was mixed into urine-agar and then, the dishes were incubated at 37ºC for 72 h. All of the dishes were observed at 24h, 48h and 72h.

Finally, antimicrobial susceptibility was assessed by agar diffusion method, using antibiotics recommended by *Clinical and Laboratory Standards Institute* (CLSI) [13]. *P. mirabilis* antimicrobial susceptibility profile was performed by VITEK® 2 automatic system (bioMérieux Brasil) and extended spectrum beta-lactamases (ESBL) were identified by disk-diffusion test. The following antibiotics were analyzed: Amikacin; Gentamicin; Amoxicillin+Clavulanic Acid; Ampicillin; Cephalothin; Cefuroxime; Ceftazidime; Ceftriaxone; Cefepime; Meropenen; Ertapenem; Imipenem; Piperacillin+Tazobactam; Ciprofloxacin; Norfloxacin; Nalidixic Acid; Sulfamethoxazole + trimethoprim; Ampicillin. + Sulbactam; Aztreonam and Cefoxitin.

Statistical analysis

Statistical analysis was performed by R® program. Chi-square test was used in order to assign significant differences between groups and P>0.05 indicated this difference.

RESULTS

Genotypic characterization of *P. mirabilis* virulence factors

Virulence genes *hpmA*, *hlyA*, *zapA*, *mrpA*, *fimH*, *pmfA*, *ucaA*, *ireA*, *atfA* and *ureA* were assessed by PCR assay. It was possible to detect almost all of the genes mentioned above: *hpmA* was detected in 97.05% of urinary isolates and in 100% of another infection sources isolates; *pmfA* in 97.05% of

urinary isolates and in 100% of another infection sources isolates; *ucaA* in 82.35% of urinary isolates and in 85.29% of another infection sources isolates; *zapA* in 94.11% of urinary isolates and in 88.23% of another infection sources isolates; *mrpA* in 100% of urinary isolates and in 97.05% of another infection sources isolates. hlyA and *fimH* genes were not detected in all of the clinical isolates (independently of their infection source); on the other hand, *atfA* and *ureA* were found in all of the them (100%). Therefore, it is possible to conclude that *P. mirabilis* isolates can exhibit different percentages of virulence genes and *hlyA* and *fimH* were absent in this pathogen (Table 2).

Table 2- *P. mirabilis* virulence genes from 68 isolates of different sources of infection assessed by PCR technique.

	SAMPLE SOURCE	VIRULENCE GENES									
		hpmA	*ureA*	*hlyA*	*zapA*	*mrpA*	*fimH*	*pmfA*	*ucaA*	*ireA*	*atfA*
1	URINE	+	+	-	+	+	-	+	+	+	+
2	TISSUE	+	+	-	+	+	-	+	-	+	+
3	BLOOD	+	+	-	+	+	-	+	+	+	+
4	URINE	+	+	-	+	+	-	+	-	+	+
5	URINE	+	+	-	+	+	-	+	+	+	+
6	OCULAR GLOBE	+	+	-	+	+	-	+	+	+	+
7	URINE	+	+	-	+	+	-	+	+	+	+
8	BLOOD	+	+	-	+	+	-	+	-	+	+
9	URINE	+	+	-	+	+	-	+	+	+	+
10	TRACHEAL	+	+	-	+	+	-	+	+	+	+
11	URINE	+	+	-	+	+	-	+	-	+	+
12	URINE	+	+	-	+	+	-	+	+	+	+
13	URINE	+	+	-	+	+	-	+	-	+	+
14	URINE	+	+	-	+	+	-	-	+	+	+
15	TRACHEAL	+	+	-	+	+	-	+	+	+	+
16	URINE	+	+	-	+	+	-	+	+	+	+
17	URINE	+	+	-	+	+	-	+	+	+	+
18	BLOOD	+	+	-	+	+	-	+	-	+	+
19	BLOOD	+	+	-	+	+	-	+	-	+	+
20	TISSUE	+	+	-	+	+	-	+	+	+	+
21	URINE	-	+	-	-	+	-	+	+	+	+
22	BLOOD	+	+	-	+	-	-	+	+	+	+
23	URINE	+	+	-	+	+	-	+	-	+	+

24	INTRACAVITARY FLUID	+	+	-	+	+	-	+	+	+	+
25	URINE	+	+	-	+	+	-	+	+	+	+
26	URINE	+	+	-	+	+	-	+	+	+	+
27	BLOOD	+	+	-	+	+	-	+	+	+	+
28	URINE	+	+	-	+	+	-	+	-	+	+
29	TISSUE	+	+	-	+	+	-	+	+	+	+
30	URINE	+	+	+	+	+	-	+	+	+	+
31	BLOOD	+	+	-	+	+	-	+	-	+	+
32	URINE	+	+	-	+	+	-	+	+	+	+
33	BLOOD	+	+	-	+	+	-	+	+	+	+
34	URINE	+	+	-	+	+	-	+	+	+	+
35	URINE	+	+	-	+	+	-	+	+	+	+
36	URINE	+	+	-	+	+	-	+	+	+	+
37	CATHETER	+	+	-	+	+	-	+	+	+	+
38	URINE	+	+	-	.	+	-	+	+	+	+
39	BLOOD	+	+	-	+	+	-	+	+	+	+
40	URINE	+	+	-	+	+	-	+	+	+	+
41	URINE	+	+	-	+	+	-	+	+	+	+
42	TISSUE	+	+	-	+	+	+	+	+	+	+
43	TRACHEAL	+	+	-	+	+	-	+	+	+	+
44	URINE	+	+	-	+	+	-	+	+	+	+
45	URINE	+	+	-	+	+	-	+	-	+	+
46	URINE	+	+	-	+	+	-	+	+	+	+
47	TRACHEAL	+	+	-	+	+	-	+	+	+	+
48	SECRETION	+	+	-	+	+	-	+	+	+	+
49	URINE	+	+	-	+	+	-	+	+	+	+
50	URINE	+	+	-	+	+	-	+	+	+	+
51	URINE	+	+	-	+	+	-	+	+	+	+
52	URINE	+	+	-	+	+	-	+	-	+	+
53	BLOOD	+	+	-	+	+	-	+	-	+	+
54	BLOOD	+	+	-	+	+	-	+	+	+	+
55	URINE	+	+	-	+	+	-	+	-	+	+
56	URINE	+	+	-	+	+	-	+	+	+	+
57	URINE	+	+	-	+	+	-	+	+	+	+

58	TISSUE	+	+	-	+	+	-	+	+	+	+
59	BONE FRAGMENT	+	+	-	+	+	-	+	+	+	+
60	TISSUE	+	+	-	+	+	-	+	+	+	+
61	BONE FRAGMENT	+	+	-	+	+	-	+	+	+	+
60	LESION SECRETION	+	+	-	+	+	-	+	+	+	+
61	BLOOD	+	+	-	+	+	-	+	+	+	+
62	TRACHEAL SECRETION	+	+	-	+	+	-	+	+	+	+
63	BLOOD	+	+	-	+	+	-	+	+	+	+
64	TRACHEAL SECRETION	+	+	-	+	+	-	+	+	+	+
65	BLOOD	+	+	-	+	+	-	+	+	+	+
66	BONE FRAGMENT	+	+	-	+	+	-	+	+	+	+
67	ASCITIC FLUID	+	+	-	+	+	-	+	+	+	+
68	URINE	+	+	-	+	+	-	+	-	+	+

HEp-2 cell adherence patterns

As expected, all of the urinary isolates showed an aggregative adherence (AA) pattern. Conversely, among non urinary isolates, 17.65% of them were non adherent (NA) and 82.35% of them presented AA pattern (see Table 3). Testing was performed once again in order to confirm the results found.

Biofilm formation testing

All of the samples (100%) were able to form biofilms (Table 3) and absorbance was higher than 8T; therefore their intensity was ranked as very strong.

Table 3- Biofilm formation and adherence pattern of 68 *P. mirabilis* from different sources of infection.

SAMPLE	SOURCE	BIOFILM FORMATION	ADHERENCE PATTERN
1	URINE	+	AA
2	TISSUE	+	AA
3	BLOOD	+	AA
4	URINE	+	AA

5	URINE	+	AA
6	OCULAR GLOBE	+	AA
7	URINE	+	AA
8	BLOOD	+	AA
9	URINE	+	AA
10	TRACHEAL	+	AA
11	URINE	+	AA
12	URINE	+	AA
13	URINE	+	AA
14	URINE	+	AA
15	TRACHEAL	+	AA
16	URINE	+	AA
17	URINE	+	AA
18	BLOOD	+	AA
19	BLOOD	+	AA
20	TISSUE	+	AA
21	URINE	+	AA
22	BLOOD	+	AA
23	URINE	+	AA
24	INTRACAVITARY FLUID	+	AA
25	URINE	+	AA
26	URINE	+	AA
27	BLOOD	+	AA
28	URINE	+	AA
29	TISSUE	+	AA
30	URINE	+	AA
31	BLOOD	+	AA
32	URINE	+	AA
33	BLOOD	+	AA
34	URINE	+	AA
35	URINE	+	AA
36	URINE	+	AA
37	CATHETER	+	NA
38	URINE	+	AA
39	BLOOD	+	AA
40	URINE	+	AA
41	URINE	+	AA
42	TISSUE	+	AA
43	TRACHEAL	+	NA

44	URINE	+	AA
45	URINE	+	AA
46	URINE	+	AA
47	TRACHEAL	+	NA
48	SECRETION	+	AA
49	URINE	+	AA
50	URINE	+	AA
51	URINE	+	AA
52	URINE	+	AA
53	BLOOD	+	AA
54	BLOOD	+	NA
55	URINE	+	AA
56	URINE	+	AA
57	URINE	+	AA
58	TISSUE	+	AA
59	BONE FRAGMENT	+	AA
60	TISSUE	+	AA
61	BONE FRAGMENT	+	AA
60	LESION SECRETION	+	AA
61	BLOOD	+	AA
62	TRACHEAL SECRETION	+	AA
63	BLOOD	+	AA
64	TRACHEAL SECRETION	+	AA
65	BLOOD	+	AA
66	BONE FRAGMENT	+	NA
67	ASCITIC FLUID	+	NA
68	URINE	+	AA

Crystals Formation Testing

Some crystals were formed in 24h, but they were too small and indistinguishable; only after 48h of incubation they were able to be differentiated. From 48h to 72h no significative change was observed in size as well as in quantity of them (Figure 1). These crystals are able to protect bacterial cells against antibiotics, immunoglobulins and urease inhibitors as well as can obstruct catheters [15].

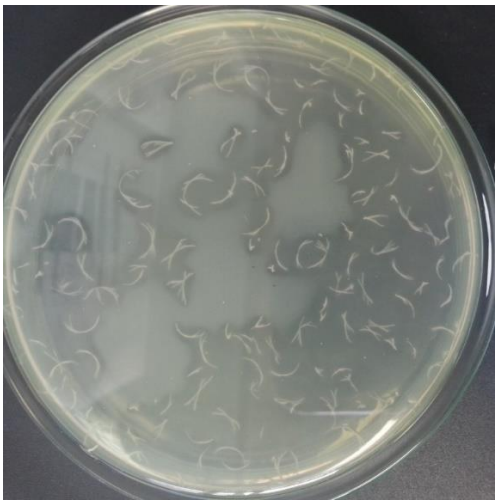

Figure 1: *In vitro* crystals formation by *Proteus mirabilis* in urine-agar médium after 72h of incubation. Crystals were X-shaped and dendritic-like

Antimicrobial susceptibility testing

Microbial resistance rates were the following (Table 3): Amikacin (35.29%), Gentamicin (50.98%), Amoxicillin+Clavulanic Acid (47.06%), Ampicillin (82.27%), Cephalothin (88.23%), Cefuroxime (84.31%), Ceftazidime (82.35%), Ceftriaxone (86.27%), Cefepime (82.35%), Meropenen (0.00%), Ertapenem (3.92%), Imipenem (1.96%), Piperacillin+Tazobactam (3.92%), Ciprofloxacin (47.05%), Norfloxacin (50%), Nalidixic Acid (76.46%), Sulfamethoxazole + trimethoprim (74.50%), Ampicillin. + Sulbactam (76.48%), Aztreonam (82.35%), Cefoxitin (52.94%). In this study, 70.58% of ESBL producers were resistant to antibiotics tested. Hence, this work reveals that most of *P. mirabilis* strains were resistant to antimicrobial agents, including those ESBL producers.

DISCUSSION

Proteus mirabilis usually expresses many virulence factors, such as adhesins, proteases, biofilm, toxins, urease and siderophores in order to stablish successfully an infection in human host [16]. By the results found, it was possible to observe that there were differences in genotypic profile between urinary and non urinary isolates. *hmpA* incidence was higher (97.05% of the isolates) than *hlyA* (0%), which was expected based on Swihart and Wech's study [17], which reveaed that *hmpA* was more common in *Proteus* isolates than *hlyA*. *mrpA* gene was found in 100% of the isolates and this result is similar to those of Barbour and coworkers [18], that found the same percentage for this gene in all of the 23 human isolates analyzed. High prevalence of the genes *hpmA*, *ireA* and *zapA* was similar to those found by Sanches and coworkers [19].

Biofilm formation is also a strategy set by *Proteus mirabilis* in order to be able to survive and develop inside of the host. It is able to form crystalline biofilms, which protect microbial cell from immune defenses of the host as well as allow its permanence inside of human [20]. In this study, 100% of the

isolates formed biofilm. This result was compatible with the one obtained by De Oliveira and coworkers [21], who also fund the same percentage for the isolates analized (urinary ones).

Adherence pattern AA was also observed for all the urinary isolates, so was for the ones analyzed by Rocha and coworkers [13], that analyzed urinary isolates from humans.

According to World Health Organization [22], antimicrobial resistance profile obtained in this study was already expected, once *P. mirabilis* present severe antimicrobial resistance to clinical antibiotics, which turns the microorganism into a global concern [22, 23]. This opportunism pathogen belongs to Morganellaceae family and presents reduced sensitivity to polymyxin and tigecycline [24].

Some studies all over the world report *P. mirabilis* resistance to a wide variety of antimicrobial agents in human hosts, once multi resistant strains as and ESBL are spread worldwide. Resistance to β-lactam, fluoroquinolones, fosfomycin, aminoglycosides and sulfonamides has been reported, so has to tetracyclines, nitrofurantoin and polymyxins [25-28].

CONCLUSION

P. mirabilis is an opportunistic bacterium able to infect humans and the main etiologic agent in nosocomial infections. This work revealed that this microorganism possesses many virulence factors, is able to form biofilms efficiently and adhere to Hep-2 cells, as well as is resistant to antibiotics. Moreover, it not was possible to observe genopytic and phenotypic differences between urinary and non urinary isolates.

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8 CONCLUSÕES

P. mirabilis é uma bactéria oportunista que coloniza animais e seres humanos, sendo considerado um dos principais agentes causais de infecções hospitalares. Os resultados deste trabalho mostram que não houve diferença significativa entre os isolados, portanto, *P. mirabilis* possui diversos fatores de virulência, além de formar biofilmes cristalinos, o que dificulta a antibioticoterapia e, apresenta considerável resistência a uma ampla gama de antimicrobianos de uso clínico.

A marioria dos estudos a respeito deste microorganismo concentram-se em isolados de origem urinária e pouco se sabe sobre o genoma deste patógeno em isolados de infecções que não a urinária; logo, não há estudos mostrando uma análise precisa explorando as ferramentas da Bioinformática em isolados de diferentes infecções em humanos, deste modo, a nossa pesquisa se destaca com esta busca inédita.