LARISSA DOS SANTOS FÁVARO

CARACTERIZAÇÃO FENOTÍPICA E MOLECULAR DE DETERMINANTES DE RESISTÊNCIA E FORMAÇÃO DE BIOFILME EM ISOLADOS DE *Acinetobacter* spp.

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Tese apresentada ao Programa de Pós-Graduação em Fisiopatologia Clínica e Laboratorial, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor.

Orientador: Prof. Dr. Emerson José Venancio

Co-orientadora: Profa. Dra. Floristher Elaine Carrara

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

— **Arthur Schopenhauer**

FÁVARO, Larissa dos Santos. **Caracterização fenotípica e molecular de determinantes de resistência e formação de biofilme em isolados de *Acinetobacter* spp**. 2023. 165f. Tese (Doutorado em Fisiopatologia Clínica e Laboratorial) – Universidade Estadual de Londrina, Londrina.

RESUMO

Nos últimos anos, espécies de *Acinetobacter* não-*baumannii* (NBA) têm se destacado como importantes patógenos responsáveis por infecções relacionadas a assistência à saúde, assim como *A. baumannii*, a espécie clinicamente mais relevante do gênero. O objetivo deste estudo foi caracterizar os determinantes de resistência aos antimicrobianos e fatores de virulência de isolados clínicos de *Acinetobacter* spp. recuperados de pacientes atendidos no Hospital Universitário de Londrina (HU) num período de dez anos (2006-2016). Um total de 750 isolados de *Acinetobacter* spp., não repetidos, foram incluídos no estudo, sendo 738 pertencentes ao complexo *A. calcoaceticus-A. baumannii* e 12 NBA. A análise genotípica revelou que *A. baumannii* foi a espécie mais comumente isolada (98,4%), seguida por *A. bereziniae* (0,8%), *A. haemolyticus* (0,1%) e *A. colistiniresistens* (0,1%). Os isolados apresentaram altas taxas de resistência para a maioria dos antimicrobianos avaliados, incluindo os carbapenêmicos. Os genes codificadores de *carbapenem-hydrolyzing class D beta-lactamases* (CHDL) adquiridos mais comumente identificados foram: *bla*$_{\text{OXA-23-like}}$ (89,2%) seguido por *bla*$_{\text{OXA143-like}}$ (1,2%) e *bla*$_{\text{OXA58-like}}$ (0,8%). O gene *bla*$_{\text{OXA-58}}$ foi detectado em cinco isolados de *A. bereziniae* e um de *A. colistiniresistens* (Ac 505/15), classificados como produtores de biofilme. A tipagem molecular destes isolados por *enterobacterial repetitive intergenic consensus-polymerase chain reaction* (ERIC-PCR) identificou quatro grupos genéticos (I-IV), sendo três agrupamentos individuais (I, II e III), e um (grupo IV) composto por três isolados de *A. bereziniae* relacionados clonalmente. A análise do contexto genético revelou que todos os isolados apresentaram IS*Aba*3 a jusante do gene *bla*$_{\text{OXA-58}}$, e os três isolados com IS*Aba*1, IS*Aba*3 e IS*Aba*125-ΔIS*Aba*3 a montante foram classificados como multirresistentes. Adicionalmente, as análises de bioinformática do sequenciamento do genoma total do isolado Ac505/15 (grupo I) revelaram a presença de genes codificadores de resistência à beta-lactâmicos, aminoglicosídeos, fenicóis, sulfonamidas e macrolídeos, bem como, várias famílias de sistemas de efluxo, ilhas genômicas, sequências de inserção e genes de virulência. Neste estudo, demonstrou-se que a resistência aos carbapenêmicos em *Acinetobacter* spp. no HU parece ser atribuída à presença de genes codificadores de CHDL. Além disso, destaca-se a necessidade da identificação correta das espécies de *Acinetobacter* e da vigilância epidemiológica a fim de evitar a disseminação de determinantes de resistência, e virulência entre patógenos hospitalares relevantes.

Palavras-chave: *Acinetobacter* spp.; Carbapenemases; CHDL; Fatores de virulência; Resistência aos antimicrobianos.

FÁVARO, Larissa dos Santos. **Phenotypic and molecular characterization of determinants of resistance and biofilm formation in *Acinetobacter* spp. isolates**. 2023. 165f. Thesis (Doctorate in Clinical and Laboratory Physiopathology) – Universidade Estadual de Londrina, Londrina.

ABSTRACT

In recent years, species of non-*baumannii Acinetobacter* (NBA) have emerged as important pathogens responsible for healthcare-associated infections, as has *A. baumannii*, the most clinically relevant species of the genus. The aim of this study was to characterize antimicrobial resistance determinants and virulence factors of clinical isolates of *Acinetobacter* spp. recovered from patients treated at the University Hospital of Londrina (HU) over a period of ten-years (2006-2016). A total of 750 non-repeated *Acinetobacter* spp. isolates were included in the study, 738 belonging to the *A. calcoaceticus-A*. *baumannii* complex and 12 NBA. Genotypic analysis revealed that *A. baumannii* was the most commonly isolated species (98.4%), followed by *A. bereziniae* (0.8%), *A. haemolyticus* (0.1%) and *A. colistiniresistens* (0.1%). The isolates showed high rates of resistance to most antimicrobials evaluated, including carbapenems. The most commonly identified acquired carbapenem-hydrolyzing class D beta-lactamases (CHDL) encoding genes were: *bla*$_{\text{OXA-23-like}}$ (89.2%) followed by *bla*$_{\text{OXA143-like}}$ (1.2 %) and *bla*$_{\text{OXA58-like}}$ (0.8%). The *bla*$_{\text{OXA-58}}$ gene was detected in five isolates of *A. bereziniae* and one of *A. colistiniresistens* (Ac 505/15), classified as biofilm producers. Molecular typing of these isolates by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) identified four genetic groups (I-IV), with three individual groups (I, II and III), and one (group IV) composed of three clonally related isolates of *A. bereziniae*. Analysis of the genetic context revealed that all isolates had IS*Aba*3 downstream of the *bla*$_{\text{OXA-58}}$ gene, and the three isolates with IS*Aba*1, IS*Aba*3 and IS*Aba*125-ΔIS*Aba*3 upstream were classified as multiresistant. Additionally, bioinformatics analyzes of the total genome sequencing of the Ac505/15 isolate (group I) revealed the presence of genes encoding resistance to beta-lactams, aminoglycosides, phenicols, sulfonamides and macrolides, as well as several families of efflux systems, genomic islands, insertion sequences and virulence genes. In this study, it was demonstrated that resistance to carbapenems in *Acinetobacter* spp. in HU seems to be attributed to the presence of genes coding for CHDL. In addition, the need for correct identification of *Acinetobacter* species and epidemiological surveillance is highlighted in order to avoid the dissemination of determinants of resistance and virulence among relevant hospital pathogens.

Key words: *Acinetobacter* spp.; Antimicrobial resistance; Carbapenemases; CHDL; Virulence factors.

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LISTA DE ABREVIATURAS E SIGLAS

AAC	***A**minoglycoside-**Ac**etyltransferase* (acetiltransferase)
AAD	***A**minoglycoside-**Ad**enyltransferase* (adeniltransferase)
Ab	***A**cinetobacter **b**aumannii*
ACB	***A**cinetobacter **c**alcoaceticus-Acinetobacter **b**aumannii*
ADC	***A**cinetobacter-**D**erived **C**ephalosporinase*
AEHU	**A**mbulatório de **E**specialidades do **H**ospital **U**niversitário de Londrina
AIM	***A**ustralian **im**ipenemase*
AK	***A**mi**k**acin*
AME	***A**minoglycoside-**M**odifying **E**nzyme* (enzima modificadora de aminoglicosídeo)
AmpC	Cefalosporinase cromossômica do tipo AmpC
ANIb	**A**verage **N**ucleotide **I**dentify based on **b**last
ANT	***A**minoglycoside-**N**ucleotidyl**t**ransferase* (nucleotidiltransferase)
APH	***A**minoglycoside-**Ph**osphotransferase* (fosfotransferase)
AR	***A**ntimicrobial **R**esistance*
ARI-1	***A**cinetobacter **R**esistant to **I**mipenem*
Arm	***A**minoglycoside **R**esistance **M**ethylase*
Ata	***A**cinetobacter **T**rimeric **A**utotransporter adhesin*
ATCC	***A**merican **T**ype **C**ulture **C**ollection*
β	Beta
BL	**B**eta-**L**actamases
Bap	***B**iofilm **A**ssociated **P**rotein* (proteína associada ao biofilme)
BEL	***B**elgium **E**xtended beta-**L**actamase*
BGNNF	**B**actérias **G**ram-**N**egativas **N**ão **F**ermentadoras
Bla	Gene codificador de **Beta**-**La**ctamase
BLAST	***B**asic **L**ocal **A**lignment **S**earch **T**ool*
CAESAR	***C**entral **A**sia and Eastern **E**uropean **S**urveillance of **A**ntimicrobial **R**esistance*
CAM	***C**entral **A**lberta **M**etallo-beta-lactamase*
CAPES	**C**oordenação de **A**perfeiçoamento de **Pe**ssoal de Nível **S**uperior
CAZ	***C**eft**az**idime*

CBC	**C**omplexo ***B**urkholderia **c**epacia*
CCIH	**C**omissão de **C**ontrole de **I**nfecção **H**ospitalar
CDC	***C**entre for **D**isease **C**ontrol and prevention*
CEP	***C**ef**ep**ime*
CGE	***C**enter for **G**enomic **E**pidemiology*
CHDL	***C**arbapenem-**H**ydrolyzing class **D** beta-**L**actamases*
CIM	**C**oncentração **I**nibitória **M**ínima
CIP	***Cip**rofoxacin*
CLSI	***C**linical and **L**aboratory **S**tandards **I**nstitute*
CMM	***C**linical and **M**olecular **M**icrobiology Department*
CNISP	***C**anadian **N**osocomial **I**nfection **S**urveillance **P**rogram*
COL	***Col**istin*
Cpa	***C**ollagen-binding **P**rotein of group **A***
CR	***C**arbapenem **R**esistance*
CRAb	***C**arbapenem-**R**esistant **A**cinetobacter **b**aumannii*
CTX-M	***C**efo**tax**ime **M**unich*
DAP	1,3-**d**i**a**mino**p**ropano
DDST	***D**ouble-**D**isk **S**ynergy **T**est*
DIM	***D**utch **im**ipenemase*
EARS-Net	***E**uropean **A**ntimicrobial **R**esistance **S**urveillance **Net**work*
ECDC	***E**uropean **C**entre for **D**isease prevention and **C**ontrol*
EDTA	***E**thylene-**D**iamine-**T**etraacetic **A**cid* (Ácido etileno-diamino-tetracético)
EF	***Eff**luents*
ER	**E**xtensivamente **R**esistente aos antimicrobianos
ERIC-PCR	***E**nterobacterial **R**epetitive **I**ntergenic **C**onsensus*-PCR
ESAC	***E**xtended-**S**pectrum **A**mp**C***
ESBL	***E**xtended-**S**pectrum **b**eta-**L**actamases*
ES-OXA	***E**xtended-**S**pectrum **Oxa**cillinases*
ETE	**E**stações de **T**ratamento de **E**sgoto
FAO	***F**ood and **A**griculture **O**rganization*
FDA	***F**ood and **D**rug **A**dministration*
FIM	***F**lorence **im**ipenemase*
g	**G**rama
GAR	***G**enes encoding **A**ntimicronial **R**esistance*

GCRAM	**G**enes **C**odificadores de **R**esistência aos **A**ntimicrobianos
GEN	***Gen**tamicin*
GES	***G**uiana **E**xtended **S**pectrum*
GIM	***G**erman **Im**ipenemase*
GLASS	***GI**obal **A**ntimicrobial Resistance **S**urveillance **S**ystem*
GyrB	*DNA **gyr**ase subunit **B*** (DNA girase subunidade B)
HAI	***H**ealthcare-**A**ssociated **I**nfections*
HMB	***H**amburg **M**etallo-**b**eta-lactamase*
HU	**H**ospital **U**niversitário de Londrina
ICU	***I**ntensive **C**are **U**nit*
IMP	***Imip**enemase*
INICC	***I**nternational **N**osocomial **I**nfection **C**ontrol **C**onsortium*
IPM	***Imip**enem*
IRAS	**I**nfecções **R**elacionadas a **A**ssistência à **S**aúde
IS	***I**nsertion **S**equence* (Sequência de inserção)
IS*Aba*	***I**nsertion **S**equence **A**cinetobacter **ba**umannii*
ITU	**I**nfecção do **T**rato **U**rinário
KHM	***K**yorin University **H**ospital i**m**ipenemase*
KPC	***K**lebsiella **p**neumoniae **C**arbapenemase*
L	**L**itro
LEMMRA	**L**aboratório **E**special de **M**icrobiologia **M**olecular e **R**esistência aos **A**ntimicrobianos
LEV	***Lev**ofloxacin*
Lip	**Lip**ase
LMC	**L**aboratório de **M**icrobiologia **C**línica
LPS	**L**ipo**p**olis**s**acarídeos (*lipopolysaccharide*)
Lpx	**Lip**o**x**igenase
MATE	***M**ultidrug **A**nd **T**oxic compound **E**xtrusion*
MBL	**M**etalo-**b**eta-**L**actamase
Mcr	*Plasmid-**M**ediated **C**olistin **R**esistance*
MDR	***Multid**rug-**R**esistant*
MEM	***Me**ropene**m***
MFS	***M**ajor **F**acilitator **S**uperfamily*
MIC	***M**inimal **I**nhibitory **C**oncentrations*

mL	**M**ili**l**itro
MR	**M**ultir**r**esistente aos antimicrobianos
NaCl	Cloreto de sódio
NBA	**N**on-***b**aumannii **A**cinetobacter* (*Acinetobacter* não *baumannii)*
NCBI	***N**ational **C**enter for **B**iotechnology **I**nformation*
NDM	***N**ew **D**elhi **M**etallo-beta-lactamase*
NmcA	***N**ot **m**etalloenzyme **c**arbapenemase **A***
NSBL	***N**arrow-**S**pectrum **b**eta-**L**actamases*
NS-OXA	***N**arrow-**S**pectrum **Oxa**cillinases*
OMP	***O**uter **M**embrane **P**rotein* (Porina de membrana externa)
OMS	**O**rganização **M**undial da **S**aúde
OMV	***O**uter **M**embrane **V**esicle* (Vesícula de membrana externa)
OIE	*World **O**rganization for An**i**mal H**e**alth*
Opr	***O**uter **Pr**otein*
OXA	**Oxa**cilinase
OXA-23 ACB	Isolado do complexo **ACB** portador do gene *bla*$_{\textbf{OXA-23}}$
OXA-58 NBA	Isolado **NBA** portador do gene *bla*$_{\textbf{OXA-58}}$
PAA	**P**rogramas de **A**dministração **A**ntimicrobiana
ParC	*DNA Topoisomerase IV subunit A* (DNA topoisomerase subunidade A)
PAV	**P**neumonia **A**ssociada à **V**entilação mecânica
PBP	***P**enicillin **B**inding-**P**roteins* (Proteína ligadora de penicilina)
PCR	***P**olymerase **C**hain **R**eaction* (Reação em cadeia da polimerase)
PDR	***P**an**d**rug-**R**esistant*
PER	***P**seudomonas **E**xtended **R**esistant*
Pmt	***P**hosphoethanolamine **M**ethyl**t**ransferase*
PPR	**P**roteínas de **P**roteção **R**ibossômica
PROPPG	**Pró**-Reitoria de **P**esquisa e **P**ós **G**raduação
PTZ	***P**iperacillin-**Taz**obactam*
Qnr	***Qui**n**o**lone **R**esistance*
QS	*Quorum Sensing*
PACE	***P**roteobacterial **A**ntimicrobial **C**ompound **E**fflux*
PBS	***P**hosphate **B**uffered **S**aline*
PCR	***P**olymerase **C**hain **R**eaction*
PEtN	***P**hospho**et**hanolamine* (Fosfoetanolamina)

PME	***P**seudo**m**onas aeruginosa **E**xtended-spectrum beta-lactamase*
PNAG	**P**oli-β-1,6-**N**-**A**cetil**g**lucosamina
PPSUS	**P**rograma de **P**esquisa para o **SUS**
PR	**P**an **R**esistentes aos antimicrobianos
PSE	***P**seudomonas **S**pecific **E**nzyme*
RAM	**R**esistência aos **A**ntimicrobianos
REC	***R**esearch **E**thics **C**ommittee*
RNAr	**RNA ribosomal**
RND	***R**esistance-**N**odulation-**D**ivision*
RpoB	***RNA **Po**lymerase Subunit **B*** (RNA polimerase subunidade B)
SAM	***A**m**S**ulbactam*
SFC	***S**erratia **f**onticola **C**arbapenemase*
SHV	***S**ulp**h**ydryl-**V**ariable beta-lactamase*
SIM	***S**eoul **Im**ipenemase*
SME	***S**erratia **m**arcescens **E**nzyme*
SMR	***S**mall **M**ultidrug **R**esistance*
SPM	***S**ão **P**aulo **M**etallo- beta-lactamase*
SST1	**S**istemas de **S**ecreção **T**ipo I
SST2	**S**istemas de **S**ecreção **T**ipo II
SST3	**S**istemas de **S**ecreção **T**ipo III
SST4	**S**istemas de **S**ecreção **T**ipo IV
SST5	**S**istemas de **S**ecreção **T**ipo V
SST6	**S**istemas de **S**ecreção **T**ipo VI
STP	***S**ewage **T**reatment **P**lants*
SUS	**S**istema **Ú**nico de **S**áude
SUT	***T**rimethoprim-**Su**lfamethoxazole*
TET	***Tet**racycline*
TEM	***Tem**oniera beta-lactamase*
TIG	***T**igecycline*
Tn	**T**ra**n**sposon
TSB	***T**ryptic **S**oy **B**roth*
UEL	**U**niversidade **E**stadual de **L**ondrina
UFC	**U**nidade **F**ormadora de **C**olônia
UPGMA	***U**nweighted **P**air **G**roup **M**ethod with **A**rithmetic averages*

UTI	**U**nidades de **T**erapia **I**ntensiva
UTI	***U**rinary **T**ract **I**nfection* (Infecção do trato urinário)
VEB	***V**ietnam **E**xtended-spectrum **b**eta-lactamase*
VIM	***V**erona **I**ntegron-encoded **M**etallo- beta-lactamase*
WHO	***W**orld **H**ealth **O**rganization*
XDR	*E**x**tensively **D**rug-**R**esistant*
2-MPA	**2**-***M**ercapto**p**ropionic **A**cid* (Ácido 2-mercaptopropiônico)

SUMÁRIO

1 INTRODUÇÃO

Nos últimos anos, a resistência antimicrobiana (RAM) tem aumentado expressivamente em escala global. Apesar dos relatos na natureza, o surgimento e a disseminação da RAM em microrganismos patogênicos ocorreram em resposta ao uso dos antimicrobianos na prática clínica. Este uso contribuiu para a evolução e disseminação da resistência ao fornecer pressões seletivas sem precedentes, especialmente em microrganismos da microbiota de humanos e animais domésticos (CHRISTAKI; MARCOU; TOFARIDES, 2020; ASLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022). A RAM é considerada um problema de saúde pública mundial que ameaça a eficácia terapêutica e tende a aumentar a morbidade, mortalidade, tempo de hospitalização, incidência e custos. Embora medir o custo global da RAM seja difícil, é indiscutível que o ônus financeiro é consideravelmente alto (MCEWEN; COLLIGNON, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020; SAMREEN; MALAK; ABULREESH, 2021).

Redes de vigilância epidemiológica na Europa (*European Antimicrobial Resistance Surveillance Network* - EARS-Net) e na Ásia (*Central Asia and Eastern European Surveillance of Antimicrobial Resistance* - CAESAR) detectaram um aumento significativo na prevalência das bactérias RAM durante a última década. De acordo com o *Centers for Disease Control and Prevention* (CDC), a cada ano nos Estados Unidos, pelo menos 2 milhões de pessoas são infectadas com bactérias resistentes e pelo menos 23 mil pessoas morrem como resultado dessas infecções (CHRISTAKI; MARCOU; TOFARIDES, 2020; ASLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021). Atualmente, infecções causadas por bactérias RAM causam pelo menos 700 mil mortes anualmente. Em 2016, a Organização Mundial da Saúde (OMS) publicou um relatório afirmando que esse número deverá aumentar para 10 milhões de mortes por ano até 2050, caso nenhum plano de ação eficaz seja implementado. Consequentemente, as infecções causadas por bactérias RAM devem se tornar a principal causa de morte no mundo, superando diabetes, doenças cardíacas e câncer. Além disso, estimativas recentes do uso de antibióticos levarão à morte de aproximadamente 444 milhões de pessoas antes de 2100 (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; SAMREEN; MALAK; ABULREESH, 2021; JONGH *et al.*, 2022).

Visando combater a ameaça da RAM, foi criada a abordagem *One Health* que envolve esforços interdisciplinares e colaborativos para alcançar a saúde ideal na tríade humano-animal-ambiente. Isto por meio de um plano de ação baseado em evidências, vigilância e notificação da RAM, rastreamento da dinâmica de transmissão de patógenos multirresistentes aos antimicrobianos (MR), conscientização e adequação de políticas públicas (ASLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021). Adotando esta abordagem, agências globais como o *Global Antimicrobial Resistance Surveillance System* (GLASS) da OMS, *Food and Agriculture Organization* (FAO) e *World Organization for Animal Health* (OIE), por meio da aliança FAO-OIE-OMS, tomaram a iniciativa para mitigar esta ameaça global, em colaboração com organizações públicas e privadas. Seguindo as diretrizes deste Compromisso Tripartite, vários países implementaram planos de ações nacionais, adotando estratégias que tendem a apresentar resultados a médio e longo prazo (ASLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021).

Dentro desse contexto, dois principais fatores têm contribuído para o aumento epidêmico global da RAM: 1) o uso inadequado ou excessivo de antimicrobianos na saúde humana e animal, e em atividades agrícolas, gerando uma pressão seletiva em ambientes clínicos e naturais; 2) a rápida disseminação local e global de bactérias resistentes e determinantes de resistência dentro e entre esses setores, por diferentes vias, como por exemplo por controle de infecção ineficaz, contaminação ambiental e movimento geográfico de humanos e animais infectados (MCEWEN; COLLIGNON, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020; ASLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021).

Adicionalmente, estudos têm evidenciado que genes codificadores de RAM (GCRAM) ocorrem naturalmente, sendo encontrados em abundância em diferentes espécies bacterianas na natureza, compreendendo, assim, o resistoma ambiental. O resistoma consiste em determinantes de resistência que carregam o potencial de causar resistência clinicamente relevante, se transferidos e/ou expressos em patógenos que causam infecções humanas (CHRISTAKI; MARCOU; TOFARIDES, 2020; ALLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022). Caracterizar o resistoma ambiental, analisando a abundância e o padrão de resistência dos microrganismos, particularmente os associados a elementos genéticos móveis, é importante para entender o papel do

ambiente como fonte de novos determinantes de resistência, bem como prever a situação da RAM local (LARSSON; FLACH, 2022).

Além dos GCRAM, os antimicrobianos são amplamente disseminados em águas superficiais, efluentes, estações de tratamento de esgoto, vegetais, solo, resíduos animais e sistemas de aquicultura, sendo considerados poluentes emergentes do meio ambiente. Esta contaminação é causada pelo tratamento inadequado de resíduos industriais, residenciais, hospitalares e pecuária, bem como escoamento de campos agrícolas contendo esterco de gado e lixiviados de aterros, que podem conter bactérias, determinantes de resistência e compostos que incluem metais pesados, biocidas e antimicrobianos (SAKKAS *et al.*, 2019; SANTOS *et al.*, 2020; ASLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022). A exposição de bactérias a estes compostos, mesmo em concentrações subinibitórias, podem selecionar microrganismos resistentes a importantes antimicrobianos utilizados na terapia de infecções humanas e animais. A pressão seletiva exercida possibilita a transferência e aquisição de uma grande variedade de GCRAM em elementos genéticos móveis entre diferentes espécies bacterianas. Contribuindo, assim, para a aquisição, manutenção e disseminação de GCRAM e fatores de virulência dentro de comunidades bacterianas, bem como maior dificuldade de prevenir e tratar infecções bacterianas pela ocorrência de bactérias MR (MCEWEN; COLLIGNON, 2018; KARKMAN *et al.*, 2018; SAKKAS *et al.*, 2019; MATAR; ANDREMONT; BAZZI, 2020; CHRISTAKI; MARCOU; TOFARIDES, 2020; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022).

No ambiente hospitalar, bactérias ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) são patógenos responsáveis por infecções graves e com opções terapêuticas limitadas, que exibem crescentes números de isolados MR e virulentos (SAKKAS *et al.*, 2019; MULANI *et al.*, 2019; OLIVEIRA *et al.*, 2020). Diante do aumento das taxas de RAM, em 2017, a OMS publicou uma lista com patógenos que representam ameaça à saúde pública, os quais constituem prioridade global para a pesquisa, descoberta e desenvolvimento de novos antimicrobianos. Dentre os patógenos listados, *A. baumannii* resistente aos carbapenêmicos foi categorizado como patógeno de prioridade crítica para pesquisa e desenvolvimento de novos antibióticos (MULANI *et al.*, 2019; OLIVEIRA *et al.*, 2020; WHO, 2017; SAMREEN; MALAK; ABULREESH, 2021).

Os carbapenêmicos são considerados antimicrobianos de escolha no tratamento de infecções graves por bactérias Gram-negativas MR. No entanto, os crescentes relatos de resistência a estes fármacos, bem como isolados MR, têm limitado as opções terapêuticas disponíveis. Assim, à medida que a disponibilidade de novos antimicrobianos avança lentamente, uma alternativa tem sido a utilização de antibióticos em combinação, como a associação de carbapenêmicos e inibidores de beta-lactamase, tigeciclina, bem como a retomada do uso de polimixinas, que apresentam atividade de amplo espectro contra patógenos resistentes aos carbapenêmicos, porém com uso clínico limitado devido nefrotoxicidade e neurotoxicidade (MCEWEN; COLLINGNON, 2018; MATAR; ANDREMONT; BAZZI, 2020; AZAM; KHAN, 2019; DOI, 2019; NORDMANN; POIREL, 2019; NGUYEN; JOSHI, 2021; YOON; JEONG, 2021).

O monitoramento da RAM em isolados de *Acinetobacter* spp. não sensíveis aos carbapenêmicos e/ou ceftazidima é realizado no Hospital Universitário de Londrina (HU) desde 2006 pelo Laboratório Especial de Microbiologia Molecular e Resistência aos Antimicrobianos (LEMMRA). Um primeiro estudo avaliou 91 isolados recuperados entre 2006 e 2009, destes 61,2% apresentaram o gene *bla*$_{\text{OXA-23}}$ e um isolado apresentou uma nova variante do gene *bla*$_{\text{OXA-143}}$ caracterizada como *bla*$_{\text{OXA-231}}$ (GIONCO, 2012). Um segundo estudo, realizado por Romanin (2018), descreveu altas taxas de resistência aos carbapenêmicos e prevalência de isolados produtores de carbapenemases (92,2%) em isolados de *A. baumannii* recuperados de hemocultura. Do total de isolados avaliados (n=103), foi observada a prevalência dos genes *bla*$_{\text{OXA-23}}$ e *bla*$_{\text{OXA-253}}$ em 97,9% e 1,9% dos isolados, respectativamente. Outro estudo, realizado por Fávaro (2019), observou que dos 282 isolados de *A. baumannii* recuperados de material respiratório, 76,3% eram MR e apresentaram altas taxas de resistência aos antimicrobianos avaliados, incluindo os carbapenêmicos (100,0%). Esta taxa foi atribuída à predominância de isolados portadores do gene *bla*$_{\text{OXA-23}}$ (93,6%).

Baseado nas recomendações mundiais a respeito da vigilância da RAM, sabendo da importância clínica de *Acinetobacter* spp. em infecções associadas às instituições de saúde, bem como à limitação terapêutica que estes patógenos apresentam na instituição devido à RAM, este estudo visa caracterizar isolados clínicos de *Acinetobacter* spp. quanto ao perfil de sensibilidade, à presença de

determinantes de resistência aos carbapenêmicos e de fatores de virulência importantes no desenvolvimento de infecções.

2 REVISÃO DE LITERATURA

2.1 BACILOS GRAM-NEGATIVOS NÃO FERMENTADORES

As bactérias Gram-negativas não fermentadoras (BGNNF) são caracterizadas pela incapacidade de fermentar açúcares para produzirem energia para suas funções celulares vitais. São representantes deste grupo *Pseudomonas* spp., *Acinetobacter* spp., o complexo *Burkholderia cepacia* (CBC), *Stenotrophomonas maltophilia,* além de alguns gêneros isolados com menor frequência no ambiente hospitalar, como *Achromobacter, Alcaligenes*, *Brevimundas, Elisabethkingia*, *Flavobacterium*, *Ralstonia*, entre outros (GAJDÁCS; BURIÁN; TERHES, 2019; SOMILY *et al.*, 2021).

As BGNNF são amplamente encontradas na natureza, e reconhecidas, também, como importantes patógenos de infecções relacionadas a assistência à saúde (IRAS): infecção do trato urinário, pneumonia, bacteremia, meningite, infecções de pele e tecidos moles, entre outras. Estes microrganismos acometem, principalmente, imunossuprimidos, idosos e pacientes em terapia intensiva (GAJDÁCS; BURIÁN; TERHES, 2019; CHOU *et al.*, 2020; SOMILY *et al.*, 2021). Apesar da antibioticoterapia ser a forma mais comum e eficaz de combatê-los, os níveis crescentes de resistência a várias classes de antibimicrobianos, levando ao aumento de isolados MR, extensivamente resistentes (ER) ou mesmo pan-resistentes (PR), tem feito com que BGNNF sejam reconhecidos como um problema de saúde global e um desafio terapêutico na clínica médica (GAJDÁCS; BURIÁN; TERHES, 2019; CHOU *et al.*, 2020; SAMREEN; MALAK; ABULREESH, 2021; SOMILY *et al.*, 2021).

2.2 CARACTERÍSTICAS GERAIS DE *ACINETOBACTER* SPP.

Atualmente, o gênero *Acinetobacter* compreende 108 espécies com nomes válidos (https://lpsn.dsmz.de/search?word=acinetobacter; último acesso em 06 de abril de 2023), na sua maioria, microrganismos ambientais (VÁLQUEZ-LÓPEZ *et al.*, 2020). Descrito pela primeira vez, em 1911 por Beijerinck, após identificação de um patógeno originalmente nomeado *Micrococcus calcoaceticus*, este gênero teve por muito tempo seus membros classificados erroneamente como pertencentes a

diferentes gêneros como *Achromobacter*, *Alcaligenes, Micrococcus, Moraxella, Neisseria*, entre outros. O gênero *Acinetobacter* (do grego *akinetos*|=|imóvel) foi proposto, em 1954, por Brisou e Prevot para distinguir os microrganismos Gram-negativos não produtores de pigmentos e imóveis na família Achromobactereae (ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018).

Acinetobacter spp. são cocobacilos Gram-negativos não fermentadores da glicose, imóveis, estritamente aeróbicos, catalase-positivos, oxidase-negativos, indol-negativos e citrato-positivos. Geralmente, se apresentam aos pares na fase exponencial de crescimento e, normalmente, menores e quase esféricos na fase estacionária (ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018). Podem ser encontrados em diferentes nichos ecológicos, como água, solo, esgoto, lodo ativado, alimentos, animais, seres humanos, incluindo ambientes hospitalares (AL ATROUNI *et al.*, 2016; ALMASAUDI, 2018).

Considerado um dos principais patógenos causadores de IRAS no mundo, *Acinetobacter* spp. está associado a uma variedade de infecções que incluem pneumonia associada à ventilação mecânica (PAV), meningite, bacteremia, infecções de feridas, do trato uinário (ITU) e associadas à cateter (ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018; NGUYEN; JOSHI, 2021). Idade avançada, neonatos com baixo peso, realização de procedimentos cirúrgicos, ventilação mecânica, hospitalização prolongada, comorbidades, imunossupressão e uso prévio de antimicrobianos de amplo espectro são considerados fatores de risco para colonização ou infecção por *Acinetobacter* spp. (ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018). As infecções são responsáveis pelo aumento da mortalidade em pacientes críticos, com uma taxa de cerca de 30,0% (ALMASAUDI, 2018).

2.3 ESPÉCIES DE *ACINETOBACTER* SPP.

Das espécies clinicamente relevantes, *A. baumannii* destaca-se, sendo responsável por uma ampla gama de infecções hospitalares e adquiridas na comunidade. Esta espécie pertence ao complexo *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) juntamente com outras espécies estritamente relacionadas: *A. nosocomialis, A. pittii, A. seifertii, A. dijkshoorniae*, *A. calcoaceticus* and *A. oleivorans*, as quais são difíceis de serem distinguidas por meio de métodos bioquímicos convencionais utilizados na rotina laboratorial (AL ATROUNI *et al.*, 2016;

HARDING; HENNON; FELDMAN, 2018; KURIHARA *et al.*, 2020; ALMEIDA *et al.*, 2021). Destas, *A. calcoaceticus* é considerado um organismo ambiental isolado do solo e raramente associado a infecções (VEERARAGHAVAN, 2019; TAVARES *et al.*, 2020; BARAKA *et al.*, 2021).

Nos últimos anos, espécies não pertencentes ao complexo ACB, denominadas de *Acinetobacter* não *baumannii* (NBA), que incluem *A. baylyi, A. beijerinckii, A. bereziniae, A. guillouiae, A. haemolyticus, A. junii, A. lwoffii, A. parvus, A. sepcticus, A. schindleri, A. soli, A. ursingii,* entre outras, surgiram como importantes patógenos responsáveis por IRAS, incluindo PAV, bacteremia e ITU. Concomitante aos frequentes relatos de RAM e isolados MR, foram reportados nestes patógenos genes codificadores de importantes determinantes de resistência e fatores de virulência, causando grande preocupação em todo o mundo (AL ATROUNI *et al.*, 2016; EZE; CHENIA; ZOWALATY, 2018; TAVARES *et al.*, 2020; BARAKA *et al.*, 2021). Além disso, várias espécies de NBA são consideradas importantes reservatórios ambientais de determinantes genéticos de resistência, os quais podem ser transferidos para outras espécies, resultando em isolados clinicamente relevantes (BARAKA *et al.*, 2021).

Dentre os principais NBA detectados no HU estão as espécies A. *bereziniae, A. colistiniresistens, A. haemolyticus, A. radioresistens e A. ursingii* (FÁVARO, 2019). Destes, ressaltamos a importância clínica da espécie *A. colistiniresistens*, anteriormente denominada genomospecie 13BJ/14TU. Esta espécie intrinsecamente resistente à colistina apresenta concentração inibitória mínima (CIM) entre 16 a 64 µg/mL para colistina e 4 a 32 µg/mL para polimixina B, enquanto espécies clinicamente mais relevantes são sensíveis às polimixinas (CIM inferior a 2 µg/mL). Desta forma, a aquisição de mecanismos de resistência a diversas classes de antimicrobianos nesta espécie é preocupante visto a possibilidade de causar infecções intratáveis (NEMEC *et al.*, 2017).

2.4 IDENTIFICAÇÃO DE *ACINETOBACTER* SPP.

A identificação precisa das espécies de *Acinetobacter* é clinicamente importante, pois estas podem diferir significativamente em suas características biológicas e patológicas, perfil de resistência, mecanismos de resistência aos antimicrobianos e virulência e, consequentemente, na evolução clínica do paciente (PARK *et al.*, 2019; VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO

et al., 2020). Os isolados clínicos são tradicionalmente identificados em laboratórios de microbiologia com base nas características morfológicas e bioquímicas. No entanto, a identificação da espécie usando esses métodos ainda deixa a desejar em termos de facilidade, rapidez, consistência e confiabilidade (VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020).

O desafio na identificação e diferenciação das espécies de *Acinetobacter* levou ao surgimento dos diversos sistemas comerciais automatizados de identificação que estão disponíveis e são amplamente utilizados nas rotinas dos laboratórios de microbiologia, baseados em diferentes tecnologias, que incluem o sistema *MicroScan WalkAway* (fluorômetro ou fotômetro), e o sistema Vitek 2® (cartões para identificação) (VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020). De acordo com a literatura, o cartão de identificação do sistema Vitek 2® consegue identificar *A. baumannii* com até 99,0% de precisão, e identifica corretamente as espécies *A. junii, A. haemolyticus* e *A. johnsonii*, mas não é capaz de discriminar as espécies pertencem ao complexo ACB. Isso ocorre porque esse sistema de identificação utiliza um banco de dados já existente que não é abrangente, bem como os substratos utilizados para identificação não foram projetados especificamente para a identificação de espécies de *Acinetobacter* (VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020).

Diante desse cenário, vários métodos moleculares têm sido desenvolvidos para a classificação e identificação de espécies de *Acinetobacter* que incluem análises baseadas em sequências de DNA como amplificação de rRNA 16S, *bla*$_{\text{OXA-51}}$ e *rpo*B, que são aplicados principalmente em ambientes de pesquisa e apresentam maior poder discriminatório sobre os métodos convencionais (VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020).

2.5 EPIDEMIOLOGIA

Os microrganismos do grupo ESKAPE são patógenos responsáveis por causar a maioria das IRAS (KARLOWSKY *et al.*, 2017; OLIVEIRA *et al.*, 2020). Segundo levantamento realizado no HU, estes patógenos representaram 43,0% (n=54622) dos isolados recuperados de pacientes atendidos no período entre setembro de 2006 a dezembro de 2016. Esses microrganismos foram obtidos principalmente de *swabs* de vigilância (29,8%), secreções (27,9%) e urina (21,6%), de

pacientes internados em unidades de terapia intensiva (UTI) (36,4%), ambulatório (10,8%) e enfermaria (10,6%). Destes, 59,8% dos pacientes tiveram alta hospitalar, e 39,5% foram a óbito. Entre os microrganismos ESKAPE, *A. baumannii* foi o segundo patógeno mais isolado (20,8%), precedido pela *K. pneumoniae* (30,0%). Um total de 11.624 isolados (9,2%) de *Acinetobacter* spp. foram recuperados no HU, no período previamente descrito, dos quais 11.271 foram identificados como pertencentes ao complexo ACB e 253 como NBA.

Estudos de vigilância realizados pelo *International Nosocomial Infection Control Consortium* (INICC) em UTIs de hospitais de diferentes continentes têm reportado altas taxas de resistência aos carbapenêmicos em isolados de *Acinetobacter* spp. Num estudo realizado em 242 hospitais de 45 países da América Latina, Europa, Mediterrâneo Oriental, Sudeste Asiático e Pacífico Ocidental, no período de 2012 a 2017, foram relatadas as seguintes taxas de resistência aos carbapenêmicos (imipenem e/ou meriopenem): 92,8% dos isolados de *A. baumannii* recuperados de PAV, 80,8% dos isolados causadores de ITU associada a cateter e 73,4% de agentes de infecção da corrente sanguínea associada a cateter venoso central (ROSENTHAL *et al*., 2020a).

Na vigilância realizada no período de 2013 a 2018, foi observado que 91,5% dos isolados de *Acinetobacter* spp. relatados em IRAS, em adultos e crianças, eram resistentes a imipenem (ROSENTHAL *et al*., 2021). Já na vigilância realizada em hospitais de 141 cidades de 42 países da África, Américas, Mediterrâneo Oriental, Europa, Sudeste Asiático e regiões do Pacífico Ocidental, no período de 2013-2019, foi observado que 63,1% e 73,4% dos isolados de *A. baumannii* de infecção de corrente sanguínea relacionada a cateter venoso periférico e central, respectivamente, eram resistentes a imipenem ou meropenem (ROSENTHAL *et al*., 2020b).

Na América Latina, Labarca *et al.* (2016) avaliaram isolados de *A. baumannii* recuperados no período de 2002 e 2013, e obtiveram uma taxa de prevalência de 14,0%, sendo esta taxa superior à observada na Europa Ocidental (6,0%) e na América do Norte (4,0%). Em outro estudo realizado no mesmo período, foi observado que 55,0% dos isolados de *A. baumannii* avaliados apresentaram resistência aos carbapenêmicos (LABARCA *et al.*, 2016; RODRIGUEZ; NASTRO; FAMILIETTI, 2018). Na vigilância *Tigecycline Evaluation and Surveillance Trial* (TEST), que monitora globalmente as atividades *in vitro* da tigeciclina e um painel de antimicrobianos comercializados contra isolados Gram-positivos e Gram-negativos

clinicamente importantes, realizada entre 2004 e 2009, a taxa de resistência ao meropenem na América Latina (73,0%) foi superior à observada na América do Norte (40,0%), Europa (47,0%) e a Ásia-Pacífico (69,0%).

No Programa de Vigilância Antimicrobiana - SENTRY, a resistência ao imipenem em *Acinetobacter* spp. recuperados no período de 2006 a 2009 foi detectada em 26,0% dos isolados da América Latina, em 12,0% nos EUA, 21,0% na Europa e 41,0% na Ásia-Pacífico (LABARCA *et al.*, 2016). Altas frequências de CRAb são relatadas na América do Sul e, de acordo com o SENTRY, as taxas de resistência a carbapenêmicos de isolados de *A. baumannii* no Brasil aumentaram aproximadamente 60,0% em apenas uma década, 1997-1999 a 2008-2010 (12,6 % para 71,4%) e, também, aumentaram consideravelmente em outros países da América Latina (CAYÔ, 2017; SILVA *et al.*, 2018; CAMARGO *et al.*, 2020). Estudos relataram as taxas de resistência aos carbapenêmicos na Argentina (78,0% - imipenem/81,0% meropenem) e no Brasil (31,0-73,0% - imipenem/56,0-73,0% meropenem) entre as mais altas do mundo (LABARCA *et al.*, 2016; RODRIGUEZ; NASTRO; FAMILIETTI, 2018). Revisão sistemática realizada por Yu *et al*. (2022), utilizando artigos publicados entre 2015 e 2020, relatou que mais de 50,0% dos isolados de *Acinetobacter* spp. nos Estados Unidos, América do Sul, Índia e China eram resistentes aos carbapenêmicos. O aumento de CRAb tem sido associado à alta prevalência de isolados produtores de carbapenemases como OXA-23 e OXA-58, e a associação de seus genes codificadores à sequências de inserção e/ou transposons (KU *et al.*, 2015; JEAN; HARNOD; HSUEH, 2022; YU *et al*., 2022).

2.6 RESISTÊNCIA AOS ANTIMICROBIANOS

Os mecanismos de resistência bacteriana podem ser intrínsecos, adquiridos ou adaptativos. Entre os principais temos a inativação ou modificação de antimicrobianos através da produção de enzimas que destroem ou alteram o antibiótico, tornando-o ineficaz; diminuição do acúmulo de antimicrobianos por alteração da permeabilidade da membrana externa ou aumento do efluxo ativo dos antimicrobianos através da superfície celular; e alteração do alvo ou dos sítios de ligação dos antimicrobianos, como, por exemplo, alteração das proteínas de ligação à penicilina (PBP) ou alteração das proteínas de ligação ao ribossomo (RUPPÉ;

WOERTHER; BARBIER, 2015; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020).

Avanços recentes na ecologia microbiana levaram ao conceito de resistoma antimicrobiano, um reservatório ambiental de GCRAM e seus precursores em bactérias patogênicas e não patogênicas. Esse conceito ajuda a explicar como a resistência intrínseca pode se desenvolver e se disseminar entre as bactérias (MORRISON; ZEMBOWER, 2020; ALLAM *et al.*, 2021). As bactérias que vivem no solo estão, constantemente, expostas a diferentes compostos e desenvolveram mecanismos para interagir com outros microrganismos. Algumas das moléculas que elas produzem para sua "proteção" levaram à evolução de elementos de resistência altamente específicos, mesmo na ausência de produção inata de antimicrobianos (MORRISON; ZEMBOWER, 2020; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022).

Desta forma, a resistência intrínseca, mediada cromossomicamente, é um fenômeno natural que pode ocorrer independente da exposição a antimicrobianos e pode ser prevista a partir da identidade de um organismo, sendo encontrada dentro do genoma de um grupo de bactérias ou dentro de uma espécie bacteriana. Por exemplo, a resistência intrínseca de todas as *K. pneumoniae* à ampicilina, todos os *E. faecium* e *E. faecalis* às cefalosporinas e todos os *A. colistiniresistens* às polimixinas (RUPPÉ; WOERTHER; BARBIER, 2015; NEMEC *et al.*, 2017; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; ASLAM *et al.*, 2021).

A resistência adquirida, por sua vez, normalmente, é o tipo de resistência mais alarmante no ambiente hospitalar, pois bactérias inicialmente sensíveis podem tornar-se resistentes aos antimicrobianos. Dentre os diversos fatores que desempenham um papel nesta resistência, o principal é o uso excessivo e inadequado de antimicrobianos (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022). Esta resistência pode ocorrer por meio de mutações genéticas bacterianas ou pela aquisição de DNA exógeno carreando genes codificadores de resistência. Embora a aquisição de novos GCRAM por patógenos seja preocupante, de grande importância, também, são as mudanças no contexto genético destes genes adquiridos. Estas alterações podem modificar a expressão dos GCRAM, oportunidades de co-seleção, virulência e aumentar o seu potencial de

disseminação (RUPPÉ; WOERTHER; BARBIER, 2015; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; SAMREEN; MALAK; ABULREESH, 2021; LARSSON; FLACH, 2022).

As mutações ocorrem durante o processo de reprodução bacteriana, por meio de alterações genéticas aleatórias. A exposição a antimicrobianos cria uma pressão evolutiva sobre as bactérias e confere uma vantagem seletiva de sobrevivência para aquelas que adquiriram estas mutações. Como resultado, um aumento na expressão de genes codificadores de mecanismos de resistência intrínseca, como sistemas de efluxo, que promovem a extrusão de antimicrobianos e outras substâncias do interior das células bacterianas; alterações de permeabilidade, por perda, modificação do tamanho, condutância, ou redução de porinas da membrana externa, impedindo que um antibiótico entre ou se acumule dentro da célula bacteriana; ou modificações de alvos requeridos pelo antimicrobiano, de tal forma que, mesmo que este entre na célula, ele não se liga ao alvo para matar ou inibir o crescimento bacteriano (RUPPÉ; WOERTHER; BARBIER, 2015; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; ASLAM *et al.*, 2021).

A aquisição de material genético exógeno ocorre através de um processo conhecido como transferência horizontal de genes, no qual o material genético de uma célula é transferido para outra, por meio de transdução, transformação e conjugação (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; SAMREEN; MALAK; ABULREESH, 2021). A transdução envolve a transferência de material genético entre uma célula doadora e uma célula receptora por um vírus conhecido como bacteriófago. A transformação consiste na captação direta de material genético livre no ambiente, geralmente oriundo de uma célula bacteriana lisada. No entanto, apenas algumas bactérias são naturalmente competentes. A conjugação, por sua vez, é provavelmente o mecanismo mais importante de transferência horizontal de genes, no qual ocorre o compartilhamento de DNA extracromossomal diretamente entre as células. Este processo de transferência lateral de genes, envolvendo plasmídeos, é auxiliado por uma estrutura da célula doadora chamada pili sexual. O pili adere à superfície da célula receptora, aproximando as duas células e estimulando a formação de um poro, que conecta ambas as células e é por onde a fita do plasmídeo é carreada (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020).

Estes mecanismos permitem que as bactérias não apenas alterem seu material genético e desenvolvam resistência a múltiplos antimicrobianos, mas também compartilhem rapidamente esse material entre diferentes espécies bacterianas. Além desses mecanismos clássicos de transferência horizontal de genes, existe uma variedade de outros elementos genéticos móveis que podem transferir características de resistência bacteriana, como ilhas genômicas, sequências de inserção, transposons e integrons (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020).

Por fim, a resistência adaptativa consiste na resistência a um ou mais antimicrobianos induzida por um sinal ambiental específico, que incluem estresse, estado de crescimento, pH, concentrações de íons, condições de nutrientes e níveis sub-inibitórios de antibióticos. Estas mudanças ambientais promovem modulações na expressão gênica de sistemas de efluxo e porinas permitindo que as bactérias respondam mais rapidamente a presença dos antimicrobianos. Geralmente, quando o sinal indutor é removido, a bactéria reverte ao seu estado original. Desta forma, em contraste com a resistência intrínseca e adquirida, causadas por alterações genéticas geralmente com fenótipos irreversíveis; a resistência adaptativa, resultante de alterações epigenéticas, tem caráter transitório. No entanto, em alguns casos, a resposta aos estímulos ambientais pode não reverter completamente após remoção do estímulo, levando a um aumento gradual da CIM ao longo do tempo (CHRISTAKI; MARCOU; TOFARIDES, 2020).

Atualmente, os elevados índices de RAM, bem como as altas taxas de isolados de *Acinetobacter* MR, tornaram o tratamento de infecções causadas por estes patógenos um grande desafio. Isto se deve à notória plasticidade genômica deste microrganismo, que permite o acúmulo de determinantes de resistência às principais classes de antimicrobianos utilizados na prática clínica, aliado aos seus mecanismos intrínsecos e adquiridos (LEE *et al.*, 2017; AZAM; KHAN, 2019; PANG *et al.*, 2019; OLIVEIRA *et al.*, 2020; FIGUEREDO *et al.*, 2021; MORRISON; ZEMBOWER, 2020; YOON; JEONG, 2021).

2.6.1 Beta-Lactamases

A produção de beta-lactamases (BL) é um importante mecanismo de resistência em bactérias Gram-negativas, responsável pela hidrólise da ligação amida

do anel beta-lactâmico dos antimicrobianos, tornando-os ineficazes. A primeira BL foi descrita, na década de 1940, em um isolado de *E. coli,* um ano antes do início do uso clínico da penicilina (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; ALLAM *et al.*, 2021). Atualmente, 7.812 BL foram relatadas em diversas espécies bacterianas (http://bldb.eu/BLDB.php?prot=A, acesso em 27 de fevereiro de 2023). Essas enzimas podem ser codificadas cromossomicamente ou, mais frequentemente, serem encontradas em elementos genéticos móveis como plasmídeos, transposons e sequências de inserção (ALMASAUDI, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020; HALAT; MOUBARECK, 2020).

As BL podem ser classificadas de acordo com suas propriedades funcionais e moleculares, conforme proposto por Bush-Jacoby-Medeiros e Ambler, respectivamente (Figura 1) (MORRISON; ZEMBOWER, 2020; HALAT; MOUBARECK, 2020). A classificação de Bush-Jacoby-Medeiros é um esquema bioquímico baseado em propriedades que incluem pontos isoelétricos, perfis de substrato e características do inibidor, que classifica as BL funcionalmente em grupos 1 a 3, de acordo com a última atualização realizada em 2010. A classificação de Ambler, sistema mais comumente utilizado, é baseada na homologia dos aminoácidos e classifica as BL em quatro classes principais: A, B, C e D. As classes A, C e D (serino carbapenemases) apresentam o aminoácido serina no seu sítio ativo, enquanto a classe B (metalo-beta-lactamases) inclui metaloenzimas com um sítio ativo de zinco (ALMASAUDI, 2018; MORRISON; ZEMBOWER, 2020; HALAT; MOUBARECK, 2020).

Com o intuito de superar as ações das BL, inibidores como ácido clavulânico, sulbactam e tazobactam, foram introduzidos na prática clínica. Esses inibidores atuam ligando-se ao sítio ativo da enzima com o objetivo de impedir a ligação e hidrólise do antimicrobiano beta-lactâmico (MORRISON; ZEMBOWER, 2020).

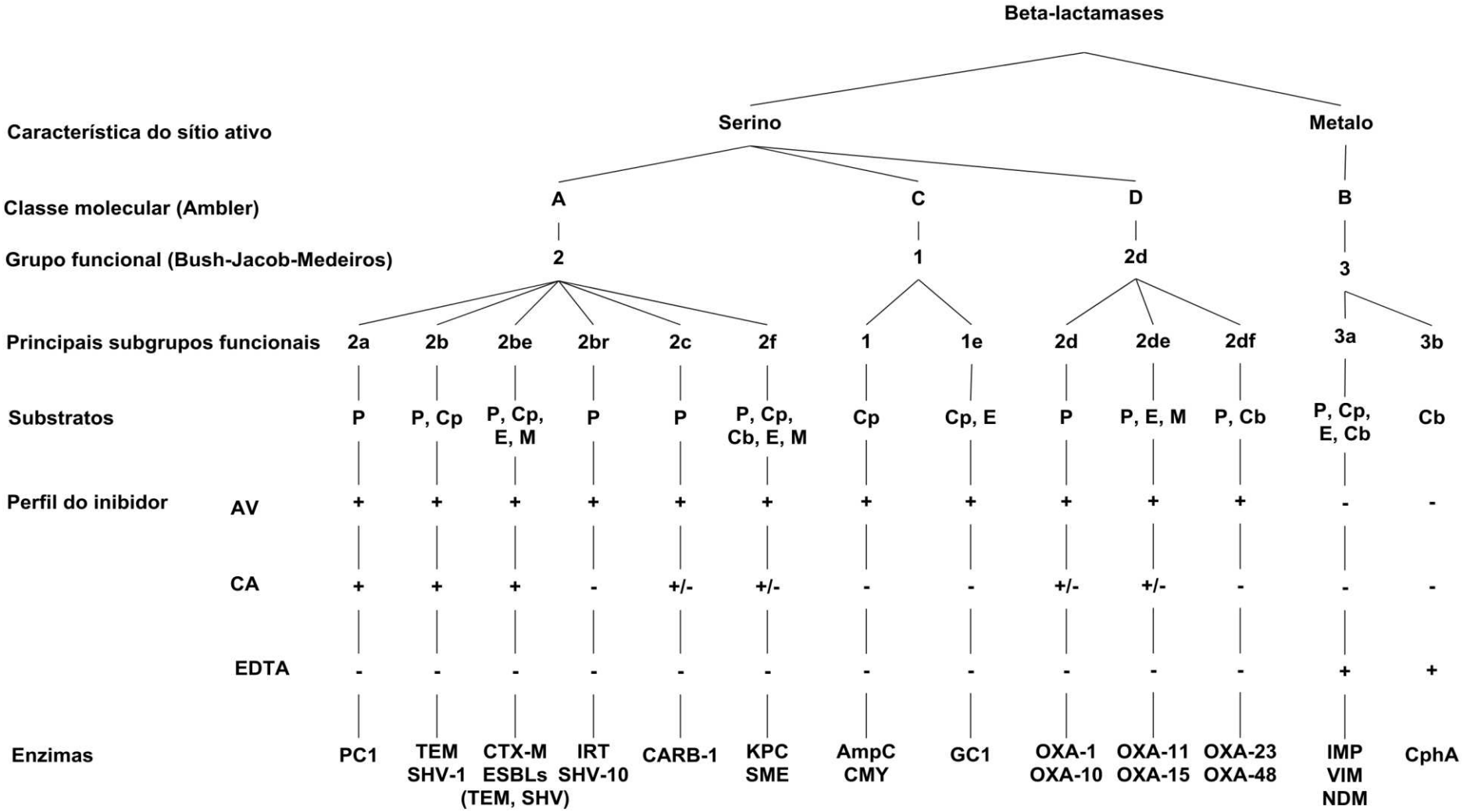


Figura 1 Relações moleculares e funcionais entre beta-lactamases

Fonte: Adaptado de BUSH, 2018.

AV, avibactam; CA, ácido clavulânico; EDTA, ácido etileno-diamino-tetracético; Cb, carbapenêmico; Cp, cefalosporina; E, cefalosporina de espectro extendido; M, monobactâmico; P, penicilina.

2.6.1.1 Carbapenemases

As carbapenemases são BL com capacidade de hidrolisar uma ampla variedade de beta-lactâmicos, incluindo os carbapenêmicos. Estas enzimas pertencem aos grupos funcionais 2d, 2f e 3 de Bush-Jacoby-Medeiros, e às classes A, B, D e, menos frequentemente, à classe C de Ambler (BONOMO *et al.*, 2018; MORRISON; ZEMBOWER, 2020; HALAT; MOUBARECK, 2020). As carbapenemases são mundialmente distribuídas em diferentes gêneros bacterianos e podem ser codificadas por genes cromossômicos e por plasmídeos. Alguns tipos de carbapenemases são associadas a regiões ou países específicos, apesar da epidemiologia das mesmas, em muitos países, ainda não ser bem documentadas. No entanto, com a globalização, a associação entre um determinado mecanismo de RAM e uma região/país pode ser modificada, sendo necessária a vigilância constante (BONOMO *et al.*, 2018; HALAT; MOUBARECK, 2020).

As principais questões associadas à produção de carbapenemases são implicações clínicas, devido ao comprometimento da atividade dos antimicrobianos de último recurso usados no tratamento de infecções graves. Até o momento, as carbapenemases mais eficazes, em termos de hidrólise de carbapenêmicos e distribuição geográfica, são as enzimas *Klebsiella pneumoniae carbapenemase* (KPC), OXA-48 e as MBLs *Verona Integron-encoded Metallo-beta-lactamase* (VIM), *Imipenemase* (IMP) e *New Delhi Metallo-beta-lactamase* (NDM) (HALAT; MOUBARECK, 2020).

2.6.1.2 Classe A de Ambler

As enzimas da classe A de Ambler apresentam o aminoácido serina na posição 70 do sítio ativo. Estas enzimas podem ser classificadas em *narrow-spectrum beta-lactamases* (NSBLs), *extended-spectrum beta-lactamases* (ESBLs) e enzimas com atividade de carbapenemase, as quais podem ser codificadas cromossomicamente ou, mais frequentemente, por elementos genéticos móveis como plasmídeos e transposons (HALAT; MOUBARECK, 2020; MORRISON; ZEMBOWER, 2020; YOON; JEONG, 2021). As NSBLs hidrolisam cefalosporinas de espectro limitado, e as ESBLs conferem resistência às penicilinas, cefalosporinas de amplo espectro (3ª geração) e monobactâmicos. Ambas podem ser inibidas pelo ácido

clavulânico e tazobactam, os quais se ligam ao sítio ativo da enzima BL, evitando a ligação e hidrólise do antimicrobiano (CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020).

As carbapenemases da classe A, por sua vez, são capazes de hidrolisar uma ampla variedade de beta-lactâmicos, incluindo penicilinas, carbapenêmicos, cefalosporinas e monobactâmicos, e podem ser inibidas por derivados de clavulanato, tazobactam e ácido borônico. As bactérias que expressam estas enzimas são caracterizadas por sensibilidade reduzida ao imipenem (RUPPÉ; WOERTHER; BARBIER, 2015; WENZLER *et al.*, 2017; ALMASAUDI, 2018; HALAT; MOUBARECK, 2020; YOON; JEONG, 2021). As carbapenemases podem ser codificadas cromossomicamente, como a *Not metalloenzyme carbapenemase A* (NmcA), *Serratia marcescens enzima-1* (SME-1), *Serratia fonticola carbapenemase-1* (SFC-1) e *Sulphydryl-Variable beta-lactamase-38* (SHV-38), e mediadas por plasmídeos, como IMI (I*mipenem hydrolyzing carbapenemase*), KPC e algumas variantes de *Guiana Extended Spectrum* (GES) (LIMA, 2019; HALAT; MOUBARECK, 2020).

2.6.1.3 Classe B de Ambler

As enzimas da classe B de Ambler, ou metaloenzimas, precisam de cátions bivalentes, geralmente íons zinco (Zn^{2+}), como cofator metálico para hidrolisar beta-lactâmicos. Considerado um dos principais mecanismos de resistência aos carbapenêmicos entre bacilos Gram-negativos, as MBLs apresentam capacidade hidrolítica frente a todos os beta-lactâmicos, exceto monobactâmicos. São resistentes aos inibidores de BL comercialmente disponíveis, no entanto, sua atividade é inibida por agentes quelantes de metais, como o ácido etilenodiaminotetraacético (EDTA) e o ácido 2-mercaptopropiônico (2-MPA) (LEE *et al.*, 2017; ALMASAUDI, 2018; HALAT; MOUBARECK, 2020; YOON; JEONG, 2021).

As MBLs mais comuns incluem as enzimas IMP, VIM, NDM, *German Imipenemase* (GIM) e *Seoul Imipenemase* (SIM), localizadas geneticamente dentro de uma variedade de integrons, onde foram incorporadas como cassetes gênicos, e quando esses integrons se associam a plasmídeos ou transposons, a transferência entre bactérias é facilitada. Atualmente, as MBLs são encontradas em diferentes espécies bacterianas Gram-negativas e estão frequentemente associadas à

resistência a diversas classes de antimicrobianos, resultando em resistência a múltiplas drogas e redução das opções de tratamento (HALAT; MOUBARECK, 2020; YOON; JEONG, 2021).

2.6.1.4 Classe C de Ambler

As enzimas da classe C de Ambler, também conhecidas como cefalosporinases cromossômicas do tipo AmpC, têm a característica de serem induzíveis pela presença de beta-lactâmicos, na maioria dos gêneros da família Enterobacteriaceae, conferindo resistência à penicilinas, cefalosporinas (1ª, 2ª, 3ª geração), monobactâmicos e cefamicinas. A indução, normalmente, diminui uma vez que a exposição ao antimicrobiano é removida, no entanto, mutações espontâneas do gene codificador desta enzima levam à uma produção contínua de AmpC (CHRISTAKI; MARCOU; TOFARIDES, 2020; HALAT; MOUBARECK, 2020; MORRISON; ZEMBOWER, 2020). Codificada, principalmente, em cromossomos, e recentemente descrita em plasmídeo, esta enzima foi relatada em várias espécies bacterianas que incluem *Enterobacter* spp., *Citrobacter freundii, Serratia* spp., *Providencia* spp., *Morganella morganii, Aeromonas* spp*., Acinetobacter* spp. e *P. aeruginosa* (HALAT; MOUBARECK, 2020; MORRISON; ZEMBOWER, 2020).

2.6.1.5 Classe D de Ambler

As enzimas da classe D de Ambler, também denominadas oxacilinases, podem ser classificadas em *Narrow-Spectrum Oxacillinases* (NS-OXA), *Extended-Spectrum Oxacillinases* (ES-OXA) e *Carbapenem-Hydrolyzing Class D beta-Lactamase* (CHDL). As NS-OXA e ES-OXA apresentam atividade hidrolítica frente a penicilinas e cefalosporinas de amplo espectro; e as CHDLs, com atividade de carbapenemases, hidrolisam penicilinas e carbapenêmicos. Estas enzimas, geralmente, não são inibidas pelo ácido clavulânico, tazobactam e sulbactam, no entanto, podem ser inibidas *in vitro* pelo cloreto de sódio (NaCl) (DJAHMI *et al.*, 2014; POTRON; POIREL; NORDMANN, 2015; RUPPÉ; WOERTHER; BARBIER, 2015; ALMASAUDI, 2018; HALAT; MOUBARECK, 2020).

O surgimento destas enzimas presumivelmente coincidiu com a introdução generalizada de flucloxacilina e meticilina para o tratamento de infecções

estafilocócicas, sendo as primeiras enzimas identificadas as OXA-1, -2 e -3, recuperadas de bactérias Gram-negativas. Posteriormente, a enzima OXA-11, primeira variante de OXA de espectro estendido, foi isolada de *P. aeruginosa* e exibiu um perfil de resistência transferível com hidrólise de ceftazidima aumentada. Logo, outras enzimas foram relatadas como OXA-13, OXA-14, OXA-15, OXA-16, OXA-17, OXA-19, OXA-28 e OXA-45 que permaneceram confinadas a *P. aeruginosa* (HALAT; MOUBARECK, 2020). Atualmente, as enzimas CHDLs são compostas por seis grupos principais que incluem os genes *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24/40-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-143-like}}$ e *bla*$_{\text{OXA-235-like}}$ (HALAT; MOUBARECK, 2020; NGUYEN *et al.*, 2020). Essas enzimas conferem fraca hidrólise dos carbapenêmicos, no entanto, podem conferir resistência se associadas a outros mecanismos e/ou sequências de inserção (IS) a montante do gene levando à sua superexpressão (NGUYEN *et al.*, 2020).

A enzima OXA-23, inicialmente denominada ARI-1 (*Acinetobacter resistente ao imipenem*), foi descrita pela primeira vez em 1985 na Escócia, mesmo ano em que o imipenem foi aprovado para uso clínico. Diversas variantes cromossomais do gene *bla*$_{\text{OXA-23-like}}$ foram relatadas na espécie *A. radioresistens*, indicando esta espécie como provável reservatório natural dessas enzimas (PATON *et al*., 1993; CASTILHO *et al.*, 2017; RODRIGUEZ; NASTRO; FAMILIETTI, 2018; HALAT; MOUBARECK, 2020). O gene *bla*$_{\text{OXA-23-like}}$ pode estar associado à IS*Aba*1 ou IS*Aba*4, compondo os contextos genéticos relatados na literatura como responsáveis por sua disseminação (Tn*2006*, Tn*2007*, Tn*2008* e Tn*2009*). Esses elementos a montante do gene podem atuar como promotores, aumentando significativamente a expressão gênica e a resistência ao carbapenem, sendo o mais eficaz deles a sequência de inserção IS*Aba*1 (CHEN *et al.*, 2017; CASTILHO *et al.* 2017; KURIHARA *et al.*, 2020; HALAT; MOUBARECK, 2020).

A OXA-143, relatada pela primeira vez, em 2004, no Brasil, é responsável por hidrolisar penicilinas e carbapenêmicos, e pode apresentar IS*Aba*1 a montante de seu gene codificador (HIGGINS *et al*., 2009; RODRIGUES-COSTA *et al.*, 2019; HALAT; MOUBARECK, 2020). Embora, acredita-se que o Brasil seja o epicentro desta enzima, a OXA-143 também foi detectada em isolados de *A. baumannii* de hospitais universitários no Irã em 2017 (HALAT; MOUBARECK, 2020). As variantes identificadas do grupo OXA-143 incluem OXA-182, OXA-231, OXA-253 e OXA-255. A OXA-231 teve sua primeira detecção em um isolado de *A. baumannii*, em 2007, no HU de Londrina, Brasil. Porém, sua real frequência ainda é

desconhecida, devido a poucos relatos até o momento (GIONCO *et al.*, 2012; HALAT; MOUBARECK, 2020).

Já a enzima OXA-58 foi descrita pela primeira vez em um isolado clínico de *A. baumannii,* em 2003, na França. Essa enzima apresenta baixa atividade hidrolítica contra penicilinas e carbapenêmicos, com capacidade de hidrolisar algumas cefalosporinas de 1ª e 4ª geração. No entanto, a presença de outros mecanismos simultaneamente permite a identificação de altos níveis de resistência aos carbapenêmicos (POIREL *et al*., 2005; EVANS; AMYES, 2014; HALAT; MOUBARECK, 2020).

O grupo enzimático OXA-24/40 foi inicialmente identificado em um isolado de CRAb recuperado do ano de 1997 na Espanha. Estas enzimas apresentam capacidade hidrolítica contra penicilinas (benzilpenicilina), cefalosporinas de 1ª geração (cefaloridina) e carbapenêmicos, e sua atividade enzimática pode ser inibida por íons cloreto e inibidores de BL como tazobactam, sulbactam e ácido clavulânico (BOU; OLIVER; BELTRAN, 2000; EVANS; AMYES, 2014).

A enzima OXA-51, por sua vez, foi primeiramente detectada em isolados clínicos de CRAb recuperados do período de 1993 à 1994 na Argentina (BROWN; YOUNG; AMYES, 2005). Estas enzimas são codificadas em cromossomos e plasmídeos, e o elevado número de variantes sugere que estas tem estado sob considerável pressão seletiva antimicrobiana resultando na detecção de novas variantes que, provavelmente, apresentam atividade hidrolítica variada. Apenas a presença do gene *bla*$_{\text{OXA-51-like}}$, devido sua baixa atividade hidrolítica, não é capaz de promover resistência aos carbapenêmicos, no entanto, quando associado a IS*Aba*1 observa-se o aumento da CIM aos carbapenêmicos (EVANS; AMYES, 2014; RUPPÉ; WOERTHER; BARBIER, 2015).

Por fim, a enzima OXA-235, CHDL intrínseca de *A. schindleri,* foi descrita pela primeira vez por Higgins e colaboradores (2013), em um estudo utilizando isolados de *A. baumannii* recuperados em hospitais dos Estados Unidos e México, entre 2005 e 2009. Além da detecção desta nova classe, foi identificada a existência de duas variantes (OXA-236 e OXA-237). Estas enzimas são capazes de hidrolisar penicilinas e carbapenêmicos, mas não cefalosporinas de amplo espectro. O gene codificador desta enzima está associado a IS*Aba*1 (HIGGINS et al, 2013; POTRON; POIREL; NORDMANN, 2015; HALAT; MOUBARECK, 2020).

2.6.2 Mecanismos de Resistência aos Antimicrobianos em *Acinetobacter* spp.

Em *A. baumannii,* a resistência intrínseca é atribuída parcialmente à baixa permeabilidade da membrana, à expressão de sistemas de efluxo e à produção de enzimas hidrolisadoras e/ou modificadoras de antimicrobianos. Além de o antimicrobiano ertapenem não apresentar atividade contra este patógeno, *A. baumannii* produz naturalmente uma cefalosporinase tipo AmpC não induzível e uma oxacilinase (OXA-51) que conferem, em níveis basais de expressão, resistência intrínseca à aminopenicilinas, cefalosporinas de 1ª e 2ª geração, e aztreonam. Quando superexpressas, estas enzimas conferem resistência a cefaloporinas de 3ª geração e carbapenêmicos, respectivamente (RUPPÉ; WOERTHER; BARBIER, 2015; MORRISON; ZEMBOWER, 2020). Concomitante a estes mecanismos, apresenta uma marcada impermeabilidade, devido mutações nas porinas ou proteínas de membrana externa (OMP); e expressa múltiplos sistemas de efluxo, que conferem resistências variáveis aos antimicrobianos (RUPPÉ; WOERTHER; BARBIER, 2015; ALMASAUDI, 2018; MORRISON; ZEMBOWER, 2020). Entre os principais sistemas de efluxo em *A. baumannii* estão os sistemas AdeABC, AbeM e AdeIJK. O AdeABC confere resistência à diversos beta-lactâmicos, incluindo carbapenêmicos, aminoglicosídeos, fluoroquinolonas, tetraciclinas e trimetoprim; o AbeM à aminoglicosídeos, fluoroquinolonas, entre outros compostos tóxicos; e o AdeIJK à tigeciclina (RUPPÉ; WOERTHER; BARBIER, 2015; LEE *et al.*, 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018).

2.6.2.1 Beta-lactâmicos

Os beta-lactâmicos são os antimicrobianos mais utilizados em todo o mundo e incluem as penicilinas, cefalosporinas, monobactâmicos e carbapenêmicos. Os carbapenêmicos, em especial, são os antimicrobianos de escolha para o tratamento de infecções por patógenos Gram-negativos MR em hospitais e instituições de longa permanência (LIMA, 2019; WANG *et al.*, 2020; BRASILIENSE *et al.*, 2021; YOON; JEONG, 2021).

A resistência aos carbapenêmicos em *A. baumannii* pode resultar da superexpressão da enzima intrínseca OXA-51-*like*, devido mutação ou presença de sequência de inserção à montante do gene, atuando como promotor. Alterações na

permeabilidade por perda ou modificação de porinas de membrana externa; superexpressão de sistemas de efluxo como AdeABC; mudanças nas porinas como CarO e OmpA, diminuindo a absorção do antimicrobiano, devido deficiência de porinas; mudanças nas PBPs 1a, 1c, 2, 3, 4, 4b e 5, diminuindo a afinidade pelo fármaco; e aquisição de genes codificadores de enzimas com atividade de carbapenemases, que são BL com o mais amplo espectro de atividade, também contribuem para este fenótipo (RUPPÉ; WOERTHER; BARBIER, 2015; ALMASAUDI, 2018; HALAT; MOUBARECK, 2020).

Diversas BL foram descritas em *Acinetobacter* spp., como *Pseudomonas Extended Resistant* (PER), *Vietnam Extended-spectrum beta-lactamase* (VEB), GES, *Temoniera beta-lactamase* (TEM), SHV, *Cefotaxime Munich* (CTX-M), *Kyorin University Hospital imipenemase* (KHM), *Australian imipenemase* (AIM), SIM, IMP, NDM, SPM, *Acinetobacter-Derived Cephalosporinase* (ADC) e OXA, conferindo resistência às penicilinas, cefalosporinas de 3ª geração e carbapenêmicos (RUPPÉ; WOERTHER; BARBIER, 2015; ALMASAUDI, 2018; REZAEI *et al.*, 2018; SOLTANI *et al.*, 2018; HALAT; MOUBARECK, 2020).

Em *Acinetobacter,* as enzimas da classe A de Ambler mais comumente identificadas são PER (-1, -2, -3, -7, -8), VEB (-1, -3, -7) e GES (-1, -5, -11, -12, -14, -22). E menos frequentemente TEM (-1, -2, -92, -116), SHV (-1, -5, -12), CTX-M (-2, -15, -43), além da enzima KPC (-2, -3, -4, -10), detectada em países como Porto Rico e Portugal (DJAHMI *et al.*, 2014; POTRON; POIREL; NORDMANN, 2015; RUPPÉ; WOERTHER; BARBIER, 2015; LEE *et al.*, 2017; SHIN; PARK, 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018; HALAT; MOUBARECK, 2020; MORRISON; ZEMBOWER, 2020; HALAT; MOUBARECK, 2020; YOON; JEONG, 2021). Entre as MBLs estão IMP (-1, -2, -4, -5, -6, -8, -10, -11, -14, -19, -24, -55), VIM (-1, -2, -3, -4, -6, -11), NDM (-1, -2, -3), SIM (-1), GIM (-1) e SPM (-1) (DJAHMI *et al.*, 2014; POTRON; POIREL; NORDMANN, 2015; LEE *et al.*, 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018; HALAT; MOUBARECK, 2020; YOON; JEONG, 2021). No Brasil, já foram detectados os genes codificadores das enzimas IMP-1 (CAYÔ, et al, 2018; BRASILIENSE *et al.*, 2021), IMP-10 (CAYÔ *et al.*, 2015) e NDM-1 (PAGANO *et al.* 2015; CHAGAS *et al.*, 2015).

Entre as enzimas da classe C foram identificadas as enzimas intrínsecas ADC (-1, -3, -4, -6, -7, -11, -26, -30, -33, -56, -57, -68, -73, -76, -77, -81), que são produzidas em níveis basais. Ao contrário do observado em outros

microrganismos Gram-negativos, em *Acinetobacter* estas enzimas não são induzíveis, sendo sua superexpressão regulada pela presença de sequências de inserção à montante do gene, promovendo resistência à penicilinas, cefalosporinas de limitado e amplo espectro. Algumas variantes da enzima ADC, como ADC-33 e ADC-56, apresentam adicionalmente atividade hidrolítica contra ceftazidima e cefepime, sendo denominadas de *Extended-Spectrum AmpC* (ESAC) (POTRON; POIREL; NORDMANN, 2015; RUPPÉ; WOERTHER; BARBIER, 2015; LEE *et al.*, 2017; WENZLER *et al.*, 2017; SHIN; PARK, 2017; ALMASAUDI, 2018; MORRISON; ZEMBOWER, 2020). Em 2014, foi relatada em um isolado de *A. baumannii* na Coréia a enzima ADC-68 que apresenta atividade de AmpC e carbapenemase. Muitos estudos mostraram que tal AmpC rara com atividade de carbapenemase pode agravar a resistência aos carbapenêmicos quando combinada com a impermeabilidade da membrana externa e/ou superexpressão de sistemas de efluxo (HALAT; MOUBARECK, 2020).

As CHDLs, por sua vez, consistem nas BL adquiridas mais prevalentes no gênero *Acinetobacter* e a principal causa de resistência aos carbapenêmicos neste patógeno, sendo elas OXA-23, OXA-24/40, OXA-58, OXA-143 e OXA-235 (HALAT; MOUBARECK, 2020). De acordo com a literatura, a enzima OXA-23 em *Acinetobacter* é a carbapenemase mais difundida no mundo e na América Latina está presente em todos os países, sendo frequentemente associada a clones endêmicos (CASTILHO *et al.*, 2017; RODRIGUEZ; NASTRO; FAMILIETTI, 2018; HALAT; MOUBARECK, 2020).

No Brasil, esse gene é considerado o principal determinante associado à resistência aos carbapenêmicos e estudos têm associado o aumento da resistência com a disseminação das cepas produtoras de OXA-23 (ROYER *et al.*, 2018; RODRIGUES-COSTA *et al.*, 2019). A segunda CHDL adquirida mais prevalente é a OXA-143, detectada principalmente em hospitais localizados na região Sudeste, em especial, no estado de São Paulo (RODRIGUES-COSTA *et al.*, 2019; HALAT; MOUBARECK, 2020). No HU de Londrina, foram relatadas as variantes OXA-231 e OXA-253 (GIONCO *et al.*, 2012; ROMANIN *et al.*, 2019).

A OXA-58 tem sido detectada em isolados clínicos, animais e ambientais de diferentes espécies de *Acinetobacter* em diversos países do mundo, que incluem isolados de *A. baumannii* no Irã, Grécia, Argentina e Líbano, e *A. pittii* na China, concomitante com o gene $bla_{\text{NDM-1}}$ (EVANS; AMYES, 2014; HALAT;

MOUBARECK, 2020). Este gene apresenta sequência genética conservada e é um dos genes mais comumente detectados em NBA (NGUYEN *et al.*, 2020; BARAKA *et al.*, 2021). Embora pouco descrito no Brasil, o gene *bla*$_{\text{OXA-58}}$ foi relatado, até o momento, em isolados clínicos e ambientais de *A. baumannii*, *A. seifertii*, *A. bereziniae* e *A. colistiniresistens* (FÁVARO *et al.*, 2019; MATOS *et al.*, 2019; PAULA-PETROLI *et al.*, 2022).

A enzima OXA-24 foi relatada em isolados de *A. baumannii* em países como Taiwan, Tailândia, Bulgária e Líbano (HALAT; MOUBARECK, 2020). No Brasil, a primeira detecção do gene ocorreu em São Paulo em um isolado clínico de *Acinetobacter* spp. em 2007 (MENDES *et al*., 2008), e desde então foi identificado em Pernambuco (CAVALCANTI *et al.*, 2013), Paraná (PAGANO, *et al.*,2017), Rio Grande do Sul (PAGANO *et al.*, 2016; PAGANO *et al.*, 2018) e Minas Gerais (AZEVEDO *et al.*, 2019).

A OXA-235, por sua vez, foi relatada recentemente pelo *Canadian Nosocomial Infection Surveillance Program* (CNISP) como responsável por quase metade de todas as carbapenemases detectadas entre espécies de *Acinetobacter* de hospitais canadenses. Além disso, a variante OXA-278 foi detectada em um isolado de *A. lwoffii* recuperado de estações de tratamento de águas residuais municipais em Cingapura, em 2019, indicando a importância dos microrganismos considederados reservatórios naturais de GCRAM (HALAT; MOUBARECK, 2020).

Na literatura, a resistência aos carbapenêmicos na espécie *A. bereziniae* foi associada à presença dos genes codificadores das MBLs do tipo IMP-1, IMP-5, IMP-19, NDM-1, VIM-2 e SIM-1; CHDLs do tipo OXA-58, bem como superexpressão do gene que codifica a enzima intrínseca OXA-228-*like*, devido mutação no promotor e presença de sequência de inserção à montante do gene. Além destes, foram detectados genes codificadores de resistência aos beta-lactâmicos (*amp*C, *bla*OXA-2, *bla*OXA-4, *bla*OXA-21) , aminoglicosídeos (aac6, aacA4, aadA1, aadB, aphA6, strA, strB), cloranfenicol (*cat*B8) e tetraciclina (*tet*A) (BONNIN *et al.*, 2012; YAMAMOTO *et al.*, 2013; ZANDER; SEIFERT; HIGGINS, 2014; ZANDER *et al.*, 2014; BROVEDAN *et al.*, 2016; GROSSO *et al.*, 2015; SUNG *et al.*, 2014). No Brasil, nesta espécie foram identificados os genes codificadores das MBLs IMP-1 e NDM-1, e da OXA-58 (CHAGAS *et al.*, 2015; FÁVARO *et al.*, 2019). Em *A. colistiniresistens*, apesar de poucos relatos, foram identificados os genes codificadores de CHDL

(*bla*$_{OXA-23}$, *bla*$_{OXA-58}$ e *bla*$_{OXA-286}$) e MBL (*bla*$_{IMP-1}$ e *bla*$_{IMP-34}$) (ZANDER *et al.*, 2014; TIAN *et al.*, 2018; SUZUKI *et al.*, 2018; BRASILIENSE *et al.*, 2021; PAULA-PETROLI, 2022).

2.6.2.2 Aminoglicosídeos

Os aminoglicosídeos, utilizados no tratamento de uma ampla gama de infecções, apresenta atividade dependente de sua ligação ao 16S rRNA da subunidade ribossômica bacteriana 30S, promovendo inibição da síntese proteica ou produção de proteínas defeituosas, tornando a bactéria disfuncional. Esses antimicrobianos são, frequentemente, considerados fármacos de último recurso na prática clínica devido às suas múltiplas toxicidades, incluindo nefrotoxicidade e ototoxicidade (ALMASAUDI, 2018; MORRISON; ZEMBOWER, 2020).

As enzimas modificadoras de aminoglicosídeos (AMEs), que incluem acetiltransferases (AAC), nucleotidiltransferases (ANT) ou adeniltransferases (AAD), e fosfotransferases (APH), promovem a modificação dos grupamentos amino ou hidroxila por acetilação, adenilação e fosforilação dos aminoglicosídeos, respectivamente (ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020). A modificação do antimicrobiano resulta em diminuição da afinidade deste pelo seu alvo e consiste no mecanismo de resistência aos aminoglicosídeos mais prevalente e clinicamente relevante em *Acinetobacter*. Os genes codificadores destas enzimas, geralmente, estão inseridos em elementos genéticos móveis, como integrons de classe 1. Dentre os genes identificados em *A. baumannii* estão os codificadores das enzimas AAC(3), AAC(6'), ANT(2''), ANT(3'') e APH(3') (RUPPÉ; WOERTHER; BARBIER, 2015; LEE *et al.*, 2017; ASIF, ALVI, REHMAN, 2018; ALMASAUDI, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020; MORRISON; ZEMBOWER, 2020).

Os genes codificadores de metilases, por sua vez, promovem a metilação do 16S rRNA, modificando o alvo do antimicrobiano, conferindo alto nível de resistência aos aminoglicosídeos, exceto neomicina. Estes genes, normalmente, estão inseridos em transposons presentes em plasmídeos transferíveis, possibilitando sua disseminação. Em *Acinetobacter* spp. já foram identificados os genes *arm*A, *rmt* (A, B, C, D, D2, E, F, G, H) e *npm*A. Outros mecanismos envolvidos na resistência aos aminoglicosídeos incluem alterações nas proteínas ribossômicas alvo; diminuição da permeabilidade da membrana externa, prejudicando o transporte do antimicrobiano

para o interior da célula; bem como superexpressão de sistemas de efluxo (AdeABC, AbeM), que apresentam o aminoglicosídeo como substrato, promovendo sua remoção de dentro da célula (RUPPÉ; WOERTHER; BARBIER, 2015; LEE *et al.*, 2017; SHIN; PARK, 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018).

2.6.2.3 Quinolonas

As quinolonas atuam inibindo a atividade das enzimas DNA girase ou topoisomerase IV, que são essenciais à sobrevivência bacteriana. O principal mecanismo de resistência a estes antimicrobianos consiste na alteração da estrutura destas enzimas por meio de mutações pontuais nos genes cromossômicos g*yr*A (Ser83Leu) e *par*C (Ser80Leu e Glu84Lis), que codificam a DNA-girase e a topoisomerase IV, respectivamente. Essas alterações estruturais diminuem a afinidade das quinolonas que se ligam ao complexo enzima-DNA, conferindo maiores níveis de resistência (LEE *et al.* 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018; MORRISON; ZEMBOWER, 2020; MOHAMMED *et al.*, 2021). Em *Acinetobacter* spp., as alterações em OMPs e a superexpressão de sistemas de efluxo, como AdeABC, AdeIJK, AbeM, QepAB e OqxAB também desempenham um papel importante neste fenótipo (ALMASAUDI, 2018; MORRISON; ZEMBOWER, 2020; MOHAMMED *et al.*, 2021). Adicionalmente, foram descritos em *A. baumannii* os genes *qnr*A, *qnr*B e *qnr*S, que codificam proteínas de proteção ribossômica (PPR). Estas proteínas, mediadas cromossomicamente ou por plasmídeos, se ligam a DNA-girase e a topoisomerase IV impedindo a ligação das quinolonas à estas enzimas (RUPPÉ; WOERTHER; BARBIER, 2015; SHIN; PARK, 2017; ASIF, ALVI, REHMAN, 2018; ALMASAUDI, 2018; LUPO; HAENNI; MADEC, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020).

2.6.2.4 Tetraciclinas

As tetraciclinas se ligam, de maneira reversível, à subunidade 30S do ribossomo bacteriano impedindo a ligação do tRNA e, por consequência, a adição de aminoácidos, impedindo a síntese proteica. Dentre os mecanismos de resistência a estes antimicrobianos, em *A. baumannii*, têm os sistemas de efluxo TetA, que tem

como substrato a tetraciclina; o TetB, que controla o efluxo de tetraciclina e minociclina; bem como o sistema AdeABC, responsável por extrusar também a tigeciclina, pertencente às glicilglicinas, que são tetraciclinas modificadas. Adicionalmente, o gene *tet*(M) codifica uma proteína de proteção ribossomal, que protege o ribossomo da ação da tetraciclina, doxiciclina e minociclina (LEE *et al.*, 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018).

2.6.2.5 Polimixinas

As polimixinas são peptídeos catiônicos que atuam na membrana externa bacteriana, onde interagem com os lipopolissacarídeos (LPS), promovendo a retirada de íons Ca^{2+} e Mg^{2+} responsáveis pela estabilização desta membrana, resultando na perda da integridade desta membrana, e consequente morte celular bacteriana (SRINIVAS; RIVARD, 2017; ASIF, ALVI, REHMAN, 2018). Estes antimicrobianos foram descritos pela primeira vez em 1947, no entanto, seus efeitos adversos que incluem neurotoxicidade, nefrotoxicidade, apnéia, ataxia, parestesisas, convulsões, entre outros, levaram ao seu desuso (REBELO et al, 2018; ASIF, ALVI, REHMAN, 2018; ALMASAUDI, 2018). O panorama clínico atual, de opções terapêuticas extremamente limitadas pela RAM e poucas opções efetivas disponíveis para o tratamento das infecções causadas por *A. baumannii* MR, levou a renovação do interesse clínico pelas polimixinas, que assim como a tigeciclina, são consideradas os antimicrobianos de escolha para o tratamento de infecções por estes microrganismos (NGUYEN; JOSHI, 2021; YOON; JEONG, 2021).

Desde a primeira descrição de um isolado de *Acinetobacter* spp. resistente à colistina na República Tcheca em 1999, vários relatos foram feitos no decorrer dos anos no mundo, ressaltando a habilidade deste patógeno em adquirir resistência à diversas classes de antimicrobianos. Em *Acinetobacter* spp. a resistência às polimixinas pode ser mediada por diferentes mecanismos, que incluem a hiperexpressão de sistemas de efluxo (EmrAB, Ttg2C), mutações gênicas (*pmr*CAB, *lpx*ACD, *lps*B, *vac*J, *lpt*D), bem como regulação negativa da expressão de cofatores envolvidos na síntese do LPS (biotina), que pode resultar em sua ausência, e de proteínas que atuam no transporte e/ou estabilização de precurssores da membrana externa (aminoácidos osmoprotetores) (POTRON; POIREL; NORDMANN, 2015; LEE

et al., 2017; SRINIVAS; RIVARD, 2017; SHIN; PARK, 2017; WENZLER *et al.*, 2017; ALMASAUDI, 2018; ASIF, ALVI, REHMAN, 2018).

Adicionalmente, temos a resistência à colistina adquirida mediada por plasmídeo, designada como *plasmid-Mediated Colistin Resistance* (*mcr*), que é responsável por codificar uma fosfoetanolamina transferase que promove a adição de PEtN ao lipídeo A, conferindo resistência ao antimicrobiano (CIM de 4 a 16 µg/mL). Desde o relato, em 2016, do primeiro gene *mcr-1*, detectado em isolados de *E. coli* e *K. pneumoniae* recuperados de animais e humanos na China, mais variantes do gene *mcr* foram descobertas, em várias bactérias em diferentes países no mundo. Foram detectados os genes *mcr-1* e *mcr-4.3* em *A. lwoffii,* ambientais e clínicos, na Itália; *A. baumannii* na China, Brasil e Índia; *A. nosocomialis* na África (LIU *et al*., 2016; REBELO *et al.*, 2018; CHRISTAKI; MARCOU; TOFARIDES, 2020; MARTINS-SORENSON *et al.*, 2020; RAHMAN; AHMED, 2020; ALLAM *et al.*, 2021; SAMREEN; MALAK; ABULREESH, 2021; SNYMAN *et al.*, 2021).

2.7 FATORES DE VIRULÊNCIA

Em conjunto com os determinantes de resistência, *Acinetobacter* spp. possui uma variedade de fatores de virulência que favorecem sua colonização, lesão tecidual, sobrevivência em hospedeiros, evasão do sistema imune, além de contribuírem no amplo espectro de infecções causadas por este patógeno e no tratamento cada vez mais desafiador (AZAM; KHAN, 2019; WILSON; PANDEY, 2022; FIGUEREDO *et al.*, 2021; CHEGINI *et al.*, 2020). Dentre os principais fatores de virulência descritos em *Acinetobacter* spp. estão: motilidade, produção de biofilme, cápsula, presença de proteínas de membrana externa, bem como sistemas de secreção de proteínas (AL ATROUNI *et al.*, 2016; LEE *et al.*, 2017; WENZLER *et al.*, 2017; HARDING; HENNON; FELDMAN, 2018; ALMASAUDI, 2018).

2.7.1 Motilidade

As espécies do gênero *Acinetobacter* são consideradas microrganismos imóveis. No entanto, foram descritas em duas espécies clinicamente relevantes, *A. baumannii* e *A. nosocomialis*, dois tipos de motilidade independente de

flagelo denominadas: motilidade associada à superfície e motilidade do tipo *twitching* (MEA; YONG; WONG, 2021).

A motilidade associada à superfície em meio semissólido, também conhecida como motilidade de espalhamento, consiste em um processo multifatorial e complexo influenciado pelo crescimento bacteriano, facilitado pela produção de surfactantes, presença do gene *pil*T e 1,3-diaminopropano (DAP). Além disso, esta motilidade pode ser regulada pelo *quórum sensing*, sistemas de dois componentes e condições extracelulares, como salinidade, luz, ferro, uso de antimicrobianos e exposição à álcool (HARDING; HENNON; FELDMAN, 2018; MEA; YONG; WONG, 2021). A motilidade do tipo *twitching,* também conhecida como motilidade de contração, é mediada pelo pili do tipo IV que após fixação em uma superfície, se retrai, promovendo a translocação da célula em direção ao ponto de fixação. Os genes *pil*A, *pil*B, *pil*C, *pil*F, *pil*M, *pil*N, *pil*O, *pil*P, *pil*Q, *pilZ* e *pilW* foram identificados como envolvidos na montagem do pili, enquanto *pil*R, *pil*S, *pil*T e *pil*U envolvidos na contração. Além de atuar na motilidade bacteriana, este pili participa de processos como a transformação natural e a adesão à superfícies biótica e abiótica (HARDING; HENNON; FELDMAN, 2018; MEA; YONG; WONG, 2021).

2.7.2 Biofilme

O biofilme consiste em comunidades bacterianas, aderidas a superfícies abiótica ou biótica, envoltas envoltos por uma matriz polimérica extracelular, composta por polissacarídeos, lipídios, proteínas, DNA extracelular e biossurfactantes. A formação do biofilme contribui para a colonização de pacientes e dispositivos médicos, e atua como barreira contra a ação de agentes antimicrobianos e desinfetantes, fornece proteção à dessecação, além de facilitar à evasão do sistema imunológico. Assim, sua formação dificulta o tratamento e contribui para a persistência do patógeno no ambiente hospitalar, bem como no hospedeiro humano, sendo responsável por infecções crônicas (MORADALI; GHODS; REHM, 2017; SHIN; PARK, 2017; EZE; CHENIA; ZOWALATY, 2018; HARDING; HENNON; FELDMAN, 2018; ASIF; ALVI; REHMAN, 2018; AZAM; KHAN, 2019; CHEGINI *et al.*, 2020; MEA; YONG; WONG, 2021; NGUYEN; JOSHI, 2021).

As etapas de formação e maturação do biofilme podem ser influenciadas por fatores como: pilus Csu tipo I, sistema de dois componentes BfmRS

e GacSA, proteínas de membrana externa (OmpA, Bap e Pmt), sistemas de efluxo (AdeABC, AdeIJK, AdeFGH), toxinas RTX, produção de poli-β-1,6-N-acetilglucosamina (PNAG), polissacarídeos capsulares, *quórum sensing*, entre outros (SHIN; PARK, 2017; EZE; CHENIA; ZOWALATY, 2018; ASIF; ALVI; REHMAN, 2018; HARDING; HENNON; FELDMAN, 2018; SHAN *et al*., 2022). Em *Acinetobacter*, a proteína associada ao biofilme Bap (*biofilm-associated protein*) é secretada pelo sistema de secreção do tipo I e modulada pelo sistema regulador de dois componentes BfmRS. Esta proteína de superfície participa do processo de formação e maturação do biofilme, adesão à superfícies abióticas, como poliestireno e titânio, além de desempenhar um papel importante na estrutura tridimensional do biofilme e na formação de canais de água. Adicionalmente, modula a hidrofobicidade da superfície celular, aumentando-a, resultando em melhor aderência às células epiteliais (MEA; YONG; WONG, 2021; NGUYEN; JOSHI, 2021).

2.7.3 Polissacarídeos capsulares

A presença de cápsula é considerada um dos principais fatores de virulência em *Acinetobacter*, que confere proteção ao patógeno contra ameaças externas, como a entrada de antimicrobianos macromoleculares e a evasão do sistema imunológico do hospedeiro, regulado pelo sistema de dois componentes BfmRS (SHIN; PARK, 2017; HARDING; HENNON; FELDMAN, 2018; SKERNIŠKYTĖ *et al.*, 2019; TIKU, 2022). A cápsula também auxilia no processo de resistência à dessecação, através da capacidade de retenção de água. Os polissacarídeos capsulares, compostos por unidades repetidas de carboidratos, atuam como um escudo de glicano envolvendo e protegendo a bactéria de ameaças externas, possibilitando sua sobrevivência por períodos prolongados (HARDING; HENNON; FELDMAN, 2018; NGUYEN; JOSHI, 2021).

2.7.4 Proteínas de Membrana Externa

As OMPs apresentam papéis importantes na patogênese e adaptação nas células hospedeiras, bem como na resistência à antimicrobianos. Consiste em proteínas que atravessam as membranas celulares e atuam como poros pelos quais ocorre a passagem de moléculas, como nutrientes, toxinas e antimicrobianos, como

cloranfenicol e aztreonam (LEE *et al.*, 2017; SHIN; PARK, 2017; WEBER et al, 2017; ELHOSSEINY, ATTIA, 2018; MEA; YONG; WONG, 2021; NGUYEN; JOSHI, 2021; TIKU, 2022).

A OmpA (também chamada de Omp38), é uma das principais proteínas liberadas por vesículas de membrana externa (OMV), e consiste na maior e mais abundante porina presente na membrana externa de *A. baumannii*. Apresenta baixa permeabilidade quando comparada com porinas de tamanho semelhante de outras bactérias, como OprF em *E. coli*, resultando em resistência aos antimicrobianos. Esta porina é necessária para uma variedade de atividades associadas à infecção. Atua na biogênese das OMVs, adesão na formação de biofilme, evasão do sistema imune do hospedeiro e citotoxicidade. Esta é mediada pela adesão às células epiteliais pulmonares do hospedeiro, induzindo uma resposta inflamatória que pode levar à invasão celular e morte da célula hospedeira por meio da ativação da caspase-3 (MEA; YONG; WONG, 2021; NGUYEN; JOSHI, 2021; TIKU, 2022).

A Omp34 (também chamada de Omp33-36) está associada à citotoxicidade, induz apoptose, modula a autofagia, promove a persistência intracelular nas células humanas, além de resistência aos antimicrobianos. Semelhante a OmpA, contribui para a patogênese bacteriana, aderindo e induzindo a morte celular epitelial do pulmão por meio da ativação de caspases. Outras porinas identificadas em *A. baumannii* que desempenham papel na patogênese deste patógeno são Omp22, CarO e OprD-*like.* CarO, além de atuar na adesão celular e modular a resposta imune da célula hospedeira, é responsável pela resistência aos carbapenêmicos em *A. baumannii* (LEE *et al.*, 2017; SHIN; PARK, 2017; WEBER et al, 2017; ELHOSSEINY, ATTIA, 2018; MEA; YONG; WONG, 2021; TIKU, 2022).

2.7.5 Sistemas de Secreção de Proteínas

Os sistemas de secreção de proteínas, por sua vez, são responsáveis pela secreção de uma ampla gama de proteínas efetoras que são essenciais para a interação do microrganismo com o ambiente e o hospedeiro. Foram descritos em *A. baumanni* os sistemas de secreção do tipo I (SST1), tipo II (SST2), tipo IV (SST4), tipo V (SST5) e tipo VI (SST6), os quais apresentam diferentes graus de contribuição para a virulência deste patógeno (WEBER *et al.*, 2017; HARDING; HENNON; FELDMAN,

2018; MEA; YONG; WONG, 2021; TIKU, 2022). A proteína de membrana trimérica Ata foi o primeiro sistema de secreção identificado em *A. baumannii,* pertencente ao SST5, conhecido como sistema autotransportador, e está relacionada com a formação e manutenção do biofilme, bem como com a adesão a componentes da matriz extracelular/membrana basal, como o colágeno tipo I, II, III, IV, V e laminina. O SST6 é um importante sistema responsável por promover a morte de outras espécies bacterianas, por meio da injeção de toxinas letais na bactéria competidora, bem como contribui para a disseminação da resistência aos antimicrobianos. O SST2 é responsável pela secreção de várias proteínas efetoras, como as lipases LipA e LipH e a protease CpaA que apresentam, respectivamente, atividade de lipase e capacidade de degradar fibrinogênio e fator V da cascata de coagulação, impedindo a formação de coágulos, além de atuar na mediação da colonização do pulmão e na disseminação do microrganismo para outros órgãos. O SST1 é responsável pela secreção de Bap e da proteína RTX. O SST4, por sua vez, medeia a transferência horizontal de genes e, portanto, desempenha um importante papel na transferência de genes de resistência aos antimicrobianos e na plasticidade genômica (LEE *et al.*, 2017; WEBER *et al.*, 2017; ELHOSSEINY, ATTIA, 2018; HARDING; HENNON; FELDMAN, 2018; TIKU, 2022).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar os determinantes de resistência aos antimicrobianos e fatores de virulência de isolados clínicos de *Acinetobacter* spp. recuperados de pacientes atendidos no Hospital Universitário de Londrina (HU) no período de 2006 à 2016.

3.2 OBJETIVOS ESPECÍFICOS

Analisar dados clínicos e epidemiológicos de prontuários microbiológicos de pacientes com detecção de isolados de *Acinetobacter* spp. no período de 2006 à 2016;

Verificar o perfil de resistência dos isolados clínicos nos prontuários microbiológicos;

Determinar a concentração inibitória miníma aos antimicrobianos: imipenem, meropenem, polimixina B, colistina, ceftazidima e tigeciclina em isolados portadores do gene *bla*$_{\text{OXA-58}}$ pela técnica de microdiluição em caldo;

Classificar os isolados clínicos em MR, ER ou PR de acordo com os critérios estabelecidos por Magiorakos e colaboradores (2012);

Investigar a presença dos determinantes de resistência adquirida aos carbapenêmicos (*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-143-like}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{GIM}}$, *bla*$_{\text{SIM}}$, *bla*$_{\text{SPM}}$), polimixinas (*mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, *mcr*-5), quinolonas (*qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S, *qnr*Vc) e aminoglicosídeos (*rmt*A, *rmt*B, *rmt*C, *rmt*D, *rmt*E, *rmt*F and *rmt*G) por PCR multiplex e *single*;

Analisar o contexto genético dos genes *bla*$_{\text{OXA- 23-like}}$ e *bla*$_{\text{OXA-58-like}}$;

Investigar a presença do gene codificador da proteína associada ao biofilme (*bap*) por PCR *single*;

Determinar a similaridade genética entre os isolados portadores do gene *bla*$_{\text{OXA-58}}$ pela técnica *Enterobacterial Repetitive Intergenic Consensus* (ERIC)-PCR;

Avaliar a capacidade de produção de biofilme dos isolados portadores do gene *bla*$_{\text{OXA-58}}$ em placas de microtitulação de poliestireno de 96 poços de fundo chato;

Sequenciar o genoma total do isolado Ac 505/15 carreador do gene *bla*$_{\text{OXA-58}}$;

Detectar e caracterizar determinantes de resistência e de virulência do isolado Ac 505/15 por meio de ferramentas de bioinformática.

4 METODOLOGIAS

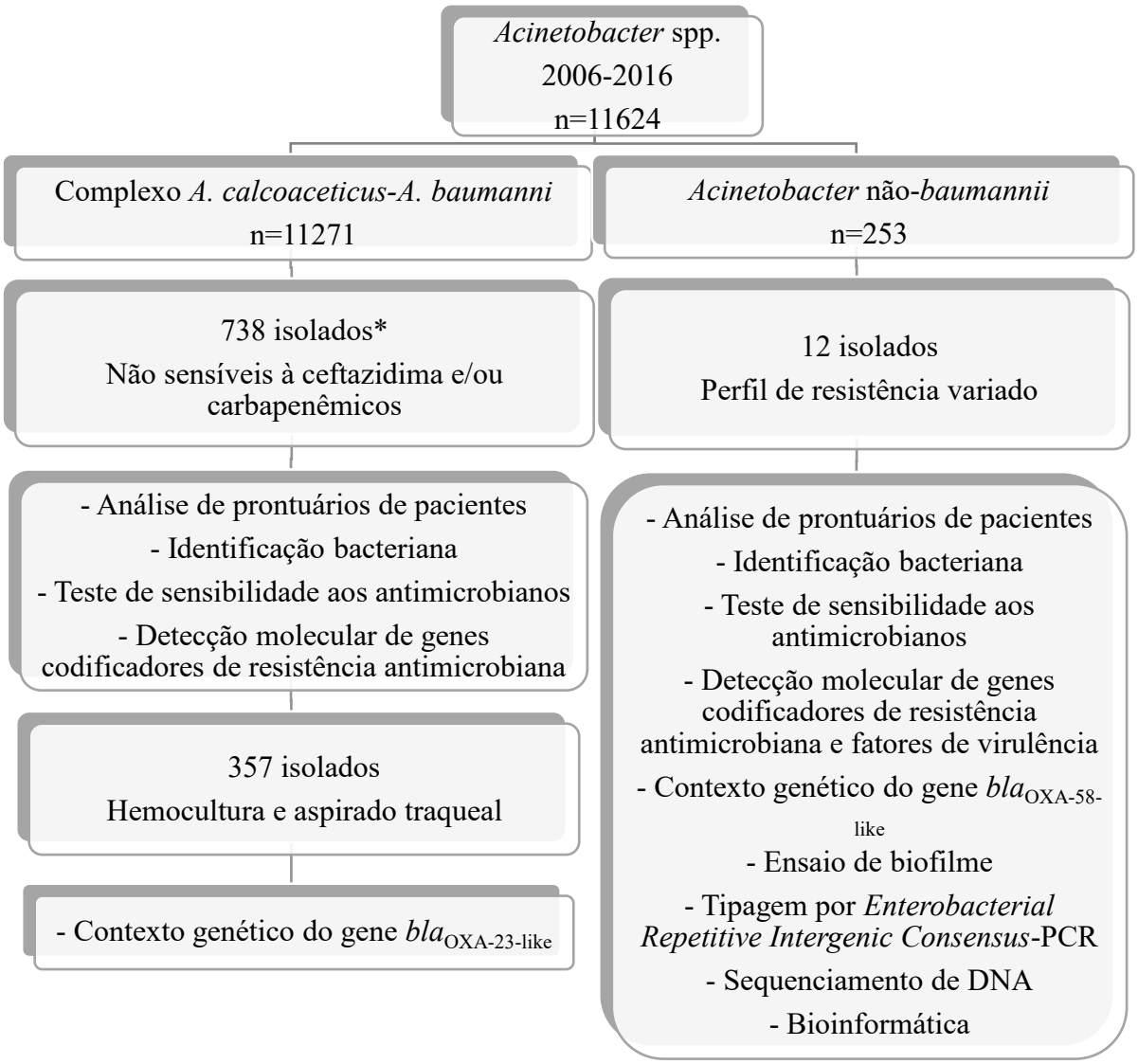
4.1 Design do Estudo e Coleção de Isolados

Os isolados de *Acinetobacter* spp. incluídos neste estudo foram recuperados no Laboratório de Microbiologia Clínica (LMC) do Hospital Universitário de Londrina (HU), um hospital escola terciário vinculado à Universidade Estadual de Londrina (UEL), localizado na cidade de Londrina, Estado do Paraná, Sul do Brasil. O HU, com 535 leitos, é um centro de referência médica para a região, prestando serviços médicos e diagnósticos gerais e de alta complexidade e assistência à saúde pública para a população residente na Região Metropolitana de Londrina e municípios circunvizinhos, que compõem a 17ª Regional de Saúde (https://saude.mppr.mp.br/arquivos/File/rs/macrorregionais.htm, acesso em 27 de fevereiro de 2023). O Comitê de Ética em Pesquisa da Universidade Estadual de Londrina [Protocolo nº 14490/2009] aprovou os protocolos experimentais.

Um total de 750 isolados de *Acinetobacter* spp. recuperados de amostras clínicas de pacientes internados no HU, estocados por conveniência entre setembro de 2006 a dezembro de 2016, foram investigados observando os seguintes critérios de inclusão: isolados não repetidos de ACB não sensíveis à ceftazidima e/ou carbapenêmicos (imipenem e/ou meropenem) (n=738) e isolados não repetidos de NBA independente do perfil de resistência apresentado (n=12) (Fluxograma 1). Os prontuários dos pacientes foram revisados e coletados os seguintes dados: idade, sexo, data de admissão hospitalar, material clínico, setor de internação. Adicionalmente, as comorbidades, motivo da internação, antibióticos prescritos e o desfecho clínico foram analisados para isolados de NBA portadores do gene *bla*$_{\text{OXA-58}}$.

4.2 Identificação Bacteriana

A identificação das espécies de *Acinetobacter* foi realizada por meio de testes convencionais, sistemas automatizados *MicroScan WalkAway* (Siemens Healthineers, Alemanha), BD Phoenix™ (Becton–Dickinson, Estados Unidos) e Vitek 2® (BioMérieux, França), e confirmada pela detecção por PCR do gene *bla*$_{\text{OXA-51}}$ ou pelo sequenciamento do gene *rpo*B (LA SCOLA *et al.*, 2006).



Fluxograma 1 *Design* do estudo

* 74 isolados recuperados no período de 2006 a 2009; 280 isolados recuperados no período de 2012 a 2014; 102 isolados MR recuperados de hemocultura no período de 2006 a 2016; 282 isolados recuperados de aspirado traqueal (≥10^6 UFC/mL) de paciente admitidos em UTI no período de 2006 a 2016.

4.3 Teste de Sensibilidade aos Antimicrobianos

A sensibilidade aos antimicrobianos foi determinada no LMC usando um ou uma combinação dos seguintes métodos: disco difusão de Kirby-Bauer, método de gradiente (por exemplo, E-test® [BioMérieux, França]) ou sistemas automatizados. Foram testados os antimicrobianos amicacina (30 µg), gentamicina (10 µg),

ciprofloxacina (5 µg), levofloxacina (5 µg), trimetoprima-sulfametoxazol (25 µg), tetraciclina (30 µg), tigeciclina (15 µg), piperacilina-tazobactam (100/10 µg), ampicilina-sulbactam (10/10 µg), ceftriaxona (30 µg), cefotaxim (30 µg), ceftazidima (30 µg), cefepima (30 µg), colistina (10 µg), imipenem (10 µg) e meropenem (10 µg) (Oxoid Ltd., Reino Unido). As concentrações inibitórias mínimas (CIMs) para ceftazidima, polimixina B, colistina, imipenem, meropenem e tigeciclina foram avaliadas por microdiluição em caldo de acordo com o *Clinical and Laboratory Standards Institute* (CLSI) (2012). *E. coli* ATCC 25922 e *P. aeruginosa* ATCC 27853 foram utilizados como organismo de controle de qualidade.

Os resultados foram analisados e interpretados usando pontos de corte do CLSI (2021), e a interpretação dos diâmetros da zona de inibição e CIM para tigeciclina foi determinada de acordo com os pontos de corte da *Food and Drug Administration* (FDA) para Enterobacteriaceae, devido o CLSI não possuir ponto de corte clínico para *Acinetobacter* spp. (CDER, 2013).

Baseado no perfil de resistência às classes de antimicrobianos utilizados na clínica para o tratamento de infecções por *Acinetobacter* spp. (Tabela 1), os isolados foram classificados como MR, ER ou PR de acordo com os critérios estabelecidos por Magiorakos *et al.* (2012) (Tabela 2).

Tabela 1 Classes de antimicrobianos utilizados para o tratamento de infecções por *Acinetobacter* spp.

Classes de antimicrobianos	Agentes antimicrobianos
Penicilinas + Inibidores de beta-lactamases	Ampicilina-sulbactam
Penicilinas Antipseudomonas + Inibidores de beta-lactamases	Piperacilina-tazobactam Ticarcilina-ácido cluvulânico
Cefalosporinas de Espectro Extendido	Cefotaxima Ceftriaxona Ceftazidima Cefepime
Carbapenêmicos	Imipenem Meropenem Doripenem
Fluoroquinolonas	Ciprofloxacin Levofloxacin
Aminoglicosídeos	Amicacina Gentamicina Tobramicina
Inibidores da via do folato	Sulfametoxazol-trimetoprim
Tetraciclinas	Tetraciclina Doxiciclina

Glicilglicinas	Minociclina
	Tigeciclina
Polimixinas	Polimixina B
	Colistina

Fonte: Adaptado de MAGIORAKOS *et al.*, 2012.

Tabela 2 Critérios para classificação do perfil de resistência dos isolados de *Acinetobacter* spp.

Perfil de Resistência	Critérios
MR	Isolados não sensíveis a pelo menos 1 agente em 3 ou mais classes de antimicrobianos utilizados no tratamento das infecções por *Acinetobacter* spp. e *Pseudomonas aeruginosa*
ER	Isolados não sensíveis a pelo menos 1 agente em todas as classes, mas sensível a pelo menos 2 ou menos classes de antimicrobianos utilizadas no tratamento das infecções por *Acinetobacter* spp. e *Pseudomonas aeruginosa*
PR	Isolados não sensíveis a todos os agentes antimicrobianos utilizados no tratamento das infecções por *Acinetobacter* spp. e *Pseudomonas aeruginosa*

Fonte: Adaptado de MAGIORAKOS *et al.*, 2012.

MR, multirresistente aos antimicrobianos; ER, extensivamente-resistente aos antimicrobianos; PR, pan-resistente aos antimicrobianos.

4.4 EXTRAÇÃO DO DNA

As 750 amostras foram cultivadas em meio *Tryptic Soy Broth* (TSB) por 18-24h a 37°C. Em seguida foram semeadas pela técnica de esgotamento em biplacas contendo os meios MacConkey (Kasvi, Brasil) e CRHOMAgar (DIFCO™, Estados Unidos) e incubadas por 18-24h.

O DNA foi extraído pelo método de fervura. Em microtubos de 1,5 mL foram adicionados 200 µL de água estéril e acrescentadas de 6 a 10 colônias bacterianas. Os microtubos contendo a suspensão celular foram submetidos a fervura por 10 minutos e, imediatamente após, em banho de gelo por 5 minutos. O lisado

celular foi centrifugado por 10 minutos a 12.000 r.p.m. e o sobrenadante transferido para um novo microtubo que foi armazenado em freezer -20 °C (DONALD *et al.*, 2000).

4.5 Detecção Molecular de Genes Codificadores de Resistência aos Antimicrobianos e de Fatores de Virulência

Os 750 isolados foram investigados quanto à presença dos seguintes genes codificadores de carbapenemases: CHDLs (*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-143-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-48-like}}$), MBLs (*bla*$_{\text{IMP-like}}$, *bla*$_{\text{VIM-like}}$, *bla*$_{\text{SIM-like}}$, *bla*$_{\text{GIM-like}}$, *bla*$_{\text{SPM-like}}$, *bla*$_{\text{NDM-like}}$), *bla*$_{\text{GES-like}}$ e *bla*$_{\text{KPC-like}}$, por PCR (Tabela 3). Os determinantes de resistência adquirida à colistina (*mcr-1* a *mcr-5*), quinolonas (*qnr*A*, qnr*B*, qnr*C*, qnr*D*, qnr*S*, qnr*Vc) e aminoglicosídeos (*rmt*A*, rmt*B*, rmt*C*, rmt*D*, rmt*E*, rmt*F*, rmt*G) e o fator de virulência *bap* foram investigados em isolados portadores do gene *bla*$_{\text{OXA-58}}$ (Tabela 3). As reações de PCR foram realizadas utilizando o *Top Taq Master Mix* (Qiagen, Estados Unidos) seguindo as instruções do fabricante.

4.6 Contexto Genético dos Genes *BLA*$_{\text{OXA-58-like}}$ e *BLA*$_{\text{OXA-23like}}$

O mapeamento por PCR das regiões *upstream* e *downstream* ao gene *bla*$_{\text{OXA-58-like}}$ (n=6) foi realizado para os isolados de NBA, conforme descrito por Villalón *et al.* (2013). O contexto genético de 357 isolados do complexo ACB portadores do gene *bla*$_{\text{OXA-23-like}}$, recuperados de hemocultura (n=93) e aspirado traqueal (n=264), foi avaliado para a presença de Tn*2006*, Tn*2007*, Tn*2008* e Tn*2009* usando mapeamento por PCR (Tabela 3).

Tabela 3 Oligonucleotídeos utilIzados para a caracterização molecular de isolados de *Acinetobacter* spp.

Oligonucleotídeos	Sequência (5' – 3')	TA[a]	Tamanho do fragmento (bp)	Referência
Ac696F	TAYCGYAAAGAYTTGAAAGAAG	60°C	857	LA SCOLA *et al.*, 2006
Ac1598R	CGBGCRTGCATYTTGTCRT			
OXA-23 F	GATCGGATTGGAGAACCAGA	52ºC	501	WOODFORD *et al.*, 2006
OXA-23 R	ATTTCTGACCGCATTTCCAT			
OXA-24 F	GGTTAGTTGGCCCCCTTAAA		246	
OXA-24 R	AGTTGAGCGAAAAGGGGATT			
OXA-51 F	TAATGCTTTGATCGGCCTTG		353	
OXA-51 R	TGGATTGCACTTCATCTTGG			
OXA-58 F	AAGTATTGGGGCTTGTGCTG		599	
OXA-58 R	CCCCTCTGCGCTCTACATAC			
OXA-143 F	TGGCACTTTCAGCAGTTCCT	52ºC	149	HIGGINS; LEHMANN; SEIFERT, 2010
OXA-143 R	TAATCTTGAGGGGGCCAACC			
NDM F	GCAGCTTGTCGGCCATGCGGGC	60ºC	782	DOYLE *et al.*, 2012
NDM R	GGTCGCGAAGCTGAGCACCGCAT			
KPC F	TGTCACTGTATCGCCGTC		900	
KPC R	CTCAGTGCTCTACAGAAAACC			
OXA-48 F	GCGTGGTTAAGGATGAACAC		438	
OXA-48 R	CATCAAGTTCAACCCAACCG			
GES F	ATGCGCTTCATTCACGCAC	55ºC	846	WANG *et al.*, 2006
GES R	CTATTTGTCCGTGCTCAGG			
IMP F	GAATAG(A/G)(A/G)TGGCTTAA(C/T)TCTC	53ºC	188	MENDES *et al.*, 2007
IMP R	CCAAAC(C/T)ACTA(G/C)GTTATC			
VIM F	GTTTGGTCGCATATCGCAAC		382	
VIM R	AATGCGCAGCACCAGGATAG			
GIM F	TCAATTAGCTCTTGGGCTGAC		72	
GIM R	CGGAACGACCATTTGAATGG			
SIM-1 F	GTACAAGGGATTCGGCATCG		569	
SIM-1 R	TGGCCTGTTCCCATGTGAG			

SPM-1 F	CTAAATCGAGAGCCCTGCTTG		798	
SPM-1 R	CCTTTTCCGCGACCTTGATC			
MCR-1 F	ATGCCAGTTTCTTTCGCGTG	59ºC	502	REBELO *et al.*, 2018
MCR-1 R	TCGGCAAATTGCGCTTTTGGC			
MCR-2 F	GATGGCGGTCTATCCTGTAT		379	
MCR-2 R	AGGCTGACACCCCATGTCAT			
MCR-3 F	ACCAGTAAATCTGGTGGCGT		296	
MCR-3 R	AGGACAACCTCGTCATAGCA			
MCR-4 F	TTGCAGACGCCCATGGAATA		207	
MCR-4 R	GCCGCATGAGCTAGTATCGT			
MCR-5 F	GGACGCGACTCCCTAACTTC		608	
MCR-5 R	ACAACCAGTACGAGAGCACG			
QnrAm F	AGAGGATTTCTCACGCCAGG	58ºC	580	KRAYCHETE *et al.*, 2016
QnrAm R	TGCCAGGCACAGATCTTGAC			
QnrBm F	GGMATHGAAATTCGCCACTG		264	
QnrBm R	TTTGCYGYYCGCCAGTCGAA			
QnrCm F	GCGAATTTCCAAGGGGCAAA		135	
QnrCm R	ACCCGTAATGTAAGCAGAGCAA			
QnrDm F	AGGTGTAGCATGTATGGAAAAGC		691	
QnrDm R	ACATTGGGGCATTAGGCGTT			
QnrSm F	GCAAGTTCATTGAACAGGGT		428	
QnrSm R	TCTAAACCGTCGAGTTCGGCG			
QnrVCm F	GAGYTKTATGGTTTAGAYCCTCG		71	
QnrVCm R	TGTTCYTGYTGCCACGARCA			
RmtA F	ATGAGCTTTGACGATGCCCTA	55ºC	756	HIDALGO *et al.*, 2013
RmtA R	TCACTTATTCCTTTTTATCATG			
RmtB F	ATGAACATCAACGATGCCCT		769	
RmtB R	CCTTCTGATTGGCTTATCCA			
RmtC F	CGAAGAAGTAACAGCCAAAG		711	
RmtC R	ATCCCAACATCTCTCCCACT			
RmtD F	CGGCACGCGATTGGGAAGC		401	
RmtD R	CGGAAACGATGCGACGAT			
RmtE F	ATGAATATTGATGAAATGGTTGC		818	
RmtE R	TGATTGATTTCCTCCGTTTTTG			
RmtF F	GCGATACAGAAAACCGAAGG		589	

RmtF R	ACCAGTCGGCATAGTGCTTT			
RmtG F	AAATACCGCGATGTGTGTCC	55ºC	250	BUENO *et al.*, 2013
RmtG R	ACACGGCATCTGTTTCTTCC			
F-Bap-C	ATGCCTGAGATACAAATTATTGCCAAG GATAATC	56ºC	561	KODORI *et al.*, 2017
R-Bap-C	AGGTGCTGAAGAATCATCATTAC			
ISAba1F	GTTATATCTTATCTTAAACA	56°C		MERKIER *et al.*, 2008
ISAba1R	GCTCACCGATAAACTCTCT			
ISAba2A	AATCCGAGATAGAGCGGTTC	50°C		POIREL; NORDMANN, 2006
ISAba2B	TGACACATAACCTAGTGCAC			
ISAba3A	CAATCAAATGTCCAACCTGC			
ISAba3B	CGTTTACCCCAAACATAAGC			
ISAba4A	ATTTGAACCCATCTATTGGC			CORVEC *et al.*, 2007
ISAba4B	ACTCTCATATTTTTTCTTGG			
Tn2006 Int-P3	GTCTATCAGGAACTTGCGCG	62ºC		CHEN *et al.*, 2017
Tn2006 Int-P4	GCAAGGCTTTAGATGCAGAAGA	62ºC		
Tn2007 Int-P6	ATTTGAACCCATCTATTGGC	56ºC		
Tn2007 Int-P7	ACTCTCATATTTTTTCTTGG	56ºC		
Tn2006/8 Int-P5	GGCTCATTACAGTCAGGTACAAGT	62ºC		
Tn2009 Int-P1	ATCCTGATGCTCGCAATCGT	62ºC		
Tn2009 Int-P8	CTGTCTGCGAACACATTCAC	62ºC		
ERIC1R	GTGAATCCCCAGGAGCTTACA	40ºC	-	SILBERT *et al.*, 2004
ERIC2	AAGTAAGTGACTGGGGTGAGCG			

[a] Temperatura de anelamento

4.7 Sequenciamento de DNA pelo Método de Sanger

Os amplicons obtidos nas reações de PCR foram purificados com o *PureLink PCR Purification Kit* (Invitrogen, Estados Unidos) e sequenciados pelo método de Sanger. As reações de sequenciamento foram preparadas com *BigDye Terminator Kit* (Applied Biosystems, Canadá) e realizadas na máquina ABI 3500xL Genetic Analyzer (Applied Biosystems, Canadá). Os resultados do cromatograma foram confirmados com Bioedit v7.0.5 (Ibis Therapeutics, Canadá) e as sequências de DNA foram analisadas usando o algoritmo BLAST no banco de dados NCBI *GenBank*.

4.8 Ensaio de Biofilme

A capacidade de formar biofilme foi avaliada em isolados portadores do gene *bla*$_{\text{OXA-58-like}}$. Foram preparados inóculos padrões com densidade celular ajustada para 1x10^{7} UFC/mL em caldo *Mueller-Hinton*. Em placas de microtitulação de poliestireno de 96 poços de fundo chato foram adicionados 200 µL do inóculo padrão e incubados por 24h a 37°C. Após o período de incubação, as células planctônicas foram removidas em lavagens com *Phosphate Buffered Saline* (PBS) e as células sésseis foram fixadas com metanol gelado durante 15 minutos e coradas com solução de cristal violeta (1,0%). Após lavagem e secagem das placas, o cristal violeta foi ressuspendido com etanol-acetona (80:20 v/v) e a suspensão foi transferida para uma nova placa, a qual foi utilizada para leitura em espectrofotômetro a 570nm (GEORGESCU *et al.*, 2016). Os experimentos foram realizados em quintuplicatas em três diferentes ocasiões de acordo com Romanin (2018). A formação de biofilme foi interpretada como ausente, fraca, moderada e forte segundo os critérios estabelecidos por Stepanovic *et al.* (2000).

4.9 Tipagem Molecular

A clonalidade dos isolados portadores do gene *bla*$_{\text{OXA-58like}}$ foi investigada utilizando a técnica de *Enterobacterial Repetitive Intergenic Consensus* (ERIC)-PCR segundo Silbert *et al.* (2004). Os amplicons foram analisados em géis de agarose a 2,0% corados com brometo de etídeo à 0,5 mg/mL e visualizados por

exposição à luz ultravioleta (260nm) no transiluminador LPIX (Loccus Biotecnologia, Brasil). Os padrões das bandas foram analisados com o software *BioNumerics* (Applied Maths, Bélgica). Adotou-se o método de agrupamento de pares não ponderados com médias aritméticas (UPGMA) e coeficiente de Dice com 2,0% de tolerância para a similaridade dos isolados. O ponto de corte de 90,0% foi aplicado para o agrupamento dos isolados.

4.10 Sequenciamento Shotgun e Análise de Bioinformática

O sequenciamento Shotgun do isolado Ac505/15 foi realizado em uma plataforma *Illumina MiSeq* (Illumina Inc., Estados Unidos). O genoma foi montado pelo *SPAdes* versão 3.13.1 e a anotação foi realizada usando o NCBI *Prokaryotic Genome Annotation Pipeline* v. 4.8 (https://ncbi.nlm.nih.gov/genome/annotation_prok/).

Para confirmar a identidade do isolado, foi realizada uma análise comparativa da sequência dos genes que codificam as subunidades β da RNA polimerase e da DNA girase, avaliando a semelhança com sequências concatenadas de isolados de *A. colistiniresistens.* Foi avaliada a relação dos genomas de *A. colistiniresistens* disponíveis no NCBI por meio da análise do pan-genoma e da árvore filogenética usando PATRIC v. 3.6.12. Além disso, a identidade nucleotídica média com base no *blast* (ANIb) foi calculada usando o programa *JSpecies* (http://imedea.uib-csic.es/jspecies).

Os genes codificadores de resistência aos antimicrobianos adquiridos foram identificados usando PATRIC v. 3.6.9 (https://patricbrc.org/), *ResFinder* v. 4.1, *KmerResistance* v. 2.2 (https://cge.cbs.dtu. dk/services/) e as sequências disponíveis no NCBI. As Ilhas genômicas e os elementos móveis foram detectados usando *IslandViewer4* (https://www.pathogenomics.sfu.ca/islandviewer), *MobileElementFinder* v. 1.0.3 (https://cge.cbs.dtu.dk/services/MobileElementFinder/) e *ISfinder* (https://www-is.biotoul.fr/). Famílias de sistemas de efluxo foram identificadas usando CARD v. 3.1.3, PATRIC e os dados disponíveis no NCBI. Os sistemas de secreção foram detectados usando o *Pathogenicity Island Database* (http://www.paidb.re.kr), PATRIC e os dados de anotação. A análise da sequência Multilocus foi realizada usando a ferramenta online CGE (https://cge.cbs.dtu.dk/services/MLST/).

5 RESULTADOS

Os resultados obtidos neste trabalho foram apresentados e discutidos em dois artigos científicos apresentados a seguir, um submetido e um publicado, respectivamente, a periódicos indexados nas principais bases de dados de saúde.

Artigo A (SUBMETIDO)

Carbapenem-hydrolyzing class D beta-lactamases codifying genes in *Acinetobacter* spp. from a Brazilian teaching Hospital: Insights into the role of non-*baumannii Acinetobacter* carrying *bla*$_{\text{OXA-58}}$

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Revista: Currenty Microbiology

Qualis: B1

Artigo B (PUBLICADO)

Molecular and phenotypic characteristics of a *bla*$_{\text{OXA-58}}$-carrying *Acinetobacter colistiniresistens* bloodstream isolate from Brazil

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Qualis: A3

4.1 ARTIGO A - CARBAPENEM-HYDROLYZING CLASS D BETA-LACTAMASES CODIFYING GENES IN *ACINETOBACTER* SPP. FROM A BRAZILIAN TEACHING HOSPITAL: INSIGHTS INTO THE ROLE OF NON-*BAUMANNII ACINETOBACTER* CARRYING *BLA*OXA-58

Carbapenem-hydrolyzing class D beta-lactamases codifying genes in *Acinetobacter* spp. from a Brazilian teaching Hospital: Insights into the role of non-*baumannii Acinetobacter* carrying *bla*OXA-58

Abstract

The aim of this study was to analyze the distribution of carbapenemases encoding genes in clinical isolates of *Acinetobacter* spp. recovered at the Hospital Universitário de Londrina (HU), Southern Brazil, during a ten-year period (2006-2016). A total of 750 isolates of *Acinetobacter* spp. were genetically analysed for their species, carbapenemases genes and antimicrobial resistance profiles. Molecular typing by ERIC-PCR, genetic context structures and ability to produce biofilm were determined for non-*baumannii Acinetobacter* (NBA). *A. baumannii* (Ab) was the most common specie isolated (98.4%), followed by *A. bereziniae* (0.8%) and *A. haemolyticus* (0.1%). High resistance rates were observed for most of the antimicrobials tested; the acquired Carbapenem-hydrolyzing class D beta-lactamases (CHDL) genes identified were: *bla*OXA-23-like (89.2%), *bla*OXA143-like (1.2%) and *bla*OXA58-like (0.8%). The metallo-beta-lactamase, KPC or GES genes were not detected. ERIC-PCR analysis of *bla*OXA-58 harboring isolates identified four genetic groups (I-IV). The analysis of the genetic context revealed that all isolates presented IS*Aba*3 downstream of the *bla*OXA-58 gene, and the three isolates had IS*Aba*1, IS*Aba*3 and IS*Aba*125-ΔIS*Aba*3 upstream were classified as multidrug-resistant. In this study, we demonstrate that resistance to carbapenems in *Acinetobacter* spp. seems to be attributed to the presence of CHDL genes capable of persisting for a long time in the HU. Also, the epidemiological scenario documented of *bla*OXA-58-like genes highlights the real need of constant genetic and laboratorial vigilance for these priority pathogens in hospital.

Key words: *Acinetobacter* spp.; Carbapenem resistance; CHDL; non-*baumannii Acinetobacter*; OXA-58.

Acknowledgments

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Declarations

Funding This work was supported by grants from Programa de Pesquisa para o SUS: Gestão Compartilhada em Saúde (PPSUS)/Fundação Araucária/SESA-PR/MS/CNPq (convênio 035/2017 – n. 48045), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Pró-Reitoria de Pesquisa e Pós Graduação (PROPPG) of the Universidade Estadual de Londrina (UEL). This work was part of the PhD thesis of L. S. F.

Conflicts of interest The authors report no conflicts of interest in this work.

Ethics approval This study was approved by the Research Ethics Committee (REC), Health Sciences Center, Universidade Estadual de Londrina (Protocol No. 14490/2009). Informed consent was waived by the REC since the study was observational and patient care was not influenced.

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material Not applicable.

Code availability Not applicable.

Authors' contributions LSF, SBPP, and FECM designed the study and coordinated the experiment. LSF and SBPP carried out the experiment, drafted the manuscript, and analyzed the data. LSF, SBPP, FECM, and EJV did the necessary editing of the manuscript. All authors read and approved the manuscript.

Introduction

Acinetobacter species comprises a diverse group of bacteria that has global attention due to the constant development of antimicrobial resistance (AR). Particularly, *Acinetobacter baumannii* (Ab) is the agent of severe healthcare-

associated infections responsible for high mortality and high transmission rates. Although carbapenem-resistant *A. baumannii* (CRAb) has been listed as critical priority pathogen by World Health organization (WHO), other non-*baumannii Acinetobacter* (NBA) species have increased in frequency in the hospital environment and emerged as important pathogens harboring potential antimicrobial resistance genes (PARK *et al.*, 2019; KURIHARA *et al.*, 2020; TAVARES *et al.*, 2020; ALMEIDA *et al.*, 2021; BARAKA *et al.*, 2021). For a long time, the carbapenems were the frontline antimicrobials for treatment of severe infections by Gram-negative bacteria. However, the carbapenem resistance (CR) due to carbapenemases limited them efficacy. In *Acinetobacter,* the CR is often attributed to the acquisition and production of oxacilinases (OXA) known as carbapenem-hydrolyzing class D beta-lactamases (CHDL) (XIN *et al.*, 2019; RAMIREZ; BONOMO; TOLMASKY, 2020; WANG *et al.*, 2020; BRASILIENSE *et al.*, 2021).

Currently, there are six subclasses of CHDLs associated with *Acinetobacter* spp.: OXA-51-like, OXA-23-like, OXA-24-like, OXA-58-like, OXA-143-like and OXA-235-like enzymes (RODRIGUEZ; NASTRO; FAMILIETTI, 2018; NGUYEN *et al.*, 2020). CHDLs, characterized by their wide variety of amino acid sequences and their hydrolyzing profiles exhibit weak carbapenem hydrolysis. They can confer resistance mediated by the combination of natural low permeability and IS*Aba* elements located upstream of the gene. Moreover, CHDL genes are present on the chromosome or plasmid and are generally associated with transposons (Tn) or insertion sequences (IS) (NGUYEN *et al.*, 2020).

The emergence of carbapenemases among diverse Gram-negative bacilli throughout the world is a public health concern. The Latin America countries have been considered an endemic geographic area for several these enzymes and *Acinetobacter* spp. are their main carriers. The OXA-23, OXA-24, OXA-58 and OXA-72 were the most frequently isolated carbapenemases in this region being that *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ genes are found in plasmids, which increased the probability of horizontal transmission (RODRIGUEZ; NASTRO; FAMILIETTI, 2018; YU *et al.*, 2022).

Therefore, the aim of this study was to analyze the distribution of CHDL codifying genes and antimicrobial resistance profile in a collection of clinical isolates of *Acinetobacter* spp. recovered in a ten-years at the Hospital Universitário de Londrina (HU). In addition, a special focus is given to the molecular epidemiology of

NBA isolates carrying the *bla*$_{\text{OXA-58}}$ gene, because it is the first detection of this determinant in HU and NBA species.

Materials and Methods

Design of Study, Isolate Collection and Setting

We conducted a retrospective study for a collection of 750 *Acinetobacter* spp. clinical isolates recovered from HU inpatients among September 2006 to December 2016. The analyses included carbapenem and/or ceftazidime non susceptible *A. calcoaceticus-A. baumannii* (ACB) complex, and NBA regardless the resistance profile presented. Only one isolate per patient was studied. Demographic and clinical data of patients were retrospectively registered from electronic medical reports and computerized hospital data (LABHOS/LAC/HU System). The HU is a 535-bed tertiary hospital affiliated to Universidade Estadual de Londrina (UEL), located in Londrina city, Paraná State, Southern Brazil. It acts as a reference center providing general and high-complexity medical and diagnostic services and public health care, thus covering a population of about 850.000 peoples residing in the Londrina Metropolitan Region and other Brazilian States (https://saude.mppr.mp.br/arquivos/File/rs/macrorregionais.htm, accessed on February 27, 2023). The Clinical and Molecular Microbiology Department (CMM) provides conventional, automated and molecular identification of microorganisms, determination of antimicrobial resistance profiles and evaluation of resistance genes. The isolates were collected during routine diagnostic procedures and the Research Ethics Committee of UEL [Protocol No. 14490/2009] approved the experimental protocols.

Bacterial Identification and Antimicrobial Susceptibility Testing

All isolates were initially identified using automated systems (either the MicroScan WalkAway [Siemens Healthineers, Germany], BD Phoenix™ [Becton–Dickinson, United States] or Vitek 2® [BioMérieux, France]). Identification at species level of ACB complex was confirmed by the presence of *bla*$_{\text{OXA-51}}$ gene. For isolates not presenting *bla*$_{\text{OXA-51}}$ gene, *rpo*B-sequencing was performed (LA SCOLA *et al.*, 2006). All the confirmed isolates were kept at – 20ºC during the study period.

Antimicrobial susceptibility tests were determined at the CMM using combination of the methods: automated systems (as above described), Kirby-Bauer

disk diffusion and gradient method (e.g. E-Test [bioMérieux, France]). The following antimicrobials were tested: ampicillin–sulbactam (SAM), piperacillin-tazobactam (PTZ), amikacin (AK), gentamicin (GEN), ceftazidime (CAZ), cefepime (CEP), imipenem (IPM), meropenem (MEM), ciprofloxacin (CIP), levofloxacin (LEV), trimethoprim-sulfamethoxazole (SUT), tetracycline (TET), tigecycline (TIG) and colistin (COL) by using the broth microdilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines. Minimal inhibitory concentrations (MICs) for ceftazidime, polymyxin B, colistin, imipenem, meropenem, and tigecycline were evaluated by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI, 2012). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were utilized as reference control in all antibiotic susceptibility tests. In addition, the MIC value for tigecycline was determined by antibiotic gradient strips, Etest (bioMérieux, France). The results were interpreted using CLSI breakpoints (2021), and the interpretation of the inhibition zone diameters and MIC of tigecycline was done by using the United States Food and Drug Administration breakpoints for Enterobacteriaceae because CLSI and BrCAST do not have published clinical breakpoints for *Acinetobacter* spp. (CDER, 2013).

Molecular Detection of Genes Encoding Antimicrobial Resistance and Virulence Factors

The presence of carbapenemase genes (CHDL, MBL, GES and KPC) was performed by polymerase chain reaction (PCR) in all isolates as previously reported (Table S1) (WANG *et al.*, 2006; WOODFORD *et al.*, 2006; MENDES *et al.*, 2007; HIGGINS; LEHMANN; SEIFERT, 2010; DOYLE *et al.*, 2012). The selected acquired resistance determinants to colistin, quinolones, and aminoglycosides and the virulence factor *bap* were searched in accordance with previously established PCR conditions for isolates carrying $bla_{\text{OXA-58}}$ gene (OXA-58 NBA) (REBELO *et al.*, 2018; HIDALGO *et al.*, 2013; BUENO *et al.*, 2013; KODORI *et al.*, 2017; KRAYCHETE *et al.*, 2016).

Genetic Context of $bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-23-like}}$ Genes

The PCR mapping of upstream and downstream regions of $bla_{\text{OXA-58-like}}$ gene was performed for the NBA isolates as previously described by Villalón *et al.* (2013) (Table S1). The genetic context of 357 ACB isolates harboring $bla_{\text{OXA-23-like}}$ (OXA-23 ACB) recovered from blood (n=93), and tracheal aspirate (n=264) of patients admitted

to ICUs were assessed for the occurrence of transposons: Tn*2006*, Tn*2007*, Tn*2008*, and Tn*2009,* using PCR mapping (Table S1) (CHEN *et al.*, 2017).

DNA sequencing

The amplicons obtained in PCR (Table S1) were purified using PureLink PCR Purification Kit (Invitrogen, United States) and nucleotide sequences were determined by Sanger method carried out on genetic DNA sequencer (ABI 3500xL Genetic Analyzer [Applied Biosystems, Canada]). Multiple alignments and calculation of sequence identities were performed by the Bioedit v7.0.5 (Ibis Therapeutics, Canada), and BLAST algorithm in the NCBI GenBank database.

Biofilm Assay

The capacity to form biofilm was evaluated for OXA-58 NBA in a 96-well microtiter plate assay (FÁVARO *et al.*, 2019). The experiments were performed in quintuplicate on three different occasions. Biofilm formation was interpreted according to the criteria established by Stepanovic *et al.* (2000).

Enterobacterial Repetitive Intergenic Consensus Typing

The clonality of the OXA-58 NBA was assigned using the Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR technique (ROMANIN *et al.*, 2019). The amplicons were analyzed on 2.0% agarose gels, stained with 0.5 mg/mL ethidium bromide, and visualized by exposure to ultraviolet light (260 nm) in the LPIX transilluminator (Loccus Biotecnologia, Brazil). Fragments were analyzed with BioNumerics software (Applied Maths, Belgium). The unweighted pair group method with arithmetic averages (UPGMA) for clustering and Dice coefficient with 2.0% tolerance were adopted for the similarity of the isolates. The cutoff value of 90.0% was applied for the isolates clustering.

Results and Discussion

A. baumannii belongs to pathogens grouped under the acronym “ESKAPE” (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), that consists in microorganisms responsible for causing most severe healthcare-associated infections (HAIs), due to their ability to "escape" the effects of most antimicrobial agents, either through the acquisition of resistance genes and/or the ability to form biofilms. In this way, they represent the greatest risks in relation to resistance patterns that limit therapeutic alternatives available (KARLOWSKY *et al.*, 2017; OLIVEIRA *et al.*, 2020).

In this study, using the methodologies of *bla*$_{\text{OXA-51}}$ and *rpo*B gene amplification, the following species were detected among the 750 isolates evaluated: *A. baumannii* (n=738), *A. bereziniae* (n=6), *A. colistiniresistens* (n=1), *A. radioresistens* (n=1), *A. haemolyticus* (n=2), *A. ursingii* (n=1), and *Acinetobacter* sp. (n=1). Although the *bla*$_{\text{OXA-51}}$ gene has been described in isolates of *A. nosocomialis* and *A. pitti* (species of the ACB complex), several authors apply the amplification of the *bla*$_{\text{OXA-51}}$ gene as a genetic marker for the presumptive identification of *A. baumannii*, suggesting that this methodology does not interfere in the analysis and conclusion of the results, since this gene is intrinsic to this species and reports in the NBA are rare (LEE *et al.*, 2012; CASTILHO *et al.*, 2017; RODRIGUEZ; NASTRO; FAMILIETTI, 2018; VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020).

The sequencing of the *rpo*B gene, considered the gold standard methodology for the identification of *Acinetobacter* species, was used for confirmation of NBA isolates in this study once this methodology presents 98.2% accuracy by amplify highly variable interspecies regions. All the NBA evaluated, with exception of *A haemolyticus,* were initially misidentified as *A. lwoffii* (n=9) by Vitek 2® (BioMérieux) automated system, due to ID card and database limitations. In the routine of clinical laboratories, the difficulty of adopting molecular methodologies, added to the inability of conventional phenotypic tests and even of automated systems to accurately identify and differentiate *Acinetobacter* species closely related and NBA, results in an underestimation of their real prevalence, as well as of your antimicrobial resistance profile, particularly to carbapenems (PARK *et al.*, 2019; VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020).

The epidemiological analysis of the 738 *A. baumannii* isolates reveled that the highest rates of isolation of were observed in male patients (65.4%), with a median age of 55.5 years, ranging from newborn to 97 years-old (Table 1). Similar results were reported by studies carried out in different regions of Brazil: A study of Minas Gerais (Southeastern Brazil) that evaluated carbapenem-resistant *A. baumannii* (CRAb) recovered from ICU patients between December 2009 to December 2010, obtained 58.9 % from males with 61 years (±15.1); a study at Goiás hospital (Central-western Brazil) that evaluated *A. baumannii* recovered from ICU inpatients between June and December 2010, obtained 59.4% from males and 53.2 years (±19); and a study at Mato Grosso do Sul hospital (Central-western Brazil) that evaluated *A. baumannii* recovered from ICU inpatients between September 2013 and April 2015, obtained 58.5% from males and aged >60 years old (46.3%) (NEVES *et al.*, 2016; CASTILHO *et al.*, 2017; SILVA *et al.*, 2018).

The ACB samples of this study were obtained mostly of tracheal aspirate (43.5%), surveillance swab (24.7%) and blood (17.2%) (Table 1). Although it was not determined whether the study isolates were associated with infection or colonization, they were recovered from the mentioned sites and are in line with what is reported in the literature, where generally *Acinetobacter* spp. are related to infections such as ventilator-associated pneumonia (VAP), skin and soft tissue infections, bacteremia and urinary tract infections (CAYÔ, 2017; SILVA *et al.*, 2018; VIJAYAKUMAR; BISWAS; VEERARAGHAVAN, 2019; BAGUDO *et al.*, 2020; KURIHARA *et al.*, 2020).

The highest number of isolates was obtained for inpatient admitted in intensive care unit (ICU) (57.0%) following ward (29.8%) and emergency room (6.5%). This microorganism is related with severe healthcare-associated infections, mostly in ICU inpatients were risks factors for infections are the utilization of invasive devices, presence of comorbidities, length of hospital stay, and prior antimicrobials exposures (NEVES *et al.*, 2016; CASTILHO *et al.*, 2017; SILVA *et al.*, 2018; ROYER *et al.*, 2018; KURIHARA *et al.*, 2020). Talizin *et al.* (2020) in study conducted in HU, between January 2017 and January 2018, analyzing the use of polymyxins for the treatment of VAP, identified *A. baumannii* as an important etiological agent (67.6%), with mortality rate of 64.1%. The NBA, in turn, were obtained of tracheal aspirate (25.0%), blood (25.0%) and secretions (25.0%), of inpatients mainly in emergency room (33.3%) and

ward (25.0%), majority in male patients (75.0%), with ranging from 8 to 97 years-old (median age of 47 years) (Table 1).

Table 1 Demographic data of patients and clinical characteristics of *Acinetobacter* spp. isolates recovered in Hospital Universitário de Londrina from 2006-2016

	Acinetobacter calcoaceticus-Acinetobacter baumannii (n=738) N (%)	non-*baumannii Acinetobacter* (n=12) N (%)
Gender		
Male	483 (65.4)	9 (75.0)
Female	253 (34.3)	3 (25.0)
Other	2 (0.3)	
Age		
0-20 years	68 (9.2)	1 (8.3)
21-40 years	155 (21.0)	4 (33.3)
41-60 years	212 (28.7)	4 (33.3)
61-80 years	240 (32.5)	2 (16.7)
≥81 years	63 (8.6)	1 (8.3)
Ward		
Intensive care unit[a]	421 (57.0)	1 (8.3)
Ward	220 (29.8)	3 (25.0)
Emergency room[b]	48 (6.5)	4 (33.3)
Urgency	21 (2.8)	
Others	28 (3.8)	4 (33.3)
Clinical sample		
Tracheal aspirate	321 (43.5)	3 (25.0)
Surveillance swab	182 (24.7)	
Blood	127 (17.2)	3 (25.0)
Urine	39 (5.3)	1 (8.3)
Secretions/Sterile liquids[c]	27 (3.6)	3 (25.0)
Tissue[d]		
Catheter tips	20 (2.7)	2 (16.7)
	22 (3.0)	
Patient outcome		
Discharged	93 (12.6)	8 (66.7)

Death	288 (39.0)	4 (33.3)
Other	357 (48.4)	

[a]Neonatal, pediatric, adult or burn intensive care unit. [b]Ambulatory, surgical, medical, obstetric or orthopedic emergency room. [c]Secretion, abdominal secretion, surgical wound secretion, ocular secretion, auricular secretion, LCR or peritoneal fluids. [d]Tissue or bone fragment.

High levels of resistance were observed for the carbapenems (95.6-98.3%), fluroquinolones (94.6-100.0%), cephalosporins (98.5-99.7%), beta-lactam/beta-lactamase inhibitor combination (57.6-100.0%), aminoglycosides (47.8-66.2%), and trimethoprim-sulfamethoxazole (87.0%) (Table 2). Furthermore, tigecycline (72.2%) and colistin (92.8%) showed important susceptible rates (Table 2). Regarding to tigecyline, similar results were observed by Neves *et al.* (2016) and Castilho *et al.* (2017) in studies conducted in Minas Gerais and Goiás, wich obtained rates of susceptibility of 76.7% and 92.9%, respectively.

Approximate susceptibility rates to colistin (78.5%) also were observed by Camargo *et al.* (2020) in São Paulo that, similar to this study, supports the possible utilization of polymyxins and tetracycline antibiotics (minocycline, doxycycline and tigecycline) for treating *A. baumannii* infections in HU. However, recent studies have observed an increase in the rate of resistance to polymyxins (93.0%) in HU (PERDIGAO NETO *et al.*, 2020). The different resistance profiles observed in studies carried out in different regions of Brazil may be related to the pattern of antimicrobial use, as well as regional factors that include daily practices, patients and complexities associated with healthcare (CAMARGO *et al.*, 2020).

Table 2 Antimicrobial susceptibility profile of *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex recovered in Hospital Universitário de Londrina from 2006-2016

	Total	**Resistant N (%)**	**Intermediate N (%)**	**Susceptible N (%)**
Antimicrobials				
Amikacin	550	263 (47.8)	42 (7.6)	245 (44.5)
Gentamicin	545	361 (66.2)	25 (4.6)	159 (29.2)
Imipenem	736	704 (95.6)	5 (0.7)	27 (3.7)
Meropenem	706	694 (98.3)	1 (0.1)	11 (1.6)

Ciprofloxacin	534	534 (100.0)	-	-
Levofloxacin	408	386 (94.6)	18 (4.4)	4 (1.0)
Cefepime	552	545 (98.7)	5 (0.9)	2 (0.4)
Cefotaxin	244	243 (99.6)	1 (0.4)	-
Ceftriaxone	357	356 (99.7)	1 (0.3)	-
Ceftazidime	549	541 (98.5)	8 (1.5)	-
Tetracycline	129	26 (20.2)	16 (12.4)	87 (67.4)
Tigecycline	465	25 (5.4)	104 (22.4)	336 (72.2)
Ampicillin-sulbactam	543	313 (57.6)	200 (36.8)	30 (5.5)
Piperacillin-tazobactam	156	156 (100.0)	-	-
Trimethoprim-sulfamethoxazole	537	467 (87.0)	-	70 (13.0)
Colistin	346	25 (7.2)	-	321 (92.8)
Profile of resistance				
CR	711			
MDR	420			
XDR	131			

CR, carbapenem resistant; MDR, multiresistant to antimicrobials; XDR, extensively resistant to antimicrobials.

The resistance profile classification, according Magiorakos *et al*. (2012), revealed worrying rates of MDR (56.9%) and XDR (17.8%) isolates (Table 2). None PDR *A. baumannii* was observed. Royer *et al.* (2018) in a study conducted in with isolates recovered of PAV inpatients ICU in Minas Gerais reported a most prevalence of XDR isolates (78.3%). As well as Romanin *et al*. (2019) that in a study conducted in with isolates recovered of bloodstream in Paraná observed that 78.6% isolates were XDR and 21.4% MDR.

In 2017, the World Health Organization published a priority pathogens document for research on antibiotic development, and CRAb ranked first on the list as a critical-situation pathogen worldwide (KURIHARA *et al.*, 2020). In a study carried out in Latin America, between 2002 and 2013, 55.0% of the isolates of *A. baumannii* showed resistance to carbapenems (LABARCA *et al.*, 2016; RODRIGUEZ; NASTRO; FAMILIETTI, 2018). In the surveillance of the Tigecycline Evaluation and Surveillance

Trial (TEST), carried out between 2004 and 2009, the rate of resistance to meropenem in Latin America (73.0%) was higher than in North America (40.0%), Europe (47.0%) and the Ásia-Pacific (69.0%).

In SENTRY surveillance, from 2006 to 2009, resistance to imipenem was observed in 26.0% of isolates from Latin America, and in 12.0% in the USA, 21.0% in Europe and 41.0% in Ásia-Pacific (LABARCA *et al.*, 2016). High frequencies of CRAb are reported in South America, and according to the SENTRY Antimicrobial Surveillance Program for Latin America, carbapenem resistance rates of *A. baumannii* isolates in Brazil increased approximately 60.0% in just one decade, 1997-1999 to 2008-2010 (12.6% for 71.4%), and increased considerably in others Latin American countries (CAYÔ, 2017; SILVA *et al.*, 2018; CAMARGO *et al.*, 2020). Studies reported the carbapenem resistance rates in Argentina (78.0% - imipenem/81.0% meropenem) and Brazil (31.0-73.0% - imipenem/56.0-73.0% meropenem) among the highest in the world (LABARCA *et al.*, 2016; RODRIGUEZ; NASTRO; FAMILIETTI, 2018). In HU, between 2006 and 2016, the incidence of CRAb was 35.2%, and in our study, despite the limitation of the number of isolates studied, was observed a rate of CRAb of 96.3% (Table 2).

Actually, polymyxins, tigecycline, ampicillin-sulbactam, and cefiderocol are used in the treatment of CRAb infections in an attempt to overcome AR (NEVES *et al.*, 2016; CAYÔ, 2017; DOI, 2019; NOTA TÉCNICA, 2021). However, the polymyxins presented toxicity systemic, and the tigecycline, despite showing well in vitro activity against *Acinetobacter* spp., increased expression of the AdeABC efflux system can lead to resistance, and, consequently, to therapeutic failure. In addition, its use was associated with increased patient mortality. Nonetheless, due to the lack of therapeutic options, it is used with epidemiological cut-off points, since there are no official BrCAST/EUCAST cut-off points established for the treatment of infections caused by *Acinetobacter* spp. (NEVES *et al.*, 2016; CAYÔ, 2017; DOI, 2019; NOTA TÉCNICA, 2021).

The NBA isolates showed lowers levels of resistance in relation to *A. baumannii* isolates. The following resistance rates were observed: carbapenems, 25.0%; fluroquinolones, 8.3-41.7%; cephalosporins, 16.6-45.5%; beta-lactam/beta-lactamase inhibitor combination, 16.7-25.0%; aminoglycosides, 33.3%; trimethoprim-

sulfamethoxazole, 41.7%; and colistin,14.3% (Table 3). Of these, 58.3% were classified as MDR.

Table 3 Antimicrobial susceptibility profile of non-*baumannii Acinetobacter* recovered in Hospital Universitário de Londrina from 2006-2016

	Total	**Resistant N (%)**	**Intermediate N (%)**	**Susceptible N (%)**
Antimicrobials				
Amikacin	12	4 (33.3)	1 (8.3)	7 (58.3)
Gentamicin	12	4 (33.3)	-	8 (66.7)
Imipenem	12	3 (25.0)	-	9 (75.0)
Meropenem	12	3 (25.0)	-	9 (75.0)
Ciprofloxacin	12	5 (41.7)	1 (8.3)	6 (50.0)
Levofloxacin	12	1 (8.3)	3 (25.0)	8 (66.7)
Cefepime	12	4 (33.3)	1 (8.3)	7 (58.3)
Cefotaxin	11	5 (45.5)	3 (27.3)	3 (27.3)
Ceftazidime	12	2 (16.6)	4 (33.3)	6 (50.0)
Ampicillin-sulbactam	12	2 (16.7)	3 (25.0)	7 (58.3)
Trimethoprim-sulfamethoxazole	12	5 (41.7)	-	7 (58.3)
Piperacillin-tazobactam	12	3 (25.0)	-	9 (75.0)
Tetracycline	12	-	-	12 (100.0)
Colistin	7	1 (14.3)	-	6 (85.7)
Profile of resistance				
CR	3			
MDR	7			

CR, carbapenem resistant; MDR, multiresistant to antimicrobials.

Among resistance mechanisms to carbapenems, the production of carbapenemases of the type Carbapenem-Hydrolyzing class D beta-Lactamases (CHDLs) is the main cause of resistance, of which the OXA-23-like, OXA-24-like, OXA-58-like, OXA-143-like, and OXA-235-like are main and most widely acquired CHDLs spread in *Acinetobacter* species (NEVES *et al.*, 2016; CAYÔ, 2017; SILVA *et al.*, 2018; RODRIGUEZ; NASTRO; FAMILIETTI, 2018). Isolates carrying CHDL-encoding genes

have been reported from Europe, North America, South America, Australia, East Asia, and Asia (KOIRALA *et al.*, 2020). These enzymes, intrinsic or acquired, presented able weakly hydrolyze carbapenems and usually are associated with mobile genetic elements such as insertion sequence (IS). Which can lead to gene their mobilization for other NBA species and, upstream of the CHDL gene, can acts as a strong promoter, increasing significantly the expression of the gene, conferring a more pronounced carbapenems resistance profile (EVANS; AMYES, 2014; NEVES *et al.*, 2016; CAYÔ, 2017; CASTILHO *et al.*, 2017; SILVA *et al.*, 2018; RODRIGUEZ; NASTRO; FAMILIETTI, 2018).

In *A. baumannii*, the most common carbapenemase is OXA-23, while OXA-24 and OXA-58 despite distributed globally present lower frequencies (DOI, 2019). In this study, the majority isolates presented at least one acquired CHDL. Most of the *A. baumannii* isolates harbored the *bla*$_{\text{OXA-23-like}}$ gene (90.5%) and the *bla*$_{\text{OXA-143-like}}$ (1.2%), whose sequencing analysis identified the *bla*$_{\text{OXA-253}}$ (8) and *bla*$_{\text{OXA-231}}$ (1) variants (Table 4), similar to observed in other studies conducted in hospital of diverse Brazilian states (CAYÔ, 2017). Neves *et al.* (2016) related most prevalence of *bla*$_{\text{OXA-23-like}}$ (51.2%) in *A. baumannii*, followed by *bla*$_{\text{OXA-143-like}}$ (18.6%). Castilho *et al.* (2017) observed a prevalence of *bla*$_{\text{OXA-23-like}}$ (55.1%), followed by *bla*$_{\text{OXA-58-like}}$ (3.6%). Studies conducted in different continents like Europe (France), North America (United States), Asia (China, Saudi Arabia, India, Vietnam, Malasia), Africa (South Africa) reported similar results (KURIHARA *et al.* 2020).

The frequency of OXA-23 observed is in accordance with the literature, that report this carbapenemase as the most widespread in Latin America, present in all countries of the region, and frequently associated with endemic clones (CASTILHO *et al.*, 2017; RODRIGUEZ; NASTRO; FAMILIETTI, 2018). In Brazil, this gene is considered the main determinant associated with resistance to carbapenems, and studies have associated the increase of resistance with the spread of the OXA-23-producers strains (DALLA-COSTA *et al.*, 2003; NEVES *et al.*, 2016; ROYER *et al.*, 2018; RODRIGUES-COSTA *et al.*, 2019). This gene can is associated with the IS*Aba*1 or IS*Aba*4, compounding the genetic contexts reported in the literature as responsible for its widespread (Tn*2006*, Tn*2007*, Tn*2008*, and Tn*2009*). The increase in CRAb has been associated with the high prevalence of isolates containing IS*Aba*1 upstream of the *bla*$_{\text{OXA-23-like}}$ gene, inserted mainly in the Tn*2006* and Tn*2008* transposons. These

elements upstream can act as promoters increasing gene expression and resistance to carbapenem significantly (KU *et al.*, 2015; CHEN *et al.*, 2017; CASTILHO *et al.* 2017; SILVA *et al.*, 2018; ROYER *et al.*, 2018; KURIHARA *et al.*, 2020).

Table 4 Genotypic profile of *Acinetobacter* spp. recovered in Hospital Universitário de Londrina from 2006-2016

	Acinetobacter calcoaceticus-Acinetobacter baumannii (n=738) N (%)	non-*baumannii Acinetobacter* (n=12) N (%)
Genotypic profile		
bla$_{\text{OXA-51}}$/ *bla*$_{\text{OXA-23}}$	668 (90.5)	
bla$_{\text{OXA-51}}$	61 (8.3)	
bla$_{\text{OXA-51}}$/ *bla*$_{\text{OXA-143-like}}$	9 (1.2)	
bla$_{\text{OXA-58}}$		6 (50.0)
bla$_{\text{OXA-23}}$		1 (8.3)

Considering the high number of *bla*$_{\text{OXA-23-like}}$ isolates, the evaluation of the genetic context is fundamental to understanding the successful spread of this gene in HU. The mapping of 357 isolates harboring the *bla*$_{\text{OXA-23-like}}$ gene was investigated, and the presence of two different transposons was detected (CHEN *et al.*, 2017). Tn*2008* was found in 72.0% of the isolates, followed by Tn*2006* (27.2%). The Tn*2008* and Tn*2006* were most prevalent in isolates recovered from tracheal aspirate and bloodstream samples, respectively. Three isolates were not positive for Tn*2006*, Tn*2007*, Tn*2008* and Tn*2009*. Despite the Tn*2006* is the genetic environment most reported globally, the high prevalence of the Tn*2008*, in this study, suggest the successful adaptation of this mobile element in *Acinetobacter* spp. from HU (NIGRO; HALL, 2016). Brazilian studies have reported these structures in 82.0% of isolates and associate these with the dissemination of the *bla*$_{\text{OXA-23-like}}$ gene, as well as a decrease in carbapenem susceptibility of isolates (CASTILHO *et al.*, 2017; ROYER *et al.*, 2018). In addition, similar rates were observed in Iran (90.0%), United Kingdom (84.0%), and Spain (74.7%) (CASTILHO *et al.*, 2017).

The second acquired CHDL most prevalent in *A. baumannii* in Brazil, the *bla*$_{\text{OXA-143-like}}$ was first reported in this country in 2004, being detected mainly in hospitals located in the Southeast region (HIGGINS *et al.*, 2009; RODRIGUES-COSTA *et al.*, 2019). This gene can be associated with the IS*Aba*1 upstream, and this way, the OXA-143 enzyme will be able to penicillins and carbapenems (HIGGINS *et al.*, 2009; EVANS; AMYES, 2014). In HU, were reported the following variants of *bla*$_{\text{oxa-143}}$ gene: *bla*$_{\text{OXA-231}}$ and *bla*$_{\text{OXA-253}}$ (GIONCO *et al.*, 2012; ROMANIN *et al.*, 2019). The *bla*$_{\text{OXA-231}}$ gene was first described in a Brazilian strain of *A. baumannii* isolated in 2007 in HU, however, its actual frequency is still unknown, due to few reports to date (RODRIGUES-COSTA *et al.*, 2019).

Of the NBA species, *A. radioresistens* carried the species intrinsic gene, *bla*$_{\text{OXA-23-like}}$, while the *bla*$_{\text{OXA-58-like}}$ gene was detected in *A. bereziniae* (n=5) and *A. colistiniresistens* (n=1), in the years 2014 and 2015 (Fig. 1). The *bla*$_{\text{OXA-58}}$ gene was described for the first time in a clinical isolate of *A. baumannii* in France in 2003 (POIREL *et al.*, 2005; XIN *et al.*, 2019). Since then, it has been detected in clinical, animal, and environmental isolates of different species of *Acinetobacter* in diverse countries around the world. This enzyme has low hydrolytic activity against penicillins and carbapenems, and is capable of hydrolyzing some first and fourth generation cephalosporins. The presence of other mechanisms simultaneously allows the identification of high levels of resistance to carbapenems (POIREL *et al*, 2005; EVANS; AMYES, 2014).

This gene presents a remarkable conserved genetic sequence and is one of the genes most commonly detected in non-*baumannii Acinetobacter* (NGUYEN *et al.*, 2020; BARAKA *et al.*, 2021). Although little described in Brazil, unlike what has been observed in neighboring countries, the *bla*$_{\text{OXA-58}}$ gene was reported, to date, in clinical and environmental isolates of *A. baumannii*, *A. seifertii*, *A. bereziniae*, and *A. colistiniresistens* (FÁVARO *et al.*, 2019; MATOS *et al.*, 2019; PAULA-PETROLI *et al.*, 2022). The *bla*$_{\text{OXA-58}}$ gene was detected only in the NBA isolates: *A. bereziniae* (n=5) and *A. colistiniresistens* (n=1), emphasizing the role of these isolates as potential reservoirs of this determinant in our institution.

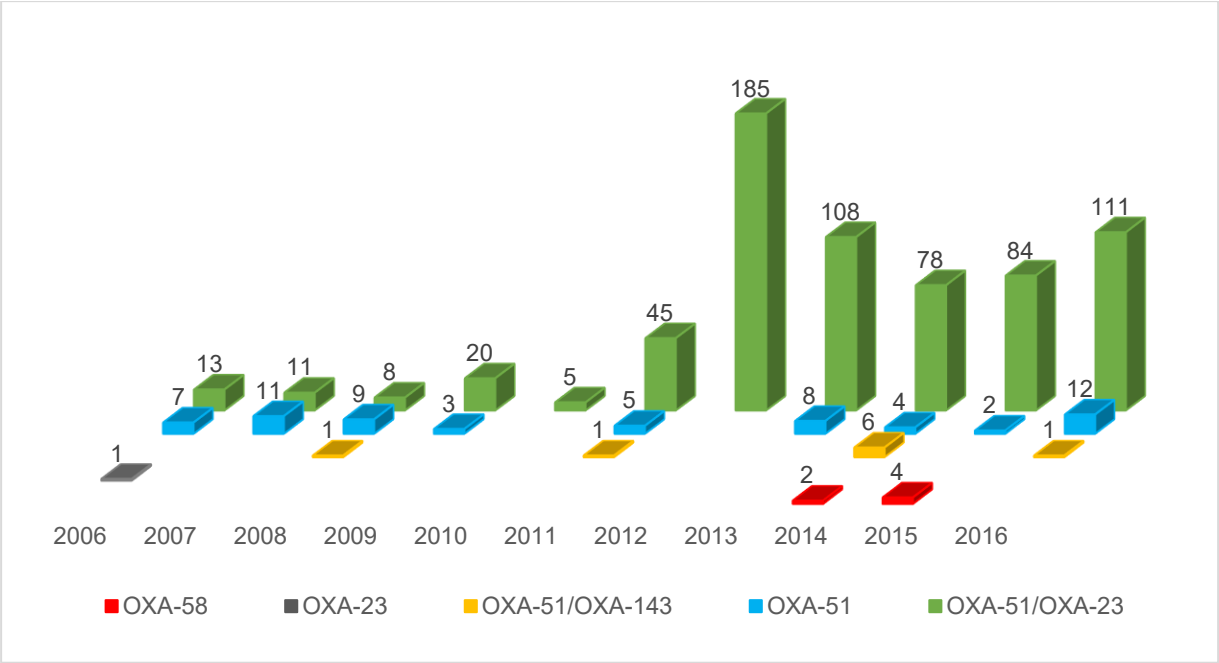

Fig. 1 Distribution of CHDL types in *Acinetobacter* spp. clinical isolates recovered in Hospital Universitário de Londrina from 2006-2016.

The *bla*$_{\text{OXA-58}}$ gene is, usually, located flanked on both sides by IS*Aba*3 (MA *et al.*, 2020; WANG *et al.*, 2020). Overexpression of the *bla*$_{\text{OXA-58}}$ frequently results from the presence of insertion sequences such as IS*Aba*1, IS*Aba*2, IS*Aba*3, or IS18 upstream that provide strong promoters for gene expression (NGUYEN *et al.*, 2020). The IS*Aba*3 sequence upstream is frequently interrupted by other IS, providing an alternative promoter that enhances the genic expression and consequently the level of carbapenems resistance (MA *et al.*, 2020; NGUYEN *et al.*, 2020; WANG *et al.*, 2020). Additionally, this structure can be present in plasmids, which plays an important role in mobilizing this determinant of resistance between different bacterial species by horizontal transfer of genes (MATOS *et al.*, 2019; LITERACKA *et al.*, 2021).

In the analysis of the genetic context, all isolates presented the IS*Aba*3 sequence downstream of the *bla*$_{\text{OXA-58}}$ gene. The isolates belonging to the clusters I-III showed IS*Aba*3, IS*Aba*1, and IS*Aba*125-ΔIS*Aba*3 upstream of the gene, respectively, and were classified with MDR (Table 5). While in the isolates belonging to the cluster IV were not identified IS upstream of *bla*$_{\text{OXA-58}}$. The failure to amplification of the IS upstream of these isolates suggests that the element may be interrupted at its 5' end by another element, as previously related. Interestingly, the isolates in which

they were not identified IS upstream of *bla*$_{OXA-58}$ were susceptible to carbapenems, while most of the isolates presenting IS were resistant (Table 5).

Previous studies observed similar contexts as observed in this study (IS*Aba*1 and IS*Aba*125-ΔIS*Aba*3) in isolates with reduced susceptibility to carbapenems (GUSATTI *et al.*, 2012; EVANS *et al.*, 2010; WU *et al.*, 2015). Different, as observed by Nguyen *et al.* (2020), in our study the intact IS*Aba*3 upstream of the gene was not associated with genic overexpression and increase of carbapenem resistance. The susceptibility to carbapenems of isolates, it is turned, can be explained by the poor hydrolytic activity of enzyme, whose coding gene is often weakly expressed (MA *et al.*, 2020).

The molecular typing of *bla*$_{OXA-58}$-harboring clinical isolates revealed the presence of four genetic groups (I-IV) (Fig. 2). The five *A. bereziniae* isolates were included in clusters II, III, and IV, while the *A. colistiniresistens,* because it is a different species and shows great diversity, was included in cluster I. The I, II, and III groups were individual clusters composed of only one MDR isolate each. The Ac 505/15 isolate (cluster I) was susceptible to most antimicrobial, including carbapenems, similar to reported in previous studies (BRASILIENSE *et al.*, 2021). Furthermore, presented a high MIC for polymyxins due to an intrinsic characteristic of this specie described in the literature (NEMEC *et al.*, 2017). The Ac 644/15 (cluster II) and Ac 374/14 (cluster III) isolates showed resistance to carbapenems, aminoglycosides, fluoroquinolones, cephalosporins, trimethoprim-sulfamethoxazole, and piperacillin-tazobactam, and additionally, Ac 644/15, resistance to polymyxins. The IV group was composed of three isolates of *A. bereziniae* considered clonally related, of these, two isolates showed 100.0% of genetic similarity. The Ac 571/14 and Ac 531/15 isolates presented resistance to polymyxins, and Ac 571/14 was resistant also to ceftazidime. While Ac 236/15 was resistant only to colistin. All the *bla*$_{OXA-58}$-harboring isolates were susceptible to tigecycline (Table 5).

Table 5 Antimicrobial susceptibility profile, genetic similarity, genetic context and biofilm production of non-*baumannii Acinetobacter* isolates harboring *bla*$_{OXA-58}$ gene.

Strain ID	Specie ID	ERIC	Resistance profile								Genetic context of *bla*$_{OXA-58}$ gene	Biofilm
				MIC (µg/mL)						Phenotype[a]		
				MEM	IPM	CAZ	TGC	COL	PMB			
374/14	*A. bereziniae*	III	AK, CN, LEV, CIP, IPM, MEM, FEP, CAZ, CTX, SAM, SXT, TZP	4,0	16,0	16,0	≤0,125	1,0	1,0	MDR	IS*Aba*125-ΔIS*Aba*3-*bla*$_{OXA-58}$-IS*Aba*3[b]	Strong
571/14	*A. bereziniae*	IV	CIP, CAZ, CTX, TZP	≤0,25	≤0,25	16,0	≤0,125	8,0	4,0	MDR	*bla*$_{OXA-58}$-IS*Aba*3	Strong
505/15	*A. colistiniresistens*	I	AK, SXT, CTX	1	0,5	8,0	≤0,125	≥32,0	32,0	MDR	IS*Aba*3-*bla*$_{OXA-58}$-IS*Aba*3[c]	Weak
236/15	*A. bereziniae*	IV		≤0,25	0,5	8,0	≤0,125	4,0	1,0	-	*bla*$_{OXA-58}$-IS*Aba*3	Strong
644/15	*A. bereziniae*	II	AK, CN, LEV, CIP, IPM, MEM, FEP,	8,0	16,0	32,0	0,5	8,0	4,0	MDR	IS*Aba*1-*bla*$_{OXA-58}$-IS*Aba*3	Weak

			CAZ, CTX, SXT, TZP									
531/15	*A. bereziniae*	IV	AK, CN, LEV, CIP, IPM, MEM, CAZ, CTX, SAM, SXT	≤0,25	0,5	2,0	≤0,125	8,0	4,0	MDR	*bla*$_{OXA-58}$-IS*Aba*3	Strong

ERIC, enterobacterial repetitive intergenic consensus;MDR, multidrug-resistant; MIC, Minimal inhibitory concentration; MEM, meropenem; IPM, imipenem; CAZ, ceftazidime; TGC, tigecycline; COL, colistin; PMB, polymyxin B; AK, amikacin; CN, gentamicin; LEV, levofloxacin; CIP, ciprofloxacin; FEP, cefepime; CTX, cefotaxime; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam.

[a]MAGIORAKOS *et al.*, 2012

[b]FÁVARO *et al.*, 2019

[c]PAULA-PETROLI *et al.*, 2022

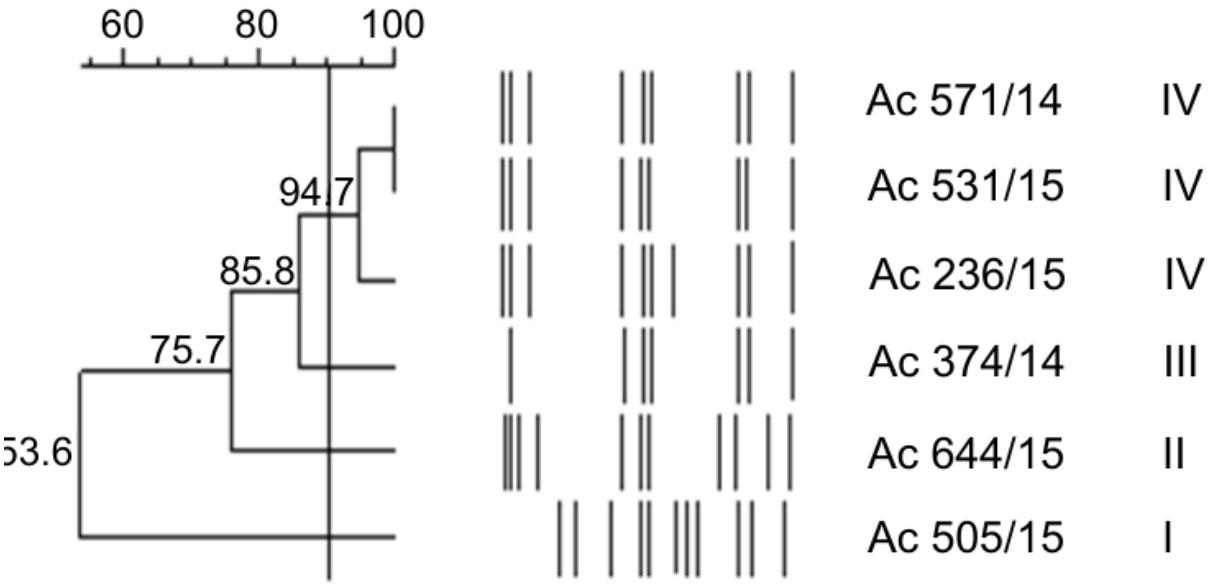

Fig. 2 Dendrogram based on ERIC-PCR of *Acinetobacter bereziniae* and *Acinetobacter colistiniresistens* isolates harboring *bla*$_{\text{OXA-58}}$ gene. Fragments were analyzed with BioNumerics v. 6.6 (Applied Maths) using Unweighted Pair Group Method with Arithmetic averages (UPGMA) for clustering and Dice coefficient with 2.0% tolerance for the isolates similarity. The use 90.0% level of similarity for determine clonally related isolates revealed the presence of four different profile.

Generally, the mechanisms of polymyxin resistance in *A. baumannii* are related to genetic alterations (*lpx*A, *lpx*C and *lpx*D) that result in the loss of lipopolysaccharide (LPS), the addition of phosphoethanolamine to lipid A reducing the negative charge of bacterial cells and, consequently, the affinity of polymyxins to their target of action, insufficient concentration of constitutional cofactors for the formation of LPS, and efflux systems (GIRARDELLO *et al.*, 2017; CARRASCO *et al.*, 2021; KYRIAKIDIS *et al.*, 2021; PARK *et al.*, 2021). In addition, although rare, there is a description of genes encoding resistance to plasmid-mediated polymyxins (*mcr*), which are a real threat to the treatment of infections by MDR isolates, due to the rapid dissemination of this determinant among different bacterial species (GIRARDELLO *et al.*, 2017; CARRASCO *et al.*, 2021). In this study, of the polymyxin resistance mechanisms mentioned, the most frequente genes related to mediated colistin resistance mechanism (*mcr*-1 to *mcr*-5) were investigated and not detected.

The biofilm formation was observed in all isolates evaluated in this study, being 66.7% considered strong and 33.3% weak biofilm producers (Table 5).

However, none NBA was positive for the presence of the *bap* gene and other resistance determinants investigated. The biofilm formation is an important virulence characteristic *of A. baumannii*, capable of increases their survival time in dry environments, conferring desiccation resistance, and is also associated with increased resistance against the host's immune response and antimicrobials (UPMANYU; HAG; SINGH, 2022; ROY *et al*., 2022).This ability is multifactorial and not totally elucidated. The biofilm-associated protein (*bap*), located in outer membrane, acts in the maturation of biofilm, providing greater thickness and volume, as well as intercellular adhesion (VÁZQUEZ-LÓPEZ *et al.*, 2020).

This study presents some limitations such as those that are inherent to retrospective analysis and the quality of data depended on the clinical records; and some molecular tests (of genetic context) were determine in the *A. baumannii* recovered from hemoculture and traqueal aspirate representing 47.6% (n=357) of the isolates. Futhermore, association of isolates with international clonal lineages was not performed on this occasion, and further MLST analysis will be relevant in the future.

Conclusion

In conclusion, we related the high incidence of antimicrobial resistance observed in this study reinforcing the idea of the enormous clinical challenge faced in the HU for the treatment of serious infections caused by CRAb (i.e., bacteremia, ventilator-associated and hospital-acquired pneumonia). Furthermore, the extensive distribution of the *bla*$_{\text{OXA-23-like}}$ gene which were mainly inserted in mobile genetic elements (Tn*2008* and Tn*2006*) may be associated with the maintenance of carbapenem resistance and increasing of MDR and XDR rates of *Acinetobacter* strains in our institution.

While carbapenem resistance in ACB is well characterized, here we show the clinical importance of NBA and the need for diligent laboratory surveillance of these *Acinetobacter* species in HU. The presence of *bla*$_{\text{OXA-58}}$ was identified from 2014 and 2015, exclusively in NBA isolates, emphasizing the role of the these microorganisms as potential novel reservoirs of this gene in the clinical setting. Notably, differences in the MICs of carbapenem and polymyxins were observed for the *bla*$_{\text{OXA-58-}}$carrying NBA isolates. Thus, carbapenem-susceptible isolates may go unnoticed, persist in the hospital environment, and direct their silent spread to other clinically relevant species. Furthermore, the presence of carbapenemase-encoding

genes in NBA with decreased susceptibility to polymyxins (i.e., *A. colistiniresistens*) represents a major concern, as it may lead co-selection of antimicrobial resistance genes limiting the therapeutic options.

This is the first report describing the genomic composition of different NBA isolates carrying *bla*$_{\text{OXA-58}}$ in southern Brazil. However, it is important to note that all the NBA have been misidentified by the Vitek 2® automated system. Therefore, it is important that clinical microbiology laboratories remain alert to the correct identification and resistance phenotypes of the various *Acinetobacter* species increasingly reported as of great clinical importance. Finally, insights and studies into differences in epidemiology, antimicrobial resistance patterns and clinical outcomes without further speciation may be considered inadequate, leading to inferior clinical care and inappropriate use of antibiotics.

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Declarations

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Conflicts of interest The authors report no conflicts of interest in this work.

Ethics approval This study was approved by the Research Ethics Committee (REC), Health Sciences Center, Universidade Estadual de Londrina (Protocol No.

14490/2009). Informed consent was waived by the REC since the study was observational and patient care was not influenced.

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material Not applicable.

Code availability Not applicable.

Authors' contributions LSF, SBPP, FECM and EJV designed the study and coordinated the experiment. LSF, SBPP, PR and BGC carried out the experiment, drafted the manuscript, and analyzed the data. LSF, SBPP, FECM, and EJV did the necessary editing of the manuscript. All authors read and approved the manuscript.

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Table S1 Oligonucleotides used for the molecular characterization of *Acinetobacter* spp.

Oligonucleotides	Sequence (5' – 3')	AT[a]	Fragment size (bp)	Reference
Ac696F	TAYCGYAAAGAYTTGAAAGAAG	60°C	857	LA SCOLA *et al.*, 2006
Ac1598R	CGBGCRTGCATYTTGTCRT			
OXA-23 F	GATCGGATTGGAGAACCAGA	52ºC	501	WOODFORD *et al.*, 2006
OXA-23 R	ATTTCTGACCGCATTTCCAT			
OXA-24 F	GGTTAGTTGGCCCCCTTAAA		246	
OXA-24 R	AGTTGAGCGAAAAGGGGATT			
OXA-51 F	TAATGCTTTGATCGGCCTTG		353	
OXA-51 R	TGGATTGCACTTCATCTTGG			
OXA-58 F	AAGTATTGGGGCTTGTGCTG		599	
OXA-58 R	CCCCTCTGCGCTCTACATAC			
OXA-143 F	TGGCACTTTCAGCAGTTCCT	52ºC	149	HIGGINS; LEHMANN; SEIFERT, 2010
OXA-143 R	TAATCTTGAGGGGGCCAACC			
NDM F	GCAGCTTGTCGGCCATGCGGGC	60ºC	782	DOYLE *et al.*, 2012
NDM R	GGTCGCGAAGCTGAGCACCGCAT			
KPC F	TGTCACTGTATCGCCGTC		900	
KPC R	CTCAGTGCTCTACAGAAAACC			
OXA-48 F	GCGTGGTTAAGGATGAACAC		438	
OXA-48 R	CATCAAGTTCAACCCAACCG			
GES F	ATGCGCTTCATTCACGCAC	55ºC	846	WANG *et al.*, 2006
GES R	CTATTTGTCCGTGCTCAGG			
IMP F	GAATAG(A/G)(A/G)TGGCTTAA(C/T)TCTC	53ºC	188	MENDES *et al.*, 2007
IMP R	CCAAAC(C/T)ACTA(G/C)GTTATC			
VIM F	GTTTGGTCGCATATCGCAAC		382	
VIM R	AATGCGCAGCACCAGGATAG			
GIM F	TCAATTAGCTCTTGGGCTGAC		72	
GIM R	CGGAACGACCATTTGAATGG			
SIM-1 F	GTACAAGGGATTCGGCATCG		569	
SIM-1 R	TGGCCTGTTCCCATGTGAG			

SPM-1 F	CTAAATCGAGAGCCCTGCTTG		798	
SPM-1 R	CCTTTTCCGCGACCTTGATC			
MCR-1 F	ATGCCAGTTTCTTTCGCGTG	59ºC	502	REBELO *et al.*, 2018
MCR-1 R	TCGGCAAATTGCGCTTTTGGC			
MCR-2 F	GATGGCGGTCTATCCTGTAT		379	
MCR-2 R	AGGCTGACACCCCATGTCAT			
MCR-3 F	ACCAGTAAATCTGGTGGCGT		296	
MCR-3 R	AGGACAACCTCGTCATAGCA			
MCR-4 F	TTGCAGACGCCCATGGAATA		207	
MCR-4 R	GCCGCATGAGCTAGTATCGT			
MCR-5 F	GGACGCGACTCCCTAACTTC		608	
MCR-5 R	ACAACCAGTACGAGAGCACG			
QnrAm F	AGAGGATTTCTCACGCCAGG	58ºC	580	KRAYCHETE *et al.*, 2016
QnrAm R	TGCCAGGCACAGATCTTGAC			
QnrBm F	GGMATHGAAATTCGCCACTG		264	
QnrBm R	TTTGCYGYYCGCCAGTCGAA			
QnrCm F	GCGAATTTCCAAGGGGCAAA		135	
QnrCm R	ACCCGTAATGTAAGCAGAGCAA			
QnrDm F	AGGTGTAGCATGTATGGAAAAGC		691	
QnrDm R	ACATTGGGGCATTAGGCGTT			
QnrSm F	GCAAGTTCATTGAACAGGGT		428	
QnrSm R	TCTAAACCGTCGAGTTCGGCG			
QnrVCm F	GAGYTKTATGGTTTAGAYCCTCG		71	
QnrVCm R	TGTTCYTGYTGCCACGARCA			
RmtA F	ATGAGCTTTGACGATGCCCTA	55ºC	756	HIDALGO *et al.*, 2013
RmtA R	TCACTTATTCCTTTTTATCATG			
RmtB F	ATGAACATCAACGATGCCCT		769	
RmtB R	CCTTCTGATTGGCTTATCCA			
RmtC F	CGAAGAAGTAACAGCCAAAG		711	
RmtC R	ATCCCAACATCTCTCCCACT			
RmtD F	CGGCACGCGATTGGGAAGC		401	
RmtD R	CGGAAACGATGCGACGAT			
RmtE F	ATGAATATTGATGAAATGGTTGC		818	
RmtE R	TGATTGATTTCCTCCGTTTTTG			
RmtF F	GCGATACAGAAAACCGAAGG		589	

RmtF R	ACCAGTCGGCATAGTGCTTT			
RmtG F	AAATACCGCGATGTGTGTCC	55ºC	250	BUENO *et al.*, 2013
RmtG R	ACACGGCATCTGTTTCTTCC			
F-Bap-C	ATGCCTGAGATACAAATTATTGCCAAG GATAATC	56ºC	561	KODORI *et al.*, 2017
R-Bap-C	AGGTGCTGAAGAATCATCATTAC			
ISAba1F	GTTATATCTTATCTTAAACA	56°C		MERKIER *et al.*, 2008
ISAba1R	GCTCACCGATAAACTCTCT			
ISAba2A	AATCCGAGATAGAGCGGTTC	50°C		POIREL; NORDMANN, 2006
ISAba2B	TGACACATAACCTAGTGCAC			
ISAba3A	CAATCAAATGTCCAACCTGC			
ISAba3B	CGTTTACCCCAAACATAAGC			
ISAba4A	ATTTGAACCCATCTATTGGC			CORVEC *et al.*, 2007
ISAba4B	ACTCTCATATTTTTTCTTGG			
Tn2006 Int-P3	GTCTATCAGGAACTTGCGCG	62ºC		CHEN *et al.*, 2017
Tn2006 Int-P4	GCAAGGCTTTAGATGCAGAAGA	62ºC		
Tn2007 Int-P6	ATTTGAACCCATCTATTGGC	56ºC		
Tn2007 Int-P7	ACTCTCATATTTTTTCTTGG	56ºC		
Tn2006/8 Int-P5	GGCTCATTACAGTCAGGTACAAGT	62ºC		
Tn2009 Int-P1	ATCCTGATGCTCGCAATCGT	62ºC		
Tn2009 Int-P8	CTGTCTGCGAACACATTCAC	62ºC		
ERIC1R	GTGAATCCCCAGGAGCTTACA	40ºC	-	SILBERT *et al.*, 2004
ERIC2	AAGTAAGTGACTGGGGTGAGCG			

[a] Annealing temperature

4.2 ARTIGO B - MOLECULAR AND PHENOTYPIC CHARACTERISTICS OF A *BLA*OXA-58-CARRYING *ACINETOBACTER COLISTINIRESISTENS* BLOODSTREAM ISOLATE FROM BRAZIL

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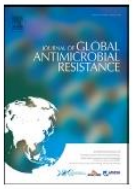

Letter to the Editor

Molecular and phenotypic characteristics of a bla_{OXA-58}-carrying *Acinetobacter colistiniresistens* bloodstream isolate from Brazil

Editor: Professor A Tsakris

Acinetobacter colistiniresistens is a cause of healthcare-associated bloodstream infections in patients with severe underlying diseases undergoing invasive medical procedures and is occasionally related to transient bacteremia [1,2]. The intrinsic colistin resistance combined with the development of resistance to relevant antimicrobials used to treat systemic infections can complicate therapy and lead to treatment failure [2]. In this species, carbapenem resistance is associated with carbapenem-hydrolyzing Class D beta-lactamase (CHDLs) ($bla_{OXA-23-like}$, bla_{OXA-58}) and metallo-β-lactamases (bla_{IMP-34}, bla_{IMP-1}) expression [2,3]. To date, *A. colistiniresistens* carrying bla_{OXA-58} has only been detected in one clinical isolate from Japan [3]. In Brazil, bla_{OXA-58} was reported in *Acinetobacter bereziniae, Acinetobacter baumannii*, and *Acinetobacter seifertii* [4,5]. Here, we report the phenotypic and molecular characteristics of a bla_{OXA-58}-carrying *A. colistiniresistens* isolated for the first time in Brazil.

In May 2015, a 48-year-old male patient was admitted to the Hospital Universitário de Londrina (Paraná state, Brazil) for diagnosis and treatment of painful oral ulcers and extensive nasal septum lesion. On admission, the patient presented with type 2 diabetes, arterial hypertension, anorexia, and weight loss in recent months. Disseminated histoplasmosis and common variable immunodeficiency were subsequently diagnosed. Itraconazole (200 mg), voriconazole (200 mg, orally, twice daily), and liposomal amphotericin B (200 mg, e.v., once a day) were prescribed. On the 17th day of hospitalization, the patient was febrile (38.6°C) and blood cultures collected were positive for Gram-negative bacillus. An improvement of clinical condition of the patient was obtained with ciprofloxacin (400 mg, twice daily) for 14 days, and the subsequent bloodstream cultures were negative. The patient was discharged after 30 days with home oral treatment with itraconazole (200 mg, 12/12 h), sulfamethoxazole-trimethoprim (400 mg-80 mg, once a day [prophylactic]), folic acid, and ferrous sulphate.

The Ac505/15, originally identified as *Acinetobacter junii* by a BD Phoenix™ Automated Microbiology system, was identified at the species level by sequencing of partial regions of the RNA polymerase β subunit (*rpo*B) gene as *A. colistiniresistens* [6]. Antimicrobial susceptibility testing was performed by disk diffusion and broth microdilution methods according to European Committee on Antimicrobial Susceptibility Testing guidelines, and the results were interpreted using the breakpoint table version 11.0 (http://www.eucast.org/clinical-breakpoints). This multidrug-resistant isolate presented resistance to amikacin, trimethoprim/sulfamethoxazole, and polymyxins (minimum inhibitory concentration of 32.0 µg/mL and ≥32.0 µg/mL for polymyxin B and colistin, respectively; Table 1). Multiplex polymerase chain reactions performed to detect class A, B, and D carbapenemases encoding genes revealed the presence of $bla_{OXA-58-like}$ [7]. Nucleotide analysis of this amplicon achieved 100% identity with known bla_{OXA-58} sequence (accession no CP033131.1).

Shotgun sequencing, performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA) using a 300-bp paired-end library, generated 4 198 572 reads (coverage of approximately 250-fold). The genome was assembled by SPAdes version 3.13.1 (using parameter-careful), generating 132 contigs. The annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline v. 4.8 (https://ncbi.nlm.nih.gov/genome/annotation_prok/). The draft genome sequence of 4 356 398 bp (with mean GC content of 41.3%, N50 value of 73 966, L50 value of 19) included 4049 coding genes and 78 RNA genes.

Comparative sequence analysis of genes encoding the RNA polymerase β-subunit and DNA gyrase subunit B [1] revealed that Ac505/15 presented identical or high similarity with concatenated sequences of *A. colistiniresistens* isolates (Supplementary Fig. S1). To assess the relationship of *A. colistiniresistens* genomes available on NCBI, the pan-genome analysis and the phylogenetic tree based on strict core gene alignment were performed using PATRIC 3.6.12 (bootstraps: 1000). In addition, average nucleotide identity based on blast (ANIb) was calculated using the JSpecies program (http://imedea.uib-csic.es/jspecies) using default settings. The *A. colistiniresistens* pan-genome of our dataset consisted of 32 535 total genes, and using their core-genome, from 1 213 425 nucleotides, a phylogenetic tree was created (Supplementary Fig. S2A). The ANIb values obtained for the 10 genomes against Ac505/15 were 98.36 for TUM15205 and TUM15258, 98.32 for TUM 15271, 98.31 for OCU_Ac7, 98.26 for TUM15188, 98.20 for TUM15240, 98.09 for NIPH2036, 97.63 for KCRI-45, 96.13 for NIPH1859, and 94.68 for NR1165 (Supplementary Fig. S2B). The values of ANIb for the comparisons between Ac505/15 and *A. colistiniresisten* strains were ≥95% for all isolates included in this analysis, which allows us to putatively classify these isolates in the same species. These results are consistent with those obtained with the phylogenetic tree based on the *A. colistiniresistens* core genome, which indicates that Ac505/15 is a close relative for which whole-genome data are available, including the representative genome (NIPH2036).

The acquired antimicrobial resistance genes were identified using the PATRIC v. 3.6.9 (https://patricbrc.org/), ResFinder (v. 4.1), KmerResistance (v. 2.2) (https://cge.cbs.dtu.dk/services/), and the sequences available in NCBI. Genomic islands and mobile elements were detected using IslandViewer4 (https://www.pathogenomics.sfu.ca/islandviewer), MobileElementFinder v. 1.0.3 (https://cge.cbs.dtu.dk/services/MobileElementFinder/), and ISfinder (https://www-is.biotoul.fr/). Genes encoding resistance to β-lactams (bla_{OXA-58}, $bla_{OXA-670}$, bla_{OXA-9}, and bla_{TEM-1A}), aminoglycosides (*aph[3′]-VIa, aac[6′]-Ib′; aac[6′]-Ij; aadA1*), phenicols (*flo*R),

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Table 1
Phenotypic and molecular characteristics of the MDR *Acinetobacter colistiniresistens* Ac505/15

	Protein/type/designation encoded
Phenotypic	
Antimicrobial susceptibility profile (MIC)	R: PB (32.0μg/mL), COL (≥32.0μg/mL), AMK, SXT; S: IPM (0.5μg/mL), MEM (1.0μg/mL), CAZ, FEP, TGC, GEN, SAM, CIP, LVX, TZP; MDR phenotype
Carbapenemase production	Negative
Biofilm formation	Weak biofilm producer
Molecular	
Resistance	OXA-58
	OXA-670 (OXA-286 family class D β-lactamase), OXA-9, TEM-1A
	Aph(3′)-Via, Aac(6')-Ib', Aac(6')-Ij, AadA1
	Sul2
	MphE, MsrE
	FloR
	AceI, CrcB
	CusA/CzcA family, CDF family Co(II)/Ni(II) efflux transporter DmeF, chromate efflux transporter ChrA, ACR3
	RND, MATE, MFS, SMR, and PACE efflux transporters (AdeA, AdeB, AdeC, MdtB/MuxB, MexI, AdeI, AdeJ, AdeK, MacB, TolC
Virulence	PgaA, PgaB, PgaC, PgaD
	OmpA
	CarO
	PilB, PilQ, PilT, Pil V, PilW, DotL
	Septicolysin (pore-forming toxin)
	TonB receptor (TonB family protein, TonB-dependent copper receptor, TonB-dependent hemoglobin/transferrin/lactoferrin family receptor, TonB-dependent receptor, TonB-dependent siderophore receptor)
	Toxin-antitoxin system (Txe/YoeB family addiction module toxin, YwqK family antitoxin, HicB, HipA, BrnA/BrnT, RelB/RelE)
	CPS biosynthesis protein, capsule assembly Wzi family protein, Wza, Wzb, Wzc
	LpxA, LpxD
	Siderophore biosynthesis and interactin protein; ferric uptake regulator family protein; ferrous iron transport protein A and B; Fe-S cluster assembly protein IscX, IscU, HscA, and HscB; iron chelate uptake ABC transporter family permease subunit; IucA/IucC family siderophore biosynthesis protein; Lípase chaperone
	PmrA, AdeR
	Secretion system (type I, II, IV, and VI) - TolC, HlyC, HlyD, F family protein, GspD, GspE, GspF, GspG, GspH, GspI, GspJ, protein M, protein B, DotU family type, TssA, TssE, TssF, TssG, TssH, TssK, TssM, TagF, Hcp, VgrG, type VI secretion system contractile sheath large and small subunit
	Hemolysin III family protein, ShlB/FhaC/HecB family hemolysin secretion/activation protein
	Phospholipase A, B, C, and D
	Septicolysin
Genetic mobile elements	Integrase, IS1, ISPa14, IS1595-like, ISAcra1, IS21, ISAba8, IS3, ISAba14, IS30, IS4, ISAba33, IS5, IS1182, ISAba23, ISAha2, ISAha3, IS6, TnpB, IS1007, IS1008, ISL3, recombinase, replication-associated recombination protein A

CPS, capsular polysaccharide; MIC, minimum inhibitory concentration.

sulphonamides (*sul2*), and macrolides (*mphE, msrE*) were detected. In addition, 44 genomic islands and 20 insertion sequences from nine families were observed. An intact *ISAba3* element upstream of bla_{OXA-58} has been associated with a lower level of resistance to imipenem compared with bla_{OXA-58} with hybrid promoters (such as IS*Aba*2, IS1, IS2, IS6 family, and IS18), which are responsible for enhancing bla_{OXA-58} expression [5,8]. In Ac505/15, the bla_{OXA-58} was flanked on either side by IS*Aba*3 as described previously [3,5], and no hybrid promoters were found, which could be correlated with the susceptibility to carbapenems and the absence of carbapanemase production presented by this isolate [5,9] (Table 1) (Supplementary Fig. S3).

Several efflux pump families were identified using CARD (v. 3.1.3), PATRIC, and the data available in NCBI (Table 1). The MdtABC multidrug efflux system and Bcr/CmlA family MFS efflux pump were associated with transposases (MdtABC with IS30 family transposase and Bcr/CmlA family MFS efflux pump with IS3 family transposase and IS5/IS1182 family transposase).

Virulence genes encoding surface adhesins (OmpA); capsular polysaccharide; phospholipases (A, C, and D); hemolysin, iron, and micronutrient acquisition systems (siderophores, zinc ABC transporter); and secretion systems (type II, IV, and IV) were detected using the Pathogenicity Island Database (http://www.paidb.re.kr), PATRIC, and the data of annotation (Table 1). Most of the elements enrolled in biofilm production described in *A. baumannii* (Wza/Wzb/Wzc complex, CsuA pili, biofilm-associated protein) were not detected in Ac505/15 and only the *pga*ABCD gene cluster was verified, which could explain the weak biofilm production of this isolate evaluated by crystal violet staining method in polystyrene plates [10–12].

Multilocus sequence typing was performed using the CGE online tool (https://cge.cbs.dtu.dk/services/MLST/). The isolate presented a novel allele, *glt*A, and therefore it belonged to a new sequence type (ST), closely related to ST198, which was previously described in an *Acinetobacter* genomic species 13BJ/14TU recovered from bloodstreams of patients at a university in South Korea [13].

Recently, the silent spread of IMP-1–producing *A. colistiniresistens* isolates was reported in our country. Here, we report the presence of bla_{OXA-58} in a carbapenem susceptible isolate. Our data emphasize the need for implementation of highly stringent control measures in order to prevent the spread of the bla_{OXA-58} to other bacterial species, as well as of *A. colistiniresistens* in the hospital environment. Accurate identification of *Acinetobacter* Spp. and the dissemination trend of *A. colistiniresistens* should be monitored to understand the clinical and microbial significance of this species in the clinical setting.

The draft genome sequence of *A. colistiniresistens* Ac505/15 has been deposited in GenBank under the accession nos.

VMTP00000000, GCA_007713425.1, PRJNA556457 for genome, assembly, and BioProject, respectively.

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Competing interests

None declared.

Ethical approval

This study was approved by the Research Ethics Committee (REC), Health Sciences Center, Universidade Estadual de Londrina (Protocol No. 14490/2009). Informed consent was waived by the REC because the study was observational and patient care was not influenced.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.01.022.

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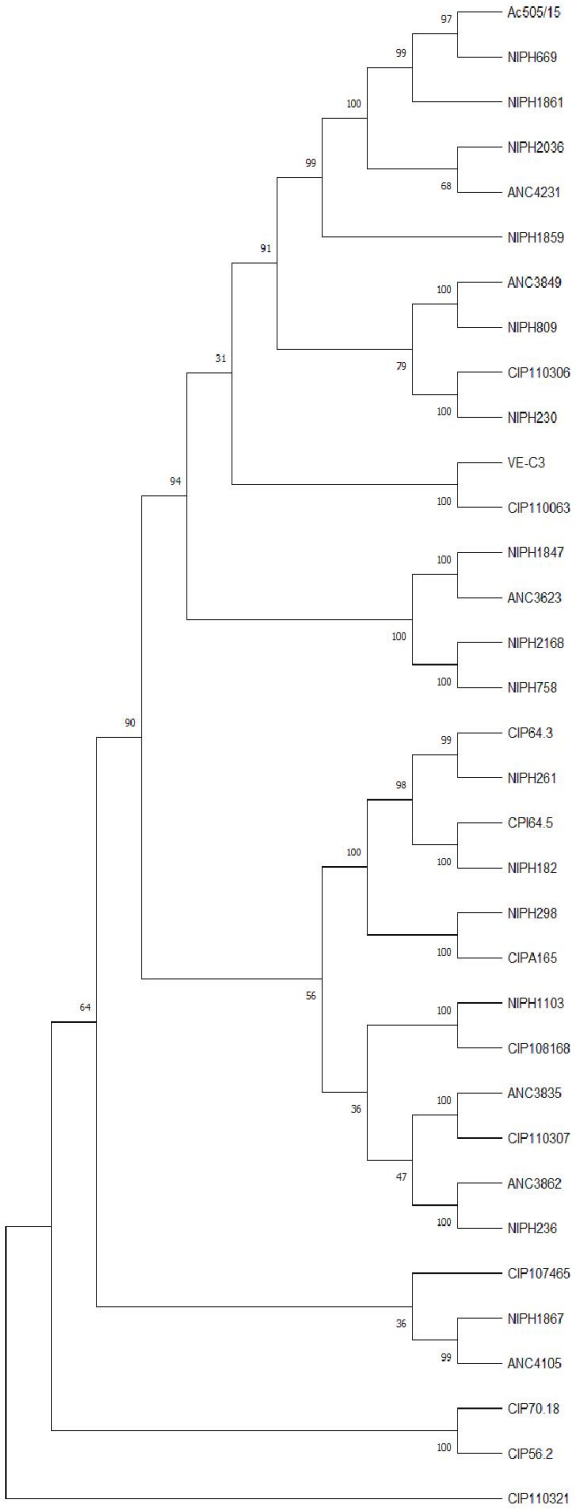

Fig 1. Analysis of 34 concatenated *rpo*B and *gyr*B sequences (1821 bp). The evolutionary history was inferred using the Neighbor-Joining method (MEGA X software). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

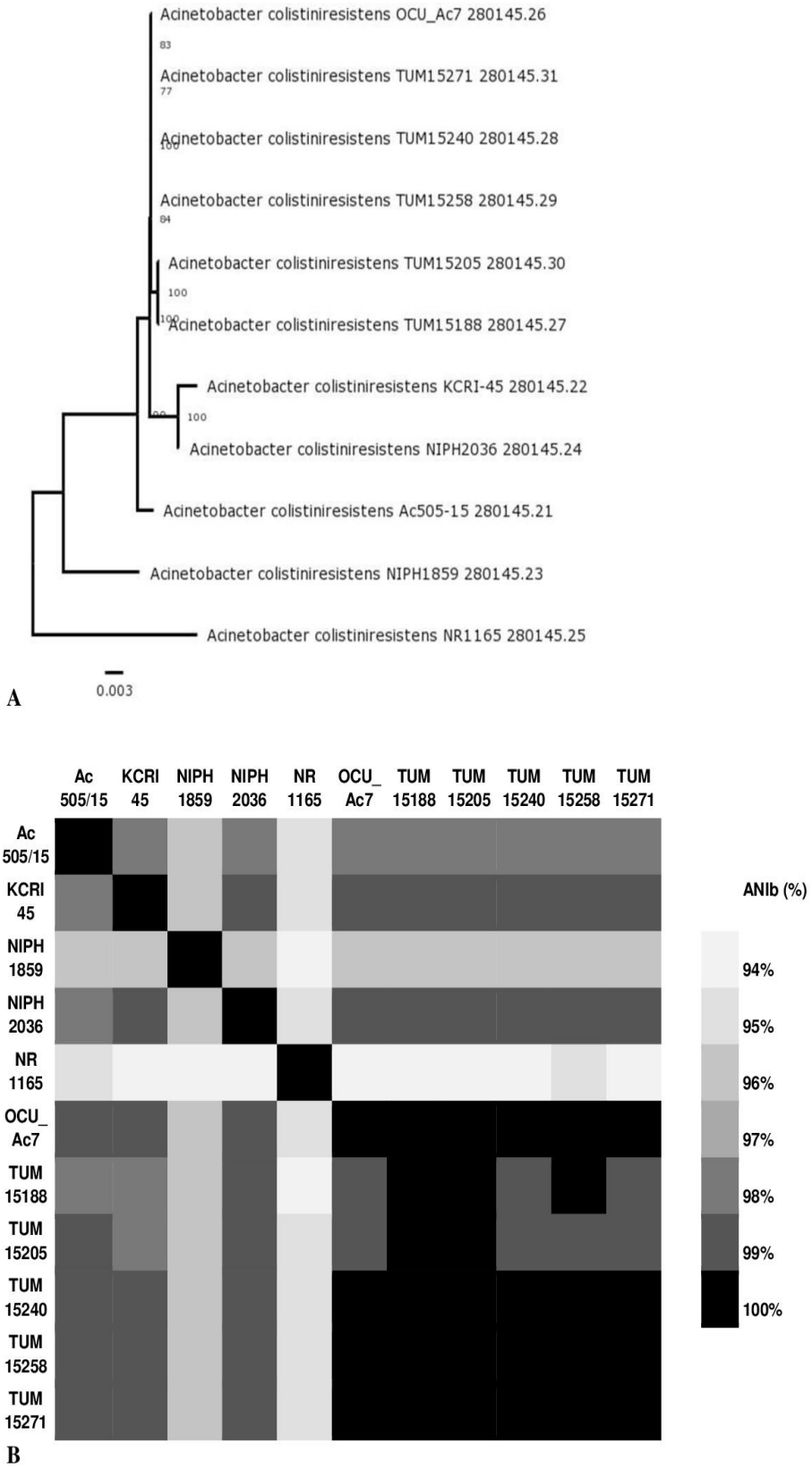

Fig 2 Phylogeny of the Ac505/15 genome and the ten *A. colistiniresistens* genomes available in the NCBI database. **A.** Phylogenetic tree based on the essential core genome, using PATRIC (v. 3.6.12). **B.** Heatmap of the Average Nucleotide Identity based on BLAST (ANIb) for each pairwise comparison. Grey color key represents the percentage of differences between genomes.

```
MK323042.1      ATAGCTCAGTGCATGATCACCAATAAACTGACGTTTACAGTCTTTGCACTGATAGTTTTG      8460
Ac505/15        ----------------TCACCAATAAACTGACGTTTACAGTCTTTGCACTGATAGTTTTG      44
                              <-----------------------------------------
                              ******************************************

MK323042.1      TTTCCCATTTACTTTGATACCATTTTTCTTTATACTATCACTGAGGCAGGTTGGACATTT      8520
Ac505/15        TTTCCCATCTACTTTGATACCATTTTTCTTTATACTATCACTGAGGCAGGTTGGACATTT      104
                ------------------------------tnpA of ISAba3---------------
                ******** **************************************************

MK323042.1      GATTGCTAGAGTTATTTGCATTTCTCTATTTTATCAAAATCCAATCGGCTTTTTCTTCAG      8580
Ac505/15        GATTGCTAGAGTTATTTGCATTTCTCTATTTTATCAAAATCCAATCGGCTTTTTCTTCAG      164
                ------------------------>
                                          -35       PAba3       -10
                ************************************************************

MK323042.1      CATACTTTTTGAAACACTACCAAATTTTAAAGTTGTATATCATGAAATTATTAAAAATAT      8640
Ac505/15        CATACTTTTTGAAACACTACCAAATTTTAAAGTTGTATATCATGAAATTATTAAAAATAT      224
                <----- IRL ISAba3 ---->
                ************************************************************

MK323042.1      TGAGTTTAGTTTGCTTAAGCATAAGTATTGGGGCTTGTGCTGAGCATAGTATGAGTCGAG      8700
Ac505/15        TGAGTTTAGTTTGCTTAAGCATAAGTATTGGGGCTTGTGCTGAGCATAGTATGAGTCGAG      284
                ************************************************************

MK323042.1      CAAAAACAAGTACAATTCCACAAGTGAATAACTCAATCATCGATCAGAATGTTCAAGCGC      8760
Ac505/15        CAAAAACAAGTACAATTCCACAAGTGAATAACTCAATCATCGATCAGAATGTTCAAGCGC      344
                ************************************************************

MK323042.1      TTTTTAATGAAATCTCAGCTGATGCTGTGTTTGTCACATATGATGGTCAAAATATTAAAA      8820
Ac505/15        TTTTTAATGAAATCTCAGCTGATGCTGTGTTTGTCACATATGATGGTCAAAATATTAAAA      404
                ************************************************************

MK323042.1      AATATGGCACGCATTTAGACCGAGCAAAAACAGCTTATATTCCTGCATCTACATTTAAAA      8880
Ac505/15        AATATGGCACGCATTTAGACCGAGCAAAAACAGCTTATATTCCTGCATCTACATTTAAAA      464
                ************************************************************

MK323042.1      TTGCCAATGCACTAATTGGTTTAGAAAATCATAAAGCAACATCTACAGAAATATTTAAGT      8940
Ac505/15        TTGCCAATGCACTAATTGGTTTAGAAAATCATAAAGCAACATCTACAGAAATATTTAAGT      524
                ************************************************************

MK323042.1      GGGATGGAAAGCCACGTTTTTTTAAAGCATGGGACAAAGATTTTACTTTGGGCGAAGCCA      9000
Ac505/15        GGGATGGAAAGCCACGTTTTTTTAAAGCATGGGACAAAGATTTTACTTTGGGCGAAGCCA      584
                ************************************************************

MK323042.1      TGCAAGCATCTACAGTGCCTGTATATCAAGAATTGGCACGTCGTATTGGTCCAAGCTTAA      9060
Ac505/15        TGCAAGCATCTACAGTGCCTGTATATCAAGAATTGGCACGTCGTATTGGTCCAAGCTTAA      644
                ************************************************************

MK323042.1      TGCAAAGTGAATTGCAACGTATTGGTTATGGCAATATGCAAATAGGCACGGAAGTTGATC      9120
Ac505/15        TGCAAAGTGAATTGCAACGTATTGGTTATGGCAATATGCAAATAGGCACGGAAGTTGATC      704
                ************************************************************

MK323042.1      AATTTTGGTTGAAAGGGCCTTTGACAATTACACCTATACAAGAAGTAAAGTTTGTGTATG      9180
Ac505/15        AATTTTGGTTGAAAGGGCCTTTGACAATTACACCTATACAAGAAGTAAAGTTTGTGTATG      764
                ************************************************************

MK323042.1      ATTTAGCCCAAGGGCAATTGCCTTTTAAACCTGAAGTTCAGCAACAAGTGAAAGAGATGT      9240
Ac505/15        ATTTAGCCCAAGGGCAATTGCCTTTTAAACCTGAAGTTCAGCAACAAGTGAAAGAGATGT      824
                ************************************************************

MK323042.1      TGTATGTAGAGCGCAGAGGGGAGAATCGTCTATATGCTAAAAGTGGCTGGGGAATGGCTG      9300
Ac505/15        TGTATGTAGAGCGCAGAGGGGAGAATCGTCTATATGCTAAAAGTGGCTGGGGAATGGCTG      884
                ************************************************************

MK323042.1      TAGACCCGCAAGTGGGTTGGTATGTGGGTTTTGTTGAAAAGGCAGATGGGCAAGTGGTGG      9360
Ac505/15        TAGACCCGCAAGTGGGTTGGTATGTGGGTTTTGTTGAAAAGGCAGATGGGCAAGTGGTGG      944
                ************************************************************

MK323042.1      CATTTGCTTTAAATATGCAAATGAAAGCTGGTGATGATATTGCTCTACGTAAACAATTGT      9420
Ac505/15        CATTTGCTTTAAATATGCAAATGAAAGCTGGTGATGATATTGCTCTACGTAAACAATTGT      1004
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MK323042.1      CTTTAGATGTGCTAGATAAGTTGGGTGTTTTTCATTATTTATAAGAATTAGAAGTTTGAG      9480
Ac505/15        CTTTAGATGTGCTAGATAAGTTGGGTGTTTTTCATTATTTATAAGAATTAGAAGTTTGAG      1064
                ************************************************************

MK323042.1      GTTAATCTATTTTTGGTAGTGTTTCAAAAAGTATGCTGAAGAAAAAGCCGATTGGATTTT      9540
Ac505/15        GTTAATCTATTTTTGGTAGTGTTTCAAAAAGTATGCTGAAGAAAAAGCCGATTGGATTTT      1124
                ************************************************************

MK323042.1      GATAAAATAGAGAAATGCAAATAACTCTAGCAATCAAATGTCCAACCTGCCTCAGTGATA      9600
Ac505/15        GATAAAATAGAGAAATGCAAATAACTCTAGCAATCAAATGTCCAACCTGCCTCAGTGATA      1184
                ************************************************************

MK323042.1      GTATAAAGAAAAATGGTATCAAAGTAGATGGGAAACAAAACTATCAGTGCAAAGACTGTA      9660
Ac505/15        GTATAAAGAAAAATGGTATCAAAGTAGATGGGAAACAAAACTATCAGTGCAAAGACTGTA      1244
                ************************************************************

MK323042.1      AACGTCAGTTTATTGGTGATCATGCACTGAGCTATCTAGGATGTAAGTCAGGTATTACTC      9720
Ac505/15        AACGTCAGTTTATTGGTGA----------------------------------------      1263
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Fig 3. The genetic environments surrounding *bla*$_{OXA-58}$. This gene was flanked by two intact IS*Aba*3 copies, this genetic context was previously observed in the pAb45063_b (sequence MK323042.1 on GenBank) described by Matos et al. (2019). The absence of hybrid promoters, suggest the lacking of a putative promoter for *bla*$_{OXA-58}$ overexpression, which could explain the susceptibility to carbapenems (MICs of 0.5 µg/mL and 1.0 µg/mL to imipenem and meropenem, respectively) and the phenotypic no-production of carbapanemase observed in Ac505/15 isolate.

Table 1. Phenotypic and Molecular Characteristics of the MDR *Acinetobacter colistiniresistens* Ac505/15

	Protein/Type/Designation encoded	Functions	Ref
Phenotypic			
Antimicrobial susceptibility profile (MIC)	R: PB (32.0µg/mL), COL (≥32.0µg/mL), AMK, SXT S: IPM (0.5µg/mL), MEM (1.0µg/mL), CAZ, FEP, TGC, GEN, SAM, CIP, LVX, TZP MDR phenotype	Antimicrobial resistance	
Carbapenemase production	Negative	Carbapenem hydrolysis	[2]
Biofilm formation capacity	Weak biofilm producer	Protection against antibiotics, host immune defense, and adverse environmental conditions.	[3, 4]
Molecular			
Resistance	OXA-58	Carbapenem resistance	[5]
	OXA-670 (OXA-286 family class D beta-lactamase), OXA-9, TEM-1A	Beta-lactam resistance	[5]
	Aph(3')-Via, Aac(6')-lb', Aac(6')-lj, AadA1	Aminoglycoside resistance	[6]
	Sul2	Sulfonamide resistance	[1]
	MphE, MsrE	Macrolide resistance	[7]
	FloR	Chloramphenicol / florfenicol resistance	[8]
	AceI, CrcB	Environmental persistence, efflux of antiseptic/ disinfectants	[3, 8]
	CusA/CzcA family, CDF family Co(II)/Ni(II) efflux transporter DmeF, Chromate efflux transporter ChrA, ACR3	Environmental persistence, efflux of heavy metals	[9]
	RND, MATE, MFS, SMR and PACE efflux transporters (AdeA, AdeB, AdeC, MdtB/MuxB, MexI, AdeI, AdeJ, AdeK, MacB, TolC	Multidrug resistance, biofilm formation	[3, 4, 5, 8]
Virulence	PgaA, PgaB, PgaC, PgaD	Protection of bacteria against innate host infection, transmembrane transporter and biofilm formation	[3, 4]
	OmpA	Antibiotic resistance, cell membrane integrity, adhesion, biofilm formation, formation of outer membranes vesicles (OMVs), cell apoptosis, complement resistance and cell invasion	[3, 10, 4]

	CarO	Carbapenem resistance, ornithine uptake	[4]
	PilB, PilQ, PilT, Pil V, PilW, DotL	Twitching motility	[3]
	Septicolysin (pore-forming toxin)	Cytolysin	[1]
	TonB receptor (TonB family protein, TonB-dependent copper receptor, TonB-dependent hemoglobin/transferrin/lactoferrin family receptor, TonB-dependent receptor, TonB-dependent siderophore receptor)	Iron uptake, host infection, bacterial survive in blood and lungs	[1]
	Toxin-antitoxin system (Txe/YoeB family addiction module toxin, YwqK family antitoxin, HicB, HipA, BrnA/BrnT, RelB/RelE)	Plasmid stabilization, regulation of growth and death under stress conditions.	[1]
	CPS biosynthesis protein, capsule assembly Wzi family protein, Wza, Wzb, Wzc	Surface colonization, biofilm formation, antimicrobial resistance, survival in adverse environments, evasion of host immune defenses	[3]
	LpxA, LpxD	LpxA, LpxC and LpxD are components of the lipid byosinthetic pathway. The absence of LpxC could explain the resistance to polymyxins.	[3, 5]
	Siderophore biosynthesis and interactin protein, Ferric uptake regulator family protein, Ferrous iron transport protein A and B, Fe-S cluster assembly protein IscX, IscU, HscA and HscB, Iron chelate uptake ABC transporter family permease subunit, IucA/IucC family siderophore biosynthesis protein, Lípase chaperone	Iron uptake	[3]
	PmrA, AdeR	Mutations in pmrAB can confer resistance to colistin. Expression regulation of the AdeABC drug efflux pump, required for biofilm formation.	[3, 5]
	Secretion system (type I, II, IV and VI) - TolC, HlyC, HlyD, F family protein, GspD, GspE, GspF, GspG, GspH, GspI, GspJ, Protein M, Protein B, DotU family type, TssA, TssE, TssF, TssG, TssH, TssK, TssM, TagF, Hcp, VgrG, type VI secretion system contractile sheath large and small subunit	Secretion of hemolysin, lipases and proteases, required for pathogenesis. Mediate horizontal gene transfer. Transport of effector proteins into eukaryotic host cells or other bacteria. Bacterial competition, control of composition of bacterial populations	[3, 10]

	Hemolysin III family protein, ShlB/FhaC/HecB family hemolysin secretion/activation protein	Attack of erythrocyte membranes and cause cell rupture	[21]
	Phospholipase A, B, C and D	Phospholipids hydrolysis, the lysis of host cell membrane	[3]
	Septicolysin	Cytolysin, and invasion of tissues and cells	[1]
Genetic mobile elements	Integrase, IS1, ISPa14, IS1595-like, ISAcra1, IS21, ISAba8, IS3, ISAba14, IS30, IS4, ISAba33, IS5, IS1182, ISAba23, ISAha2, ISAha3, IS6, TnpB, IS1007, IS1008, ISL3, recombinase, replication-associated recombination protein A	Acquisition and mobilization of genes	[4, 7]

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5 CONCLUSÃO GERAL

De modo geral, observamos que, no período avaliado, entre os membros do complexo ACB, *A. baumannii*, segundo patógeno ESKAPE mais frequente no HU, foi responsável por uma diversidade de infecções na instituição, acometendo, principalmente, indivíduos do sexo masculino e com idade avançada. Estes isolados apresentaram os genes codificadores das carbapenemases OXA-23 e OXA-143, bem como elevadas taxas de resistência à diferentes classes de antimicrobianos, sendo classificados, em sua maioria, como MR e ER. O gene *bla*$_{\text{OXA-23-like}}$, amplamente distribuído nos isolados avaliados, foi, frequentemente, associado aos elementos genéticos móveis Tn*2008* e Tn*2006*. Isso pode ter contribuído para o aumento da incidência de isolados de *A. baumannii* resistentes aos carbapenêmicos em nossa instituição.

Os NBA, embora menos frequentes que os microrganismos do complexo ACB, apresentaram isolados portadores de importantes determinantes de resistência, como o gene *bla*$_{\text{OXA-58}}$, identificado nas espécies *A. bereziniae* e *A. colistiniresistens.* A análise do contexto genético revelou que todos os isolados apresentaram IS*Aba*3 a jusante do gene *bla*$_{\text{OXA-58}}$, e os três isolados com IS*Aba*1, IS*Aba*3 e IS*Aba*125-ΔIS*Aba*3 a montante foram classificados como MR. Estes isolados MR, pela tipagem molecular por ERIC-PCR, foram agrupados em três clusters individuais (I, II e III). O grupo IV, composto por três isolados de *A. bereziniae* relacionados clonalmente, compartilhou características genotípicas e fenotípicas, que incluíam a forte formação de biofilme. Apesar da ausência do gene *bap*, todos os isolados OXA-58 NBA foram capazes de formar biofilme.

A detecção de isolados sensíveis aos carbapenêmicos portadores do gene *bla*$_{\text{OXA-58}}$ ressalta a dificuldade na detecção deste determinante de resistência, uma vez que estudos moleculares para a detecção de genes codificadores de CHDL, geralmente, são realizados em isolados que apresentam resistência aos carbapenêmicos. Além disso, a presença deste gene na espécie *A. colistiniresistens,* que apresenta sensibilidade reduzida às polimixinas, representa uma grande preocupação para a saúde pública, devido limitação das opções terapêuticas disponíveis para o tratamento de infecções por esses patógenos. Estes achados, reforçam a importância clínica das espécies de NBA como responsáveis por IRAS e, sobretudo, como reservatórios de determinantes de resistência. Demonstrando,

assim, a necessidade da identificação correta das espécies de *Acinetobacter* e a realização de vigilância epidemiológica a fim de evitar a disseminação do gene *bla*$_{\text{OXA-58}}$ e outros determinantes de resistência para espécies mais virulentas presentes no ambiente hospitalar, como *A. baumannii,* bem como determinar o papel epidemiológico desempenhado por estas espécies no cenário clínico.

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APÊNDICES

APÊNDICE A - CLINICAL AND ENVIRONMENTAL ISOLATES OF *PSEUDOMONAS* SPP. CARRYING *BLA*IMP-16 GENE: VIRULENCE FACTORS AND RESISTANCE MECHANISMS

Clinical and environmental isolates of *Pseudomonas* spp. carrying *bla*IMP-16 gene: virulence factors and resistance mechanisms

Isolados clínicos e ambientais de *Pseudomonas* spp. portadores do gene *bla*IMP-16: fatores de virulência e mecanismos de resistência

ABSTRACT

Antimicrobial resistance is a worldwide public health problem responsible for difficult-to-treat infections, increased morbidity and mortality, and high hospital costs. The antimicrobial resistance genes are emerging pollutants from the environment. The effluents are hotspots for horizontal transfer of resistance genes, favoring the dissemination among bacterial species. Thus, this study characterized six isolates of *Pseudomonas* spp. carrying the *bla*IMP-16 gene: two recovered from hospitalized patients at the Hospital Universitário de Londrina (HU) and four from Municipal Southern Region Sewage Treatment Plants.

Objective: Determine the profile of antimicrobial resistance; detect genes encoding virulence factors; and characterize phenotypic expression of these factors in clinical and environmental isolates of *Pseudomonas* spp. carrying the *bla*IMP-16 gene.

Results: Genotypic and phenotypic detection of virulence factors was predominant in clinical isolates compared to environmental isolates. By PCR, 50.0% of isolates presented codifying virulence genes as alginate, elastase, hemolytic phospholipase C, exotoxin A and Y, and the quorum sensing component. The genes encoding the alkaline protease (33.3%), exotoxin U (33.3%), and exotoxin S (16.7%) were less frequently observed. All isolate formed biofilms on polystyrene. In agar enriched with milk casein, gelatin, and ram blood, the production of proteases (50.0%, n=3), gelatinase (33.3%, n=2), and hemolysins (50.0%, n=3) was detected, respectively.

Conclusion: The detection of important virulence factors in multiresistant (MR) and/or extensively resistant (ER) isolates recovered from the hospital and the environment reinforce the need to monitor these pathogens under the One Health approach to improve the capacity to prevent, control, and treat severe infections in humans and animals, and to avoid public health risks.

Keywords: Carbapenemases, Effluent, IMP-16, *Pseudomonas* spp.

RESUMO
A resistência aos antimicrobianos é um problema de saúde pública mundial responsável por infecções de difícil tratamento, aumento da morbimortalidade e altos custos hospitalares. Os genes de resistência antimicrobiana são poluentes emergentes do meio ambiente. Os efluentes são *hotspots* para a transferência horizontal dos genes de resistência, favorecendo a disseminação entre espécies bacterianas. Assim, este estudo caracterizou seis isolados de *Pseudomonas* spp. portadores do gene *bla*$_{\text{IMP-16}}$: dois recuperados de pacientes internados no Hospital Universitário de Londrina (HU) e quatro de Estações de Tratamento de Esgotos Municipal da Região Sul.
Objetivo: Determinar o perfil de resistência aos antimicrobianos; detectar os genes de fatores de virulência; e caracterizar a expressão fenotípica desses fatores em isolados clínicos e ambientais de *Pseudomonas* spp. portadores do gene *bla*$_{\text{IMP-16}}$.
Resultados: A detecção genotípica e fenotípica de fatores de virulência foi predominante em isolados clínicos em relação aos isolados ambientais. Pela PCR, 50,0% dos isolados apresentaram genes codificadores de virulência como alginato, elastase, fosfolipase C hemolítica, exotoxina A e Y, e o componente *quorum sensing*. Os genes que codificam a protease alcalina (33,3%), exotoxina U (33,3%) e exotoxina S (16,7%) foram observados com menor frequência. Todos os isolados formaram biofilmes em poliestireno. Em ágar enriquecido com caseína de leite, gelatina e sangue de carneiro foi detectado a produção de proteases (50,0%, n=3), gelatinase (33,3%, n=2) e hemolisinas (50,0%, n=3), respectivamente.
Conclusão: A detecção de importantes determinantes de virulência em isolados multirresistentes (MR) e/ou extensivamente resistentes (ER) recuperados do hospital e do ambiente reforça a necessidade de monitorar esses patógenos sob a abordagem *One Health* para melhorar a capacidade de prevenir, controlar e tratar infecções graves em humanos e animais, e para evitar riscos para à saúde pública.

Palavras chave: Carbapenemases, Efluente, IMP-16, *Pseudomonas* spp.

1 INTRODUCTION

Antimicrobial resistance (AR) is a worldwide public health problem due to its emergence and spread on all continents. Also, it is responsible for difficult-to-treat infections and is associated with high rates of morbidity, mortality, and health care costs (PAHO, 2017). Studies have associated the increase in AR with the extensive and/or inappropriate use of antimicrobial in agriculture, livestock, and human and veterinary medicine. The spread of AR is not restricted to health care units, once AR bacteria were identified in effluents (EF) and urban sewage treatment plants (STP) (TURANO *et al.*, 2016; KARKMAN *et al.*, 2018; SAKKAS *et al.*, 2019; ORY *et al.*, 2019). These aquatic environments contain a high bacterial load and compounds such

as antibiotics, disinfectants, heavy metals, and biocides, which exert selective pressure for AR even at low concentrations, and enabling the dissemination of AR genes among bacterial species. Thus, EF and STP are hotspots for horizontal gene transfer (TURANO *et al.*, 2016; KARKMAN *et al.*, 2018; SAKKAS *et al.*, 2019; WANG; WANG; YANG, 2018).

Pseudomonas aeruginosa is a non-fermenting Gram-negative bacillus belonging to the ESKAPE (*Enterococcus faecium*, Staphylococcus aureus, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) group, widely disseminated in nature, found in aquatic environments and soil, and colonizing plants, animals, and humans. It is an important human opportunistic pathogen responsible for a variety of acute and chronic infections, mainly in immunosuppressed individuals, with cystic fibrosis and users of Intensive Care Units (BÉDARD; PRÉVOST; DÉZIEL, 2016; KARIMINIK; BASERI-SALEHI; KHEIRKHAH, 2017; PACHORI; GOTHALWAL; GANDHI, 2019; LOPES *et al.*, 2020).

The pathogenicity of *P. aeruginosa* is due to its metabolic versatility, a large repertoire of virulence determinants, a complex regulatory network of intracellular and intercellular signals, and intrinsic, acquired, and/or adaptive AR. These characteristics allow this pathogen to adapt and thrive under conditions adverse, as well as provide resistance to many classes of antimicrobial, leading to an increase in pseudomonal infections and, possibly, to therapeutic failures (KLOCKGETHER; TÜMMLER, 2017; MORADALI; GHODS; REHM, 2017; LUPO; HAENNI; MADEC, 2018; AZAM; KHAN, 2019).

The ability of *P. aeruginosa* to resist multiple antimicrobials, especially carbapenems, which are drugs of choice for the treatment of serious infections caused by this pathogen, has made the treatment of infections caused by this microorganism a big challenge. Isolates of carbapenem-resistant *P. aeruginosa* were detected in human, animal, and environmental samples, including food, surface water, and effluents (urban and hospital) (AHMED *et al.*, 2019; ORY *et al.*, 2019).

Although resistance to carbapenems in *P. aeruginosa* is mainly associated with the *opr*D gene, enzymes with the hydrolyzing activity of these antimicrobial, denominated carbapenemases also play an important role (LUPO; HAENNI; MADEC, 2018; ORY *et al.*, 2019). Metallo-beta-lactamases (MBL) is one of the leading carbapenems resistance mechanisms among Gram-negative bacilli and the carbapenemases most frequently reported in *Pseudomonas* spp., especially the

enzymes IMP and VIM (MORADALI; GHODS; REHM, 2017; LUPO; HAENNI; MADEC, 2018; KHUNTAYAPORN *et al.*, 2019; ORY *et al.*, 2019; BALLABEN *et al.*, 2021).

The first IMP-type MBL (*bla*$_{IMP-1}$) was described in 1988, in Japan, in an isolated of *P. aeruginosa* (WATANABE *et al.*, 1991). Currently, reported 97 variant IMP enzymes in different bacterial species worldwide (BALLABEN *et al.*, 2021). The IMP-16 variant was first described in Brazil in a clinical isolate of *P. aeruginosa* recovered in 2002. These isolate presented resistance to all beta-lactam tested and demonstrated a general preference for cephalosporins and carbapenems (MENDES *et al.*, 2004). This gene was occasionally reported in clinical isolates of *P. aeruginosa*, in São Paulo, Paraná and Santa Catarina, and in the species *P. putida* and *P. stutzeri* in Rio de Janeiro (MARRA *et al.*, 2006; CARVALHO-ASSEF *et al.*, 2010; SCHEFFER *et al.*, 2010; PICÃO *et al.*, 2012).

The Laboratório Especial de Microbiologia Molecular e Resistência aos Antimicrobianos (LEMMRA), located in the Ambulatório de Especialidades do Hospital Universitário de Londrina (AEHU), in Londrina city, Paraná State, Southern Brazil, belong to the Universidade Estadual de Londrina. This AR surveillance laboratory started to investigate the production of carbapenemases in clinical isolates of *Pseudomonas* spp. in 2002 and in 2012 it began to study environmental isolates under a One Health approach. In particular, this study aimed to evaluate the phenotypic and genotypic characteristics of six *Pseudomonas* spp. isolates producing the IMP-16 enzyme, recovered in the 2012-2013 period, to infer their main potential impacts on public health and control and treatment of animal and human infections.

2 METHODOLOGY

2.1 BACTERIAL ISOLATES, IDENTIFICATION, AND PROFILE OF SUSCEPTIBILITY TO ANTIMICROBIALS

This study included two clinical and four environmental isolates of *Pseudomonas* spp. carrying the *bla*$_{IMP-16}$ gene, recovered from hospitalized patients at Hospital Universitário de Londrina (HU) and from STPs of Londrina, respectively. Two environmental isolates were obtained from effluent samples collected from STP-Limoeiro, that receive the effluents discharged from the South region (where the HU is localized) and the Central region of the city of Londrina. The other two environmental

samples were obtained from the affluent of the STP in the Southern region (STP-South), where the sewage from STP-Limoeiro and other parts of the city find, and the treatment is carried out. Samples were collected at two different points in the affluent of the STP- South.

The isolates were previously identified by conventional biochemical tests and by the automated system Vitek 2® (bioMérieux, Craponne, France) in the Laboratory of Clinical Microbiology at HU. The molecular identification was confirmed by amplification of the 16S rDNA according to Spilker *et al.* (2004).

The evaluation of antimicrobial susceptibility was performed using the disk diffusion method and automated systems. The results were analyzed and interpreted according to the CLSI (documents M07-A9 and M100-S28). The isolates were classified as multidrug-resistant (MR) or extensively resistant (ER) according to the definitions proposed by Magiorakos *et al.* (2012).

2.2 DETECTION OF VIRULENCE FACTORS

The virulence encoding genes *alg*D (alginate), *apr*A (alkaline protease), *las*B (elastase), *plc*H (hemolytic phospholipase), *exo*S (exotoxin S), *exo*U (exotoxin U), *exo*Y (exotoxin Y), *tox*A (exotoxin A) e *las*I (quorum sensing autoinduter) were detected by PCR as previously described (AJAYI *et al.*, 2003; LANOTTE *et al.*, 2004; WOLSKA; SZWEDA, 2009; COTAR *et al.*, 2010; MITOV; STRATEVA; MARKOVA, 2010; SABHARWAL *et al.*, 2014).

2.3 PHENOTYPIC DETECTION OF CARBAPENEMASE, METALLO-BETA-LACTAMASE AND VIRULENCE FACTORS

In Mueller-Hinton agar, the isolates were cultured previously at 37 °C for 18h. Cell density was adjusted to $1x10^7$ UFC/mL in Mueller-Hinton broth, and this standard inoculum was used in the tests. The experiments were carried out in a triplicate on three different occasions, except when specified. *P. aeruginosa* PA01 was included in the tests.

2.3.1 CARBAPENEMASE PRODUCTION DETECTION

The carbapenemase production was analyzed by Blue Carba Test (PIRES; NOVAIS; PEIXE, 2013). A loop (approximately 5µl) of each isolate was suspended in 100µL of two solutions: test (aqueous solution of bromothymol blue at 0.04%, adjusted

to pH 6.0, 0.1mmol/liter ZnSO4, and 3mg/mL of imipenem, pH 7.0) and negative control (aqueous solution of bromothymol blue at 0.04%, pH 7.0). The solutions were incubated at 37°C for 2h. The carbapenemase production was observed when the negative control solution remained green or blue and the test solution changed its color to yellow or green. Isolates with blue or green color in both solutions was considered non-carbapenemase producers. The experiment was carried out in triplicate.

2.3.2 METALLO-BETA-LACTAMASE PRODUCTION DETECTION

The detection of MBL production was performed by the Double-Disk Synergy Test (DDST) (PICÃO *et al*., 2008). The synergy test was performed using commercially supplied Kirby-Bauer disks of imipenem (10µg), meropenem (10µg) and ceftazidime (30µg). In Muller-Hinton agar plate containing antibiotic disks and a filter disk containing an MBL inhibitor was inoculated the standard suspension, as recommended by CLSI. The antibiotic disks were aligned around disks containing the MBL inhibitors EDTA (100mM) and 2-mercaptopropionic acid at a center-to-center distance of 1.5 and 2.5cm, respectively. The plate was incubated at 37°C overnight. The expansion of the growth-inhibitory zone or appearance of a phantom zone between the antibiotic disks and the disk containing an MBL inhibitor was considered highly suggestive of MBL production.

2.3.3 PROTEASE PRODUCTION DETECTION

Protease production was evaluated using the protocol described by Sokol, Ohman, and Iglewski (1979). The standard inoculum (10µL) was added to the surface of the medium containing milk casein (2.0%), followed by incubation for 24 hours at 37 °C. Proteolytic activity was observed in the presence of a clear zone around the colony.

Colonies of each isolate were also inoculated in tubes containing nutrient agar supplemented with gelatin (12.0%) (Becton, Dickinson and Company, Sparks, USA) and incubated at 37 °C for up to 7 days. After refrigeration at 4 °C/1h, the presence of a liquid medium indicated proteolytic activity (CRUZ; TORRES, 2014).

2.3.4 DETECTION OF HEMOLYTIC ACTIVITY

Hemolytic activity in blood agar plates was evaluated by sowing 10µL of the standard inoculum on the surface of the agar medium supplemented with 5.0% of sheep erythrocytes. The plates were incubated for 24h at 37 °C, and the presence of a translucent halo around the inoculum indicated hemolytic activity (GEORGESCU *et al.*, 2016).

2.3.5 BIOFILM FORMATION

The biofilm production capacity was evaluated by adding 200µL of the standard suspension to a flat-bottomed 96-well polystyrene microtiter plate. The plates were incubated for 24h at 37 °C without shaking. After the incubation period, planktonic cells were removed, and the sessile cells were fixed with cold methanol for 15 minutes and then stained with violet crystal solution (1.0%). After washing and drying the plates, the violet crystal was resuspended using ethanol-acetone (80:20 v/v), and the suspension was transferred to a new plate for reading on a spectrophotometer at 570 nm (GEORGESCU *et al.*, 2016). The experiments were carried out in quintuplicate on three different occasions. Biofilm formation was interpreted according to the criteria established by Stepanovic and collaborators (2000).

2.4 MOLECULAR TYPING

The clonality of the *bla*$_{\text{IMP-16}}$ gene carrier isolates was determined using the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) technique according to Versalovic, Koeuth, and Lupski (1991). The amplicons were analyzed on 2.0% agarose gels stained with ethidium bromide. Fragments profiles were analyzed using GelJ software version 1.0. Those that showed ≥93.0% genetic similarity were considered to be clonally related isolates.

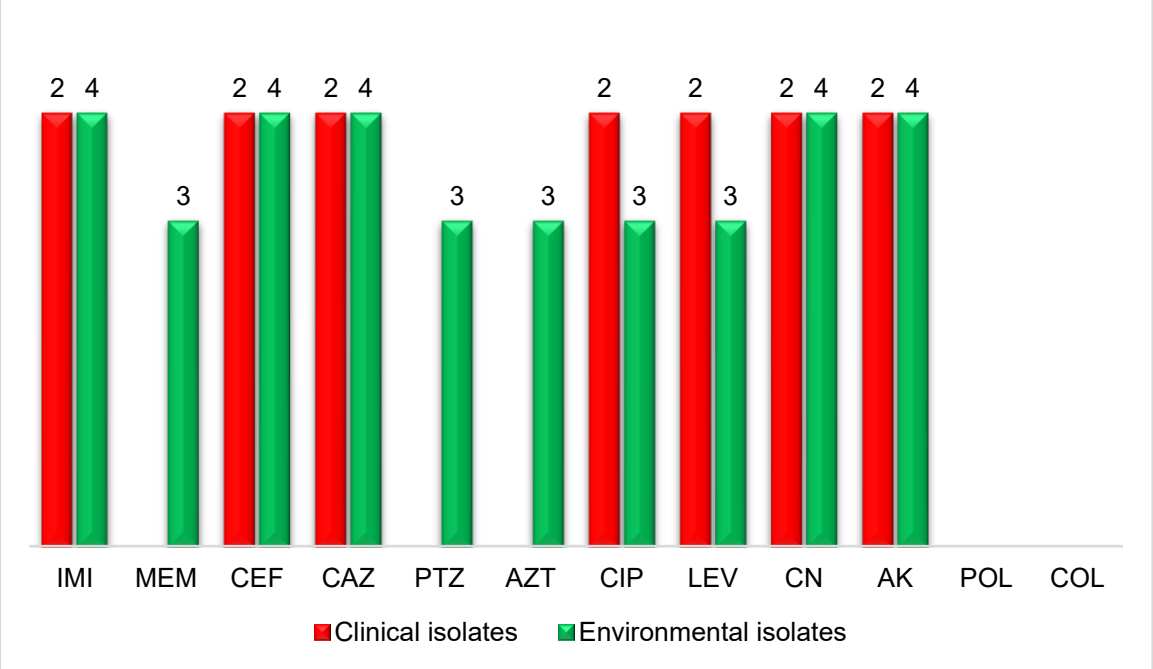
3 RESULTS AND DISCUSSIONS

The six isolates included in this study were identified by amplification and sequencing of 16S rRNA as *P. aeruginosa* (4 - 66.6%), *P. putida* (1 - 16.7%), and *P. mendocina* (1 -16.7%) and characterized as producers of carbapenemases and carriers of *bla*$_{\text{IMP-16}}$ gene. The two clinical isolates of *P. aeruginosa* were recovered from male individuals and obtained from urine and surveillance swabs, respectively.

The four environmental isolates were obtained from samples collected at STP-Limoeiro and STP-South. The diversity of *Pseudomonas* species carrying the gene encoding IMP-16 isolated in the environment suggests the interaction of environmental microorganisms with clinical isolates carrying *bla*$_{\text{IMP-16}}$, with possible horizontal gene transfer through mobile genetic elements, since resistant bacteria can be released directly into hospital and/or community sewage systems. These mobile genetic elements can still carry other genes encoding AR, contributing to the accumulation of resistance determinants, further limiting antimicrobial therapy.

The antimicrobial susceptibility test showed high resistance rates for most of the evaluated antimicrobials, including carbapenems, cephalosporins, aminoglycosides, and fluoroquinolones. Polymyxins were active against all pathogens (Figure 1). Also, clinical isolates showed sensitivity to aztreonam, meropenem, and piperacillin-tazobactam, while effluent samples showed high rates of resistance the these antimicrobials. Of the total isolates, 2 clinical and 2 environmental (66.0%) were classified as MDR and 2 environmental (33.3%) as XDR (Table 1). Additionally, the production of carbapenemase and MBL was observed in all isolates that associated with the imipenem resistance profile, suggests the expression of the *bla*$_{\text{IMP-16}}$ gene reported in these pathogens (Table 1).

Figure 1 Rates of resistance to antimicrobials used in clinical practice to treat infections caused by *Pseudomonas* spp.



IMI, imipenem; MEM, meropenem; CEF, cefepime; CAZ, ceftazidime; PTZ, piperacillin-tazobactam; AZT, aztreonam; CIP, ciprofloxacin; LEV, levofloxacin; CN, gentamicin; AK, amikacin; POL, polymyxin B; COL, colistin.

Source: Research data

Table 1 Epidemiological, phenotypic and molecular characteristics of the clinical and environmental isolates of *Pseudomonas* spp. carrying *bla*$_{IMP-16}$ gene.

	Pa2665	Pa2792	Ef26	Ef27	Ef63	Ef673
Collection location	HU	HU	STP-Limoeiro	STP-Limoeiro	STP-South	STP-South
Bacterial species	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*P. mendoncina*	*P . putida*	*P. aeruginosa*
Resistance profile	MDR	MDR	MDR	MDR	XDR	XDR
Phenotupic: Carbapenemase	■	■	■	■	■	■
Phenotupic: MBL	■	■	■	■	■	■
Phenotupic: Gelatinase	■		■			
Phenotupic: Protease	■	■	■			
Phenotupic: Hemolysin	■	■	■			
Phenotupic: Biofilm	+++	++	+++	+	++	++
Molecular detection: *Las*I	■	■	■			
Molecular detection: *Alg*D	■	■	■			
Molecular detection: *Plc*H	■	■	■			
Molecular detection: *apr*A		■	■			
Molecular detection: *Las*B	■	■	■			
Molecular detection: *Tox*A	■	■	■			
Molecular detection: *Exo*Y	■	■	■			
Molecular detection: *Exo*S			■			
Molecular detection: *Exo*U	■	■				
ERIC-PCR cluster	A	A	B	C	D	E

HU, Hospital Universitário de Londrina; STP-Limoeiro, Sewage Treatment Plant-Limoeiro; STP-South; Sewage Treatment Station in the Central and South of Londrina; MDR, multidrug-resistant to antimicrobials; XDR, extensively resistant to antimicrobials; MBL, metallo-beta-lactamase; Gaps in black, positive; +, weak biofilm producer; ++, moderately biofilm producer; +++, strong biofilm producer; ERIC-PCR, Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction.

Source: Research data

Similar results were observed in other studies carried out with clinical and environmental isolates of *P. aeruginosa* carrying the *bla*$_{IMP-16}$ gene (MARRA *et al.*, 2006; CARVALHO-ASSEF *et al.*, 2010; SCHEFFER *et al.*, 2010; MIRANDA *et al.*,

2015). A study performed at HU reported two clinical isolates with *bla*$_{IMP-16}$ gene resistant to imipenem and susceptible to meropenem recovered of HU patients approximately ten-years before the recovery of the isolates in this study (PICÃO *et al.*, 2012). Santoro, Romão, and Clementino (2012), however, reported higher resistance rates for clinical isolates of *P. aeruginosa* compared to isolates obtained from hospital wastewater.

The molecular typing of *bla*$_{IMP-16}$-harboring isolates revealed the presence of five genetic groups (A-E) (Table 1). The clinical isolates were included in cluster A, and the environmental isolates in the individual clusters B, C, D and E. Genes associated with virulence were detected only in clinical isolates Pa2665 and Pa2792 and in environmental isolate Ef26. In these isolates, the genes *exo*Y, *las*B, *plc*H, *alg*D, *tox*A, and *las*I were detected. The *apr*A, *exo*U, and *exo*S genes were detected in 33.3%, 33.3%, and 16.7% of the isolates, respectively (Table 1). Three environmental isolates did not present any of the evaluated virulence genes. The production of hemolysin and protease was observed in 3 isolates (50.0%). A total of 2 isolates (33.3%) were gelatinase producers (Table 1). All isolates were able to form biofilm on an abiotic surface, of which 1 (16.7%), 3 (50.0%), and 2 (33.3%) isolates were considered weak, moderate, and strong biofilm producers, respectively (Table 1).

Our findings show that environmental isolates presented fewer virulence factors than the clinical isolates but do not rule out the possibility that these isolates are similar to clinical isolates responsible for difficult-to-treat infections. Furthermore, the observation of AR in environmental isolates that represent densely populated areas highlights the importance of greater public health awareness. The limitation of this study was the small number of isolates, but our findings provide basic knowledge about the AR and virulence characteristics of *Pseudomonas* spp.

4 CONCLUSION

To date, the determinants of virulence, antibiotic resistance, metabolic features conferring survival, and host-associated pathogenic potential of *P. aeruginosa* are extensively investigated, but, other species of this genus remain underexplored. The analysis of resistance genes and virulence determinants of clinical and environmental isolates of *Pseudomonas* spp. improves our understanding of the potential for pathogenicity of these microorganisms. Taken together, these analyzes provide

insight into their interactions with the environment, other microorganisms, and eukaryotic hosts. Additionally, antimicrobial susceptibility surveillance assists in monitoring and preventing the spread of pathogenic MDR strains of *Pseudomonas* spp.

As seen, MDR isolates of *Pseudomonas* spp. carrying virulence factors are not limited to health care units but are also found in aquatic environments as effluents. Hospital effluents consist of a reservoir of bacteria and resistance genes that can contribute to AR due to favoring lateral gene transfer. Studies on the efficiency of effluent treatment plants show that neither primary nor secondary treatment can effectively drop MDR bacteria. These bacteria carrying resistance genes in effluents and surface waters can spread and persist in the environment, creating a stable reservoir of AR and a constant risk to the health of humans and animals. Further large-scale studies of clinical and environmental isolates of different species of *Pseudomonas* are needed to validate the virulence factors and antibiotic resistance genes of these potentially opportunistic pathogens and will provide a baseline for further studies leading to an in-depth understanding of molecular mechanisms of the pathogenesis of *Pseudomonas* spp. Thus, this research emphasizes the need to create measures to control and monitor the discharge of hospital effluents, to minimize the spread of MDR pathogens to external environments.

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APÊNDICE B- EVALUATION OF RESISTANCE AND VIRULENCE DETERMINANTS IN *BLA*GES-5-CARRYING *PSEUDOMONAS AERUGIONOSA* ISOLATED FROM ENVIRONMENTAL AND CLINICAL SAMPLES

Evaluation of resistance and virulence determinants in *bla*GES-5-carrying *Pseudomonas aerugionosa* isolated from environmental and clinical samples

Avaliação de determinantes de resistência e virulência em *Pseudomonas aerugionosa* portadoras de *bla*GES-5 isoladas de amostras ambientais e clínicas

ABSTRACT

Antimicrobial resistance (AR) is a global public health problem that threatens therapeutic efficacy and tends to increase the severity, incidence, and costs of bacterial infections. The antimicrobials and genes encoding AR are considered emerging pollutants of the environment, thus contributing to the emergence of multidrug-resistant bacteria.

Objective: Determine antimicrobial resistance profile, detect the presence of genes encoding antimicrobial resistance and virulence factors, as well as the phenotypic detection of these factors in *P. aeruginosa* isolates carrying the *bla*GES-5 gene, recovered in the period 2012 to 2016.

Results: All isolates were classified as extensively resistant to antimicrobials. High resistance rates were observed to carbapenems, fluoroquinolones, aminoglycosides, monobactams, cephalosporins, and penicillin associated with an inhibitor of beta-lactamase. The genes *bla*GES, *alg*D, *las*B, *plc*H, *apr*A, *las*I, *exo*A, *exo*Y, and *exo*S were detected. All isolates (100.0%) showed the capacity to produce proteases and hemolysins. But, gelatinase production was not observed. By enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), five genetic groups were identified (A-E), of which the E group was composed of an environmental isolate and two clinical ones, which, besides genetic similarity, shared considerable similarities about the phenotypic and molecular characteristics evaluated.

Conclusion: Our findings suggest the contribution of effluents as a reservoir of resistant pathogens in the aquatic environment, thus constituting an environmental and public health risk.

Keywords: Carbapenem-Resistant Enterobacteriaceae. Sewage. Virulence factors. *Pseudomonas aeruginosa*.

RESUMO

A resistência aos antimicrobianos (RAM) é um problema de saúde pública global que ameaça a eficácia terapêutica e tende a aumentar a gravidade, a incidência e os custos das infecções bacterianas. Os antimicrobianos e genes que codificam a RAM são considerados poluentes emergentes do meio ambiente, contribuindo assim para o surgimento de bactérias multirresistentes.

Objetivo: Determinar o perfil de resistência antimicrobiana, detectar a presença de genes codificadores de resistência antimicrobiana e fatores de virulência, bem como

a detecção fenotípica desses fatores em isolados de *P. aeruginosa* portadores do gene *bla*$_{GES-5}$, recuperados no período de 2012 a 2016.
Resultados: Todos os isolados foram classificados como extensivamente resistentes aos antimicrobianos. Altas taxas de resistência foram observadas aos carbapenêmicos, fluoroquinolonas, aminoglicosídeos, monobactâmicos, cefalosporinas e penicilinas associadas a um inibidor da beta-lactamase. Os genes *bla*$_{GES}$, *alg*D, *las*B, *plc*H, *apr*A, *las*I, *exo*A, *exo*Y e *exo*S foram detectados. Todos os isolados (100,0%) apresentaram capacidade de produzir proteases e hemolisinas. Porém, a produção de gelatinase não foi observada. Por *enterobacterial repetitive intergenic consensus-polymerase chain reaction* (ERIC-PCR) foram identificados cinco grupos genéticos (A-E), sendo o grupo E composto por um isolado ambiental e dois clínicos, que, além da similaridade genética, compartilhavam consideráveis semelhanças quanto às características fenotípicas e moleculares avaliadas.
Conclusão: Nossos achados sugerem a contribuição dos efluentes como reservatório de patógenos resistentes no ambiente aquático, constituindo assim um risco ambiental e de saúde pública.

Palavras chave: Enterobacteriacea Resistentes a Carbapenêmicos. Esgotos. Fatores de virulência. *Pseudomonas aeruginosa*

1 INTRODUCTION

Antimicrobial resistance (AR) is a worldwide public health problem that threatens therapeutic efficacy and increase the severity, incidence, and costs of bacterial infections. The inappropriate use of antimicrobial in human, animal, and agricultural health, as well as the local and global spread of resistant bacteria and resistance determinants have contributed to the increase in AR (MCEWEN; COLLIGNON, 2018).

Antimicrobials and genes encoding AR (GAR) are widely disseminated in surface water, effluents, sewage treatment plants, plants, soil, and animal waste, being considered emerging pollutants of the environment (SAKKAS *et al.*, 2019). This contamination is caused by improper treatment of industrial, residential, hospital, and agricultural waste, which may contain bacteria, resistance determinants, and compounds that include heavy metals, biocides, and antimicrobials. The exposure of bacteria to these compounds, even at subinhibitory concentrations, can select these microorganisms for resistance to important antimicrobials used in the therapy of human and animal infections. The selective pressure exerted by antimicrobial molecules favors the acquisition and transfer of mobile genetic elements between different bacterial species, thus contributing to the emergence of multidrug-resistant

(MDR) and extensively resistant (XDR) bacteria (MCEWEN; COLLIGNON, 2018; KARKMAN *et al.*, 2018; SAKKAS *et al.*, 2019).

Pseudomonas aeruginosa are Gram-negative bacilli, non-fermenting glucose, strictly aerobic, commonly found in soil and aquatic environments, and colonizing plant, animal, and human tissues (AZAM; KHAN, 2019). Considered an opportunistic pathogen, it is responsible for a variety of community and hospital infections, especially in immunocompromised patients with comorbidities, using antimicrobials, undergoing invasive procedures, and being admitted to intensive care units (AZAM; KHAN, 2019; FIGUEREDO *et al.*, 2021). It is estimated to be responsible for about 51,000 health-associated infections and 400 deaths in the United States each year, of which 13.0% are caused by MDR isolates (AZAM; KHAN, 2019; CHEGINI *et al.*, 2020). The alarming growth of MDR and extensively-resistant bacteria associated with the decrease in the development of new drugs has caused great concern, since infections caused by such pathogens do not respond, in most cases, to treatment with currently available agents (MCEWEN; COLLIGNON, 2018; SAKKAS *et al.*, 2019).

Carbapenems are the antimicrobial of choice in the treatment of serious infections caused by Gram-negative MDR bacteria. However, increasing reports of resistance to these drugs have limited the therapeutic options available. Consequently, the therapeutic alternative has been the use of antibiotics in combination, as well as the resumption of the use of polymyxins (MCEWEN; COLLIGNON, 2018; AZAM; KHAN, 2019; YOON; JEONG, 2021).

Resistance to carbapenems can occur by mechanisms that include loss or modification of outer membrane porins (OprD), overexpression of efflux systems, alteration of the level of production or binding affinity of penicillin-binding proteins (PBPs), as well as enzymatic hydrolysis by the acquisition of genes encoding carbapenemases (MOUBARECK *et al.*, 2019; YOON; JEONG, 2021). Carbapenemases are beta-lactamases belonging to Ambler classes A, B, and D, with the ability to hydrolyze practically all classes of beta-lactams available, including carbapenems. The production of class B enzymes (metallo-beta-lactamase) is the mechanisms of acquired resistance to carbapenems most described in *P. aeruginosa* in the world (MOUBARECK *et al.*, 2019), and in Brazil, the metallo-beta-lactamase SPM is the most prevalent (LIMA, 2019).

However, enzymes belonging to other classes of carbapenemases also play an important role in resistance to these antimicrobial, such as GES (Guyana-

Extended-Spectrum) type enzymes, which are an emerging group of Ambler class A carbapenemases. The first representative of the group, GES-1, was first described, in 1998, in a clinical isolate of *K. pneumoniae* in France. Since then, 56 variants (http://bldb.eu/BLDB.php?prot=A) of this enzyme have been reported worldwide in different bacterial species. The rapid dissemination of this enzyme, encoded by the *bla*$_{GES}$ gene, is partly due to the gene's association with mobile genetic elements such as class 1 integrons and plasmids (HISHINUMA *et al.*, 2018; PICCIRILLI *et al.*, 2018).

The carbapenemase activity of some variants of the GES group, including GES-2, GES-4, GES-5, GES-6, GES-13, GES-14, GES-15, GES-16, GES-18, GES-20, GES-21, GES-24, GES-25, GES-27, GES-28, GES-29, GES-30, GES-33, GES-34, GES-36, GES-37, GES-39, GES-40, GES-41, GES-42, GES-43, is attributed to a single mutation at position 170, with substitution of the aminoacid glycine for asparagine or serine (PICCIRILLI *et al.*, 2018; YOON; JEONG, 2021). Among these variants, GES-5, first described in 2014 in China, has been reported in Brazil, South Africa, Japan, Lithuania, Russia, Spain, and Turkey (HISHINUMA *et al.*, 2018; LIMA, 2019).

Associated with the resistance determinants, *P. aeruginosa* has a variety of virulence factors that favor its colonization, tissue damage, survival in hosts, evasion of the immune system, also contributing to the broad spectrum of infections caused by this pathogen and to the resulting treatment increasingly challenging (AZAM; KHAN, 2019; CHEGINI *et al.*, 2020; FIGUEREDO *et al.*, 2021). Among the virulence factors described in *P. aeruginosa*, have pili formation, flagellum, pyocyanin production, pyoverdin, biofilm, and secretory factors that include elastases, proteases, hemolysins, quorum sensing system proteins, and the effector proteins ExoS, ExoT, ExoY, and ExoU that act by disrupting cellular physiology and subverting the host's innate immune response (MORADALI; GHODS; REHM, 2017; AZAM; KHAN, 2019; ALONSO *et al.*, 2020).

In recent years, high rates of resistance to carbapenems (43.2% - imipenem/41.9% - meropenem) have been observed in clinical isolates of *P. aeruginosa* at the Hospital Universitário de Londrina (HU). Given this scenario, research has been carried out by the Laboratório Especial de Microbiologia Molecular e Resistência aos Antimicrobianos (LEMMRA) to monitor clinical and environmental isolates of *Pseudomonas* spp. producers of carbapenemases in the city of Londrina-PR since 2002. With the detection of GES-5 producing isolates, this work

characterized clinical and environmental isolates that carry this carbapenemase. This information can help the Hospital Infection Control Commission at HU in the implementation of effective *P. aeruginosa* MDR and XDR infection control measures and the establishment of appropriate protocols for antimicrobial therapies.

2 METHODOLOGY

2.1 BACTERIAL ISOLATES, IDENTIFICATION, AND PROFILE OF SUSCEPTIBILITY TO ANTIMICROBIALS

Seven *P. aeruginosa* isolates carrying the *bla*$_{\text{GES-5}}$ gene recovered between 2012 and 2016 were selected: six from patients hospitalized at the HU and one from the Sewage Treatment Plant in the southern region (STP-South) of the city. The isolates were previously identified by conventional biochemical tests and by the automated system Vitek 2® (bioMérieux, Craponne, France) in the Laboratory of Clinical Microbiology at HU. The molecular identification was confirmed by amplification of the gene encoding the 16S rRNA (SPILKER *et al.*, 2004).

The evaluation of antimicrobial susceptibility was performed using the disk diffusion method and automated systems. The results were analyzed and interpreted according to the Guidelines of the Clinical and Laboratory Standards Institute (2021). The isolates were classified as multidrug-resistant (MDR), extensively resistant (XDR), or pan-resistant (PDR) to antimicrobials (MAGIORAKOS *et al.*, 2012).

2.2 DETECTION OF GENES ENCODING ANTIMICROBIAL RESISTANCE AND VIRULENCE FACTORS

The genes encoding antimicrobial resistance and virulence factors *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{GIM}}$, *bla*$_{\text{SPM}}$, *bla*$_{\text{SIM}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-51}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-143}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{PER}}$, *bla*$_{\text{VEB}}$, *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-25}}$,*rmt*A, *rmt*B, *rmt*C, *rmt*D, *rmt*E, *rmt*G, *rmt*H, *qnr*A, *qnr*B, *qnr*C, *qnr*S, *qnr*Vc, *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, *mcr*-5, *las*B, *plc*H, *exo*S, *exo*U, *exo*Y, *tox*A, and *las*I were investigated by PCR (SABHARWAL *et al.*, 2014; PAULA, 2017). The *bla*$_{\text{GES}}$ amplicons obtained were purified and sequenced to confirm the variant.

2.3 PHENOTYPIC DETECTION OF VIRULENCE FACTORS

The isolates were previously cultured on Mueller-Hinton agar at 37 °C for 18h. Cell density was adjusted to $1x10^7$ UFC/mL in Mueller-Hinton broth, and this standard inoculum was used in the tests. The experiments were carried out in a triplicate on three different occasions, except when specified. *P. aeruginosa* PA01 was included in the tests.

2.3.1 PROTEASE PRODUCTION DETECTION

Protease production was evaluated using the protocol described by Sokol, Ohman, and Iglewski (1979). The standard inoculum (10µL) was added to the surface of plates containing Brain Heart Infusion medium enriched with milk casein (2.0%), followed by incubation for 18-24 hours at 37 °C. Proteolytic activity was observed in the presence of a clear zone around the inoculum (SOKOL; OHMAN; IGLEWSKI, 1979).

Colonies of each isolate were also inoculated in tubes containing nutrient agar supplemented with gelatin (12.0%) (Becton, Dickinson and Company, Sparks, USA) and incubated at 37 ºC for up to 7 days. After refrigeration at 4-8 ºC/1h, the presence of a liquid medium indicated proteolytic activity (DELA CRUZ; TORRES; 2012).

2.3.2 DETECTION OF HEMOLYTIC ACTIVITY

Hemolytic activity in blood agar plates was evaluated by sowing 10µL of the standard inoculum ($1x10^7$ UFC/mL) on the surface of plates containing agar medium supplemented with 5.0% of sheep erythrocytes. The plates were incubated for 18-24h at 37 °C, and the presence of a translucent halo around the inoculum indicated hemolytic activity (GEORGESCU *et al.*, 2016).

2.4 MOLECULAR TYPING

The clonality of the *bla*$_{GES-5}$ gene carrier isolates was determined using the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) technique according to Versalovic, Koeuth, and Lupski (1991). The amplicons were analyzed on 2.0% agarose gels stained with ethidium bromide. Fragments profiles were analyzed using GelJ software version 1.0. Those that showed ≥93.0% genetic similarity were considered to be clonally related isolates.

3 RESULTS AND DISCUSSIONS

The seven *Pseudomonas* spp. were identified as *P.* aeruginosa by amplification and sequencing of 16S rRNA. The clinical isolates were recovered from male patients and obtained from tracheal secretion (66.7%), tissue (16.6%), and urine (16.6%). The environmental isolate was recovered from water samples collected at the STP-South (Table 1). High rates of resistance were observed to aminoglycosides, fluoroquinolones, monobactams, cephalosporins, carbapenems, and penicillin associated with beta-lactamases inhibitors. Only polymyxins were effective against infections by these pathogens, showing 100.0% sensitivity. Clinical and environmental isolates showed similar resistance rates, and all were classified as XDR (Table 1).

Similar results were observed in studies conducted in Brazil, Japan and the United Arab Emirates, in which resistance to most tested antimicrobials and sensitivity to polymyxins was reported (FONSECA *et al.*, 2007; HISHINUMA *et al.*, 2018; MOUBARECK *et al.*, 2019). This resistance profile can be explained by the presence of *bla*$_{\text{GES-5}}$ associated with the notorious ability of *P. aeruginosa* to be intrinsically resistant to several antimicrobials and to acquire resistance mechanisms, conferring resistance to multiple classes of antimicrobials and contributing to the occurrence of difficult-to-treat infections (MORADALI; GHODS; REHM, 2017).

The quorum sensing (QS) control of gene regulation of virulence determinants, as well as more than 10.0% of genes in *P. aeruginosa* (MORADALI; GHODS; REHM, 2017; AZAM; KHAN, 2019). In this species, the Las system, the main pathway of QS, comprises an autoinducer synthase (LasI), which produces the autoinducer N-3-oxododecanoyl-L-homoserine lactone, which is exported out of the cell, imported in sequence, and bind to the LasR regulatory protein, activating the lasI gene and several other downstream genes (MORADALI; GHODS; REHM, 2017; AZAM; KHAN, 2019). In this study, 100.0% of the isolates showed the gene encoding LasI (Table 1), similar to the observed by Alonso and contributors (2020) (98.0%).

The bacterial biofilm consists of cell aggregates surrounded by an extracellular polymeric matrix, composed of polysaccharides, lipids, proteins, extracellular DNA, and biosurfactants (MORADALI; GHODS; REHM, 2017; AZAM; KHAN, 2019; CHEGINI *et al.*, 2020). Alginate has a wide range of functions that include biofilm maturation, structural stability, protection of cells against phagocytosis and opsonization, and reduction of antimicrobial diffusion through the biofilm, conferring

sessile cells up to 1000 times more AR than planktonic cells. Furthermore, it confers a viscous or mucoid phenotype to the structure, which is considered a marker of chronic infections by *P. aeruginosa*, indicative of disease progression and long-term persistence (MORADALI; GHODS; REHM, 2017; AZAM; KHAN, 2019). In this study, the *alg*D gene was observed in 100.0% of the isolates analyzed (Table 1), while other studies reported detection of this gene in 92.3-100.0% of the isolates (GEORGESCU *et al.*, 2016; ELLAPPAN; NARASIMHA; KUMAR, 2018; JAHROMI *et al.*, 2018).

P. aeruginosa, still in the acute phase, uses the type III secretion system (T3SS) to directly inject the toxins ExoS, ExoY, ExoT and ExoU into the cytoplasm of the eukaryotic host cell (AZIMI *et al.*, 2016). These proteins act by interrupting cell physiology, subverting the innate immune response of the host, and contributing to the establishment of the infection. ExoU promote acute epithelial injury in several diseases, due potent phospholipase A2 activity, causing rapid cell lysis and necroptosis in epithelial cells, neutrophils and macrophages. Neutrophil depletion has an immunosuppressive effect that makes the host more susceptible to secondary infections. Thus, *exo*U gene expression is considered a marker of disease severity and poor prognosis for the patient (SABHARWAL *et al.*, 2014; AZIMI *et al.*, 2016; GEORGESCU *et al.*, 2016; FOULKES *et al*., 2019). ExoS and ExoT exoenzymes are molecules that alter the cytoskeleton and signaling pathways, resulting in cell rounding, inhibition of phagocytosis, and eventually leading to cell death by apoptosis. Additionally, ExoS production is associated with endocytic uptake and intracellular survival. ExoY, in turn, promotes rounding of host cells and increased endothelial permeability with tissue edema formation (AZAM; KHAN, 2019; SABHARWAL *et al.*, 2014; AZIMI *et al.* 2016; GEORGESCU *et al.*, 2016; ELLAPPAN; NARASIMHA; KUMAR, 2018).

While the *exo*T and *exo*Y genes are present in the vast majority of *P. aeruginosa* clinical isolates, the *exo*S and *exo*U genes have a varied prevalence. ExoS and ExoU are almost always mutually exclusive, and the occurrence of isolates that encode both or neither of these exotoxins is rare since they provide better conditioning in different ecological niches (RUTHERFORD *et al.*, 2018). An explanation for the absence of *exo*U-positive isolates would be the presence of *exo*S (100.0%) in all isolates evaluated (Table 1). The prevalence of *exo*U was lower than that observed in other studies, which showed a rate ranging from 8.3 to 98.1% (AZIMI *et al.*, 2016; GEORGESCU *et al.*, 2016; POBIEGA *et al.*, 2016; HISHINUMA *et al.*, 2018;

RODULFO *et al.*, 2018; ALONSO *et al.*, 2020). Regarding *exo*S, the result was similar to that obtained by Pobiega and contributors (2016) (92.3%), and higher than that reported in some studies that detected this determinant in 1.9-76.0% of the isolates (AZIMI *et al.*, 2016; GEORGESCU *et al.*, 2016; ELLAPPAN; NARASIMHA; KUMAR, 2018; HISHINUMA *et al.*, 2018; RODULFO *et al.*, 2018; ALONSO *et al.*, 2020). The *exo*Y gene was detected in 100.0% of the isolates (Table 1). Higher than that reported by Azimi and contributors (2016) (55.0%), and similar to that observed in previous studies that reported the presence of this gene in 93.0-96.2% of the isolates (YOON; JEONG, 2021; DALLENNE *et al.*, 2010).

About the environmental isolate, similar rates were obtained in a study that analyzed environmental isolates of *P. aeruginosa* recovered from soil, water, plants, and moist substrates, in which the *exo*Y, *exo*S, and *exo*U genes were identified in 96.0%, 83.0%, and 16.0% of the isolates, respectively (RUTHERFORD *et al.*, 2018). In a study with isolates recovered from various niches and ecological sources (hospitals, animals, food, wastewater, among others) was observed a prevalence of 72.7% of the *exo*S gene and 30.3% of the *exo*U gene (AHMED *et al.*, 2020). The high prevalence of *exo*Y and *exo*T genes in environmental samples, in several studies, suggests an important role of these determinants in the survival of *P. aeruginosa* in natural environments. The different prevalence of *exo*S and *exo*U vary according to the origin of the isolate and are proposed as a reflection of distinct levels of pollution or contamination of natural water systems with water manipulated by humans, such as sewage, which is considered a potential reservoir of *P. aeruginosa* (RUTHERFORD *et al.*, 2018; AHMED *et al.*, 2020).

In this specie, rhamnolipid and phospholipase C (hemolytic and non-hemolytic) are soluble proteins responsible for bacterial cell invasion. Rhamnolipid acts to solubilize of phospholipids in cell membranes and pulmonary surfactant, making them more accessible to the action of phospholipases, which act in the degradation of membrane phospholipids, hemolyzing erythrocytes, and destroying surfactant lipids. The phospholipases plays an important role in the spread of infection and wound extension, increasing vascular permeability, causing damage to the target organ, as well as protecting the bacteria from the immune system and antimicrobial agents (SABHARWAL *et al.*, 2014; GEORGESCU *et al.*, 2016; JAHROMI *et al.*, 2018; AZAM; KHAN, 2019; RODULFO *et al.*, 2019; BOGIEL *et al.*, 2021). In this study, the *plc*H gene, which encodes the hemolytic toxin, was detected in 100.0% of the isolates

(Table 1). Similar rates have been described previously (ELLAPPAN; NARASIMHA; KUMAR, 2018; JAHROMI *et al.*, 2018; ALONSO *et al.*, 2020; BOGIEL *et al.*, 2021). However, the prevalence was higher than that observed by Sabharwal and contributors (2014) and Georgescu and contributors (2016). The detection of the *plc*H gene was phenotypically supported in this study by the production of hemolysin (100.0%) (Table 1).

Proteolytic activity (100.0%) was also observed (Table 1) and suggests the ability of the isolates to promote tissue damage and delay wound healing, through the degradation of extracellular matrix components that include elastin, collagen, fibrin, and fibronectin, and interfere with the host immune system (SABHARWAL *et al.*, 2014; GEORGESCU *et al.*, 2016; AZAM; KHAN, 2019; RODULFO *et al.*, 2019). The production of gelatinase, which is responsible for degrading a variety of proteins, as a component of the matrix of different cells, was not detected in this study (Table 1) (RODULFO *et al.*, 2019). Previous studies reported caseinase (91.7%) and beta-hemolysin (80.1-83.3%) as the most frequently expressed soluble virulence factors in *P. aeruginosa* isolates. As well was, highest rate of gelatinase production (66.7-85.2%) in relation to this study (GEORGESCU *et al.*, 2016; RODULFO *et al.*, 2019).

The extracellular proteases elastase B (LasB) and alkaline protease (AprA) act in the cellular invasion stage of the infection process, breaking physical barriers and damaging host cells. LasB is a metalloprotease with high proteolytic activity secreted by T2SS that acts on the degradation of structural proteins of host cells causing tissue damage and inactivating the main components of the immune system, being a crucial factor in the process of cell invasion and tissue infection (SABHARWAL *et al.*, 2014; MORADALI; GHODS; REHM, 2017; ELLAPPAN; NARASIMHA; KUMAR, 2018; AHMED *et al.*, 2020; AZHAM; KHAN, 2019; BOGIEL *et al.*, 2021). In this study, the *las*B gene was detected in 100.0% of the isolates (Table 1), corroborating with what was observed in other studies that reported high detection rates of this gene in isolates recovered from urine and respiratory tract secretions, suggesting a role for this determinant in urinary tract infections (UTI) and respiratory infections (BOGIEL *et al.*, 2021). Similar results have been previously reported (ELLAPPAN; NARASIMHA; KUMAR, 2018; JAHROMI *et al.*, 2018; AHMED *et al.*, 2020; ALONSO *et al.*, 2020; BOGIEL *et al.*, 2021). Other studies have reported lower rates, with detection of 75.0-80.8% (SABHARWAL *et al.*, 2014; POBIEGA *et al.*, 2016).

The alkaline protease, encoded by the *apr*A gene, is involved in the colonization and invasion of host tissue. With broad protease activity, this determinant is responsible for degrading crucial proteins, destroying the basal lamina causing hemorrhagic tissue necrosis, as well as inactivating complement factors of the immune system and inhibiting neutrophil phagocytosis and fibronectin, favoring their survival in the host. Additionally, it contributes to the overproduction of pyocyanin in the presence of specific proteins or peptides, increasing the virulence of the isolates (MORADALI; GHODS; REHM, 2017; AZAM; KHAN, 2019; BOGIEL *et al.*, 2021). In this study, *apr*A was detected in 100.0% of the isolates (Table 1), as observed previously (ALONSO *et al.*, 2020; BOGIEL *et al.*, 2021). Other studies have reported lower rates, with a prevalence of 16.6-61.5% (SABHARWAL *et al.*, 2014; POBIEGA *et al.*, 2016).

Exotoxin A (ToxA) inhibits protein synthesis, causes tissue damage, allows bacterial invasion, contributes to immunosuppression by reducing phagocytic activity, and causes cell death (MORADALI; GHODS; REHM, 2017; AHMED *et al.*, 2020; AZAM; KHAN, 2019). The *tox*A gene was detected in 100.0% of the evaluated isolates (Table 1). As ToxA is considered an important virulence factor in UTI and reported in several studies with high detection rates in *P. aeruginosa* isolates recovered from respiratory infections and burns, it justifies the prevalence observed in this study, whose clinical origin of the isolates were secretion tracheal, tissue and urine (SABHARWAL *et al.*, 2014). Similar results were obtained in studies conducted with clinical isolates (SABHARWAL *et al.*, 2014; JAHROMI *et al.*, 2018) and isolate recovered from different niches (clinical and environmental), which showed a prevalence of 100.0% (AHMED *et al.*, 2020).

Table 1 Epidemiological, phenotypic and molecular characteristics of the clinical and environmental isolates of *Pseudomonas aeruginosa* carrying *bla*$_{GES-5}$ gene.

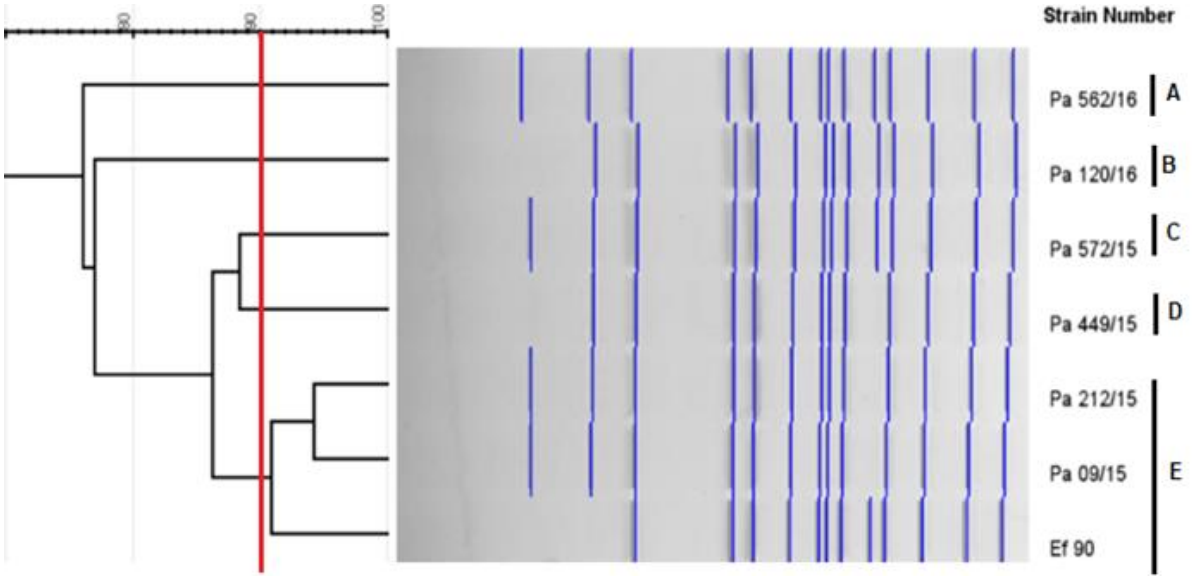
		Pa 09/15	Pa 212/15	Pa 449/15	Pa 572/15	Pa 120/16	Pa 562/16	Ef 90
Collection location		HU	HU	HU	HU	HU	HU	STP-South
Bacterial species		*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*
Patient gender		M	M	M	M	M	M	NA
Clinical material		TS	TS	Tissue	TS	TS	Urine	Water
Hospital unit		ICU	ICU-P	AMB	ICU	ICU	ID	NA
Clinical outcome		Death	Death	Discharge	Death	Death	Death	NA
Resistance profile		XDR	XDR	XDR	XDR	XDR	XDR	XDR
Phenotupic detection	Gelatinase							
	Protease	+	+	+	+	+	+	+
	Hemolysin	+	+	+	+	+	+	+
Molecular detection	*Las*I	+	+	+	+	+	+	+
	*Alg*D	+	+	+	+	+	+	+
	*Plc*H	+	+	+	+	+	+	+
	*Apr*A	+	+	+	+	+	+	+
	*Las*B	+	+	+	+	+	+	+
	*Tox*A	+	+	+	+	+	+	+
	*Exo*Y	+	+	+	+	+	+	+
	*Exo*S	+	+	+	+	+	+	+
	*Exo*U							
ERIC-PCR clusters		E	E	D	C	B	A	E

HU, Hospital Universitário de Londrina; STP-South; Sewage Treatment Station in the Central and South of Londrina; M, male; NA, not applicable; TS, tracheal secretion; ICU, intensive care unit; ICU-P, pediatric intensive care unit; AMB, ambulatory; ID, infectious diseases; ER, extensively resistant to antimicrobials; +, positive; ERIC-PCR, Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction.

Source: Research data

Genetic similarity analysis by ERIC-PCR revealed five distinct genetic groups (A-E) (Figure 1). Group E included three isolates, one environmental and two clinical, showing similarity of at least 93.0%. The other groups included only one clinical isolate each. Except for group D, in which the patient was discharged, in the other clonal groups the patients died. In group E, in addition to genetic similarity, the isolates shared great similarity in terms of phenotypic and molecular characteristics evaluated. These results alert to possible dissemination of resistance determinants in *P. aeruginosa* isolates, as well as to the fact that this pathogen can become a reservoir of the *bla*$_{GES-5}$ gene both in the HU and in the environment. Since these bacteria and their GAR can be released directly into hospital and/or community sewage systems and not be eliminated during processing in STPs, posing a risk to human and animal health (KARKMAN *et al.*, 2018).

Figure 1 Genetic relatedness of clinical and environmental isolates of *Pseudomonas aeruginosa* carrying the *bla*$_{GES-5}$.



Dendrogram generated from the ERIC-PCR amplicon patterns using the GelJ v.1.0. software and unweighted pair group method with arithmetic averages at 93.0% similarity on 7 strains of *P. aeruginosa*. The different clusters were arbitrarily designated as Clusters A-E, of which Cluster E was the largest group representing the most prevalent clone of *P. aeruginosa*.

Source: Research data

4 CONCLUSION

Phenotypic and molecular analyzes demonstrated AR determinants and virulence factors in the evaluated clinical isolates. The coexistence of these determinants is a worrying factor, which may imply therapeutic limitation, persistence, and dissemination of these isolates in the hospital environment.

In particular, an environmental isolate of *P. aeruginosa* XDR was reported, carrying the *bla*$_{\text{GES-5}}$ gene, endowed with genes encoding virulence factors and producing these determinants, sharing great similarity in terms of phenotypic and molecular characteristics evaluated with clinical isolates recovered from the HU. These results suggest that isolates with this profile are not limited to health care units, but are also contaminants of environmental samples.

Thus, the continuous monitoring of hospital effluents, the determination of the antimicrobial resistance profile, and the detection of virulence determinants in clinical and environmental isolates of *Pseudomonas* spp. are of fundamental importance in the surveillance of multidrug-resistant isolates, in the establishment of measures to control nosocomial infection as well as the spread of AR bacteria and their GAR to external environments.

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