**UNIVERSIDADE FEDERAL DA GRANDE DOURADOS**
**FACULDADE DE CIÊNCIAS DA SAÚDE**
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE**

# Mecanismos moleculares de resistência em enterobactérias e avaliação de uma nova abordagem terapêutica

**KESIA ESTHER DA SILVA**

**Dourados - MS**
**2019**

KESIA ESTHER DA SILVA

# Mecanismos moleculares de resistência em enterobactérias e avaliação de uma nova abordagem terapêutica

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Atenciosamente,

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Cecilia Carvalhaes
EAEF3798D8D047A...

**Profa. Dra. Cecília Godoy Carvalhaes**

JMI Laboraories

# DEDICATÓRIA

Dedico este trabalho aos meus pais **Sueli** e **Osiris (*in memorian*)**, por toda dedicação, apoio, incentivo e amor incondicional que me proporcionaram. Eles que serão presença constante em minha vida.

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O conhecimento amplia a vida. Conhecer é viver uma realidade
que a ignorância impede desfrutar.
(*Carlos Bernado González*)

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# LISTA DE ABREVIATURAS E SÍMBOLOS

ABC – ATP-bindingcassette

ANVISA – Agência Nacional de Vigilância Sanitária

AmpC – Cefalosporinase cromossomal

β - Beta

$Ca^{2+}$ - Cálcio

CDC – *Center for Diseases Control and Prevention*

CTX-M - Cefotaximase

DNA - Ácido Desoxirribonucleico

EDTA – Ácido Etileno-Diamino-Tetracético

ESBL – β-lactamase de expectro estendido

GES - *Guiana extended espectrum*

GIM – *German imipenemase*

*gyrA* – Girase A

IMP – *Imipenemase*

IS – *Insertion sequence* (Elemento de inserção)

IRAS – Infecções relacionadas à assistência a saúde

KPC – *Klebsiella pneumoniae carbapenemase*

L-Ara4N – L-4-aminoarabinose

LPS - Lipopolissacarídeo

$Mg^{2+}$ - Magnésio

MATE – *Multidrug and toxic compound extrusion*

MBL – Metalo-β-lactamase

MDR – multirresistente

MFS – *Major Facilitator Superfamily*

MIC – *Minimun Inhibitory Concentration* (Concentração Inibitória Mínima)

NDM - *New Delhi metallo- β-lactamase*

NMC - N*ot metalloenzyme carbapenemase*

OMP – Proteínas de membrana externa

OXA - Oxacilinase

PBP – *Penicillin Binding Protein* (Proteínas ligadoras de penicilina)

pEtN – fosfoetanolamina

PNA – *Peptide nucleic acid*

RNA – Ácido Ribonucléico

RND – *Resistance Nodulation Division*

Sap – *sensitive antimicrobial peptides*

SENTRY – *Antimicrobial Surveilance Program*

SIM - *Seul imipenemase*

SMR – *Small Multidrug-resistance*

SME – *Serratia marcescens* enzyme

SPM - *São Paulo metallo-β-lactamase*

UTI – Unidade de Terapia Intensiva

VIM - *Verona imipenemase*

$Zn^{+2}$ – Zinco

# Mecanismos moleculares de resistência em enterobactérias e avaliação de uma nova abordagem terapêutica


## RESUMO

O aumento de casos de infecção hospitalar causada por enterobactérias multirresistentes, constitui um grande desafio para a Saúde Pública e um problema de grandes proporções para o tratamento de pacientes hospitalizados em Unidades de Terapia Intensiva (UTIs). A identificação dos mecanismos genéticos envolvidos na resistência e a busca de novas terapias para o tratamento têm grandes implicações no aperfeiçoamento de medidas de redução e contenção da disseminação de microrganismos multirresistentes. O objetivo desse estudo foi caracterizar os mecanismos de resistência à polimixina em enterobactérias envolvidas em surtos de infecção hospitalar através do sequenciamento do genoma desses isolados, associando a dados clínicos e epidemiológicos dos pacientes. Além disso, o estudo avaliou a atividade antimicrobiana de um peptídeo antisense frente a uma cepa multirresistente, buscando contribuir na contenção da disseminação destas cepas. Durante Setembro/2015 a Fevereiro/2017, foram obtidos 53 isolados de enterobactérias com perfil de resistência a polimixina, coletadas de dois hospitais brasileiros. Destas cepas, 30 foram identificadas como *Klebsiella pneumoniae* e submetidas ao sequenciamento do genoma. O resultado revelou que o gene $bla_{KPC}$ foi o principal gene de resistência identificado, seguido de $bla_{SHV}$, $bla_{TEM}$, $bla_{CTX\text{-}M}$ e $bla_{OXA}$. Outros genes que conferem resistência a aminoglicosídeos, fluoroquinolonas, sulfonamidas, tetraciclinas, trimetoprim e macrolídeos também foram detectados. Todos os isolados apresentaram alta similaridade genética (acima de 96%) e a resistência a polimixina foi atribuída a três mecanismos envolvendo a inativação do gene *mgrB*, incluindo a inativação por sequências de inserção (IS) e mutações pontuais sem sentido. Foi identificada em seis cepas uma sequência de inserção de 80 pb que resulta na duplicação de 26 aminoácidos da proteína MgrB. Esta é a primeira observação deste tipo de alteração causando resistência à polimixina. A caracterização da resistência a polimixina também foi realizada em isolados de *Klebsiella aerogenes*, nesse estudo nove cepas foram isoladas de pacientes admitidos em UTIs. Foi observada uma expansão clonal dos isolados e os dados de sequenciamento de genoma revelaram que as cepas foram agrupadas em dois grupos clonais. A análise genômica mostrou que a resistência à polimixina foi mediada por três mecanismos mutacionais distintos, incluindo alterações pontuais sem sentido e

substituição de aminoácidos nas proteínas PhoP e PhoQ. Além disso, foi possível identificar uma mutação incomum no gene *soxS* resultando na codificação uma proteína truncada. Foi realizado um estudo caso-controle com 159 pacientes para explorar os potenciais fatores de risco associados à aquisição de cepas resistentes a polimixina em enterobactérias produtoras de carbapenemases isoladas de pacientes internados em UTIs adulto e neonatal, bem como descrever a mortalidade e as características clínicas dessas infecções. Nos pacientes adultos foram identificados diversos fatores de risco, incluindo insuficiência renal, uso de cateter urinário, procedimentos cirúrgicos, exposição a antibióticos carbapenêmicos e transferência entre alas hospitalares. Em neonatos uso de cateter venoso central foi o principal fator de risco identificado. A mortalidade foi significativamente maior em pacientes infectados com cepas resistentes à polimixina do que naqueles com cepas sensíveis à polimixina ($p$<0.01). O estudo dos fatores de risco também demonstrou que o uso de ventilação mecânica e exposição à polimixina estavam fortemente associados à mortalidade dos pacientes incluídos no estudo. Além disso, foi investigado *in vitro* o potencial antimicrobiano de um peptídeo anti-sentido (PNA) contra *K. pneumoniae* produtora de KPC. O PNA inibiu o crescimento bacteriano na concentração de 50 μM, com redução de 96,7% da amplificação do gene 16s. O PNA apresentou baixa atividade hemolítica nas concentrações necessárias para matar as bactérias e as análises de bioinformática demonstraram que a estrutura do PNA exibe boa estabilidade conformacional em fluidos biológicos. Com base nos resultados obtidos neste estudo, conclui-se que a identificação dos mecanismos genéticos envolvidos na resistência antimicrobiana e a identificação de fatores de risco específicos envolvidos na aquisição desses microrganismos são importantes na busca de estratégias inovadoras para o controle dessas cepas, bem como para o desenvolvimento de novas abordagens terapêuticas para o tratamento dessas infecções.

**Palavras-chave:** Enterobactérias. Resistência a polimixina. Sequenciamento de genoma. Fatores de Risco. Peptídeo anti-sentido.

# Molecular mechanisms of resistance in *Enterobacteriaceae* and evaluation of a new therapeutic approach


## ABSTRACT

The increase of hospital infections caused by multidrug-resistant *Enterobacteriaceae* is a major challenge for Public Health and a main problem for the treatment of patients hospitalized in Intensive Care Units (ICUs). The identification of genetic mechanisms involved in bacterial resistance and the search for new therapies have major implications in the reduction and control of multidrug-resistant strains. The objective of this study was to characterize the resistance mechanisms to polymyxin in *Enterobacteriaceae* involved in outbreaks by genome sequencing, associating clinical and epidemiological data of the patients. In addition, the study evaluated the antimicrobial activity of an antisense peptide against a multidrug-resistant strain, seeking to contribute in the control of the dissemination of these strains. During September/2015 to February/2017, 53 strains of *Enterobacteriaceae* with polymyxin-resistance profile were isolated from two Brazilian hospitals. Of these strains, 30 were identified as *Klebsiella pneumoniae* and submitted to genome sequencing. The results revealed that the $bla_{KPC}$ gene was the main resistance gene identified, followed by $bla_{SHV}$, $bla_{TEM}$, $bla_{CTX\text{-}M}$ and $bla_{OXA}$. Other genes that confer resistance to aminoglycosides, fluoroquinolones, sulfonamides, tetracyclines, trimethoprim and macrolides were also detected. All isolates showed high genetic similarity and polymyxin resistance was attributed to three mechanisms involving inactivation of the mgrB gene, including inactivation by insertion sequences (IS) and point mutations. An 80 bp insertion sequence was identified in six strains which results in duplication of 26 amino acids of the MgrB protein. This is the first observation of this type of change causing polymyxin resistance. The characterization of polymyxin resistance was also performed in *Klebsiella aerogenes* isolates, in this study nine strains were isolated from patients admitted to ICUs. A clonal expansion of the isolates was observed and the genome sequencing data revealed that strains were clustered into two clonal clusters. Genomic analysis showed that polymyxin resistance was mediated by three distinct mutational mechanisms, including nonsense point changes and amino acid substitution in PhoP and PhoQ proteins. In addition, we identify an unusual mutation in the *soxS* gene resulting in a truncated protein. A case-control study was conducted with 159 patients to explore the potential risk factors associated with the acquisition of polymyxin-resistant strains

producing carbapenemases isolated from patients in adult and neonatal ICUs, as well as to describe the mortality and clinical characteristics of these patients' infections. In adult patients, several risk factors were identified, including renal failure, urinary catheter use, surgical procedures, exposure to carbapenem and transfer between hospital wards. In neonates, use of central venous catheter was the main risk factor identified. Mortality was significantly higher in patients infected with polymyxin-resistant strains than in those with polymyxin-sensitive strains ($p$<0.01). The study of risk factors also showed that use of mechanical ventilation and polymyxin exposure were strongly associated with the mortality of patients included in the study. In addition, the antimicrobial potential of an antisense peptide (PNA) against KPC-producing *K. pneumoniae* was investigated *in vitro*. PNA inhibited bacterial growth at 50 μM, with a 96.7% reduction in 16s gene amplification. PNA showed low hemolytic activity at concentrations necessary to kill bacteria and bioinformatics analyzes demonstrated that the PNA structure exhibits good conformational stability in biological fluids. Based on these results, it is concluded that the identification of the genetic mechanisms involved in antimicrobial resistance and the identification of specific risk factors are important in the search for innovative strategies for the control of these strains, as well as for the development of new therapeutic approaches for the treatment of these infections.

***Keywords*:** *Enterobacteriaceae*. Polymyxin-resistance. Genome sequencing. Risk factors. Antisense peptide.

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# 1 INTRODUÇÃO

As enterobactérias (Rahn, 1937) constituem uma família de bacilos Gram-negativos com grande importância na microbiologia clínica e amplamente distribuídos na natureza, podendo ser encontradas no solo, água, e no trato gastrointestinal de humanos e animais (Al-Kharousi et al., 2016; Kaushik et al., 2018). Essas cepas se diferenciam pela capacidade de fermentação de uma série carboidratos, pela produção de toxinas, ou por suas características antigênicas (O'hara C, 2005; Singh et al., 2015). São classificadas como microrganismos oportunistas, frequentemente associadas a infecções no ambiente hospitalar e a um número significativo de mortes, principalmente por acometerem indivíduos imunodeprimidos e hospitalizados em Unidades de Terapia Intensiva (UTIs) (Martin et al., 2018). Estas cepas são responsáveis por uma grande variedade de infecções humanas graves, incluindo meningites, pneumonias e bacteremias (Clarivet et al., 2016; Li e Ye, 2017; Kaushik et al., 2018; Martin et al., 2018).

A emergência de enterobactérias resistentes a β-lactâmicos constitui um dos principais desafios aos laboratórios clínicos e equipes de saúde, uma vez que se trata de uma categoria de medicamentos muito utilizados para o tratamento de infecções na clínica médica (Jee et al., 2018; Yang e Buttery, 2018). Vários mecanismos são descritos na resistência a essa classe de antibióticos, como alteração do sítio alvo das proteínas ligadoras de penicilinas (PBPs), a impermeabilidade da membrana externa e a hiper-expressão de bombas de efluxo, porém, a produção de carbapenemases é considerada o mecanismo de resistência mais importante, sendo o mais frequentemente descrito (Iredell et al., 2016; Chopra et al., 2018; Xu e Qu, 2018). Desta forma a disseminação mundial de enterobactérias resistentes a carbapenêmicos resultou no aumento do uso de colistinas e com isso o inevitável risco da emergência de resistência a estes antibióticos (Jeannot et al., 2017).

A resistência a polimixinas se desenvolve através de mecanismos adaptativos ou mutacionais (Jeannot et al., 2017). O principal mecanismo envolve uma grande variedade de mutações gênicas que causam alterações na membrana externa das bactérias Gram-negativas e levam a modificações na estrutura química do lipopolissacarídeo (LPS). A ativação de sistemas reguladores promove a adição de 4-amino-4-desoxi-L-arabinose à porção lipídica do LPS, dificultando a ligação da polimixina na membrana externa das bactérias (Olaitan et al., 2014; De Maio Carrillho et al., 2017). Até recentemente a resistência à polimixina foi relacionada somente a mutações cromossômicas, mas recentes estudos descreveram o gene de

resistência *mcr*1 como um mecanismo de transferência horizontal para resistência à polimixina (Liu et al., 2016; Wang et al., 2017; Lepelletier et al., 2018; Wise et al., 2018).

O aumento da distribuição de genes de resistência antimicrobiana diminui as opções terapêuticas, demonstrando a urgente necessidade de desenvolvimento de estratégias alternativas de tratamento que contribuam para a diminuição das taxas de morbidade e mortalidade dos pacientes acometidos por estas infecções (Yang e Buttery, 2018). Uma das principais estratégias estudadas atualmente para o desenvolvimento de novos fármacos é a utilização de peptídeos antisense, que tem como objetivo a inibição da expressão de genes de resistência (Quijano et al., 2017). A terapia antisense consiste na introdução no interior da célula de um fragmento de DNA ou RNA capaz de se hibridizar com uma sequência complementar do mRNA, inibindo a sua tradução. Este acoplamento pode ocorrer também com o DNA nuclear, originando uma tríplice hélice, e consequentemente, impedindo a transcrição (Goyal e Narayanaswami, 2018; Shen e Corey, 2018). A inibição da transcrição ou tradução do material genético pode impedir o avanço de diversos processos infecciosos e estados patológicos (Sully e Geller, 2016; Quijano et al., 2017; Ly e Miller, 2018; Verma, 2018).

Com o aumento da resistência a carbapenêmicos as polimixinas tornaram-se os antibióticos de última escolha utilizados no tratamento de infecções bacterianas multirresistentes. Entretanto, a eminência de surtos de infecção hospitalar causados por essas bactérias preocupa a comunidade científica mundial. Diante disso, a identificação dos mecanismos genéticos envolvidos na aquisição da resistência tem grandes implicações no aperfeiçoamento de medidas de redução e contenção da disseminação desses microrganismos. Além disso, estas informações dão subsídios para pesquisa e desenvolvimento de novas drogas que possam ser utilizadas no tratamento de pacientes acometidos por essas infecções. O objetivo desse estudo foi avaliar os mecanismos de resistência de enterobactérias produtoras de β-lactamases e resistentes a polimixina e propor uma nova estratégia de tratamento para infecções causadas por esses microrganismos por meio do silenciamento de genes de resistência, utilizando a terapia antisense como estratégia.

# 2 REVISÃO DE LITERATURA

## 2.1 Infecções relacionadas à assistência a saúde

As infecções relacionadas à assistência a saúde (IRAS) são consideradas um grave problema de saúde pública, acometendo cerca de um em cada 10 pacientes hospitalizados em todo mundo, afetando pelo menos 7% dos pacientes em países desenvolvidos e 10% em países em desenvolvimento (Who, 2016). Na Europa, aproximadamente 3,2 milhões de pacientes adquirirem algum tipo de IRAS todos os anos e 37.000 morrem em consequência dessas infecções (Caselli, Brusaferro, et al., 2018). Nos Estados Unidos, são observadas taxas de 5% a 10% dos pacientes hospitalizados cada ano, resultando em 75.000 mortes (Almeida, 2015). No Brasil o cenário é ainda mais preocupante, as taxas de infecção permanecem elevadas e continuam crescendo, sendo causa significativa de altas taxas de morbidade e mortalidade, visto que cerca de 15% dos pacientes contraem algum tipo de infecção em hospitais brasileiros por ano (Fortaleza et al., 2017). Essas infecções podem ser atribuídas principalmente às condições do ambiente hospitalar em que o paciente está exposto, as quais podem se manifestar durante a internação ou após a alta (Caselli, Brusaferro, et al., 2018).

As IRAS são definidas como qualquer tipo de infecção detectada dentro de 48 horas após a admissão hospitalar, ou detectada em pacientes que tiveram contato prévio com o serviço de saúde dentro do período de um ano (Amin e Deruelle, 2015). A maioria dessas infecções manifesta-se em pacientes em estado grave, submetidos a procedimentos invasivos e a longos períodos de internação (Khan et al., 2017). A incidência das infecções hospitalares varia de acordo com a unidade de tratamento, sendo que nas Unidades de Terapia Intensiva (UTIs) encontram-se pacientes em estado mais grave e com defesa imunológica comprometida, condições clínicas predisponentes ao surgimento de infecção (Merzougui et al., 2018). Pacientes internados em UTIs apresentaram risco médio de 5 a 10 vezes maior do que pacientes internados em outros setores, atingindo de 10 a 30% dos pacientes, com taxa de mortalidade que varia de 10 a 60% (Pradhan et al., 2014; Iwuafor et al., 2016; Macvane, 2017; Mahomed et al., 2017).

Os tipos mais comuns de IRAS são: infecções urinárias, infecções de sítio cirúrgico, de corrente sanguínea e pneumonias (Wang, J. et al., 2018). Entretanto além dessas, várias outras fontes foram identificadas como contribuintes para o desenvolvimento de IRAS, incluindo o uso de dispositivos médicos (cateteres urinários, venosos e ventiladores mecânicos), transmissão entre pacientes e profissionais de saúde, sistemas de ar condicionado

contaminados, as características e o *layout* físico da unidade de saúde (Dancer, 2014). Muitos hospitais possuem dificuldade na implementação de normas de biossegurança efetivas para contenção de microrganismos, o que contribui para a ocorrência de infecções hospitalares. Deve-se também considerar um dos fatores determinantes para o surgimento das infecções a grande rotatividade dos leitos, o que favorece a limpeza e higienização inadequada, contribuindo para a proliferação de bactérias entre os pacientes (Amin e Deruelle, 2015; Khurana et al., 2018).

Além de elevar as taxas de morbidade e mortalidade, o custo com internação de um paciente com infecção hospitalar pode ser três vezes maior do que de um paciente sem infecção (Brouqui et al., 2017). Neste contexto, torna-se pertinente a realização de estudos que determinem a prevalência das infecções em ambientes hospitalares, visando identificar fatores relacionados ao surgimento destas em pacientes em situação crítica (Choudhuri et al., 2017; Khan et al., 2017). As bactérias Gram-negativas são os patógenos com maior relevância clínica e epidemiológica nos ambientes hospitalares, dentre elas as pertencentes à família *Enterobacteriaceae* (O'hara C, 2005; Jorgensen et al., 2015).

## 2.2 Enterobactérias

As enterobactérias constituem uma família de bacilos Gram-negativos amplamente distribuídos na natureza. Esses microrganismos habitam a microbiota intestinal e estão entre os patógenos mais comuns em humanos causando diversos tipos de infecções. Possuem a capacidade de crescer rapidamente em condições aeróbicas e anaeróbicas, apresentando exigências nutricionais simples. Não formam esporos e do ponto de vista bioquímico, são catalase-positivo, oxidase negativo, reduzem o nitrato e fermentam glicose. Embora apresentem características comuns, a família *Enterobacteriaceae* é extremamente diversificada (O'hara C, 2005; Singh et al., 2015)

Existem aproximadamente 42 gêneros e mais de 140 espécies de bactérias pertencentes a esta família, as quais podem ser diferenciadas a partir de aspectos morfológicos, sorológicos e bioquímicos. Todos esses aspectos são levados em consideração para a identificação desses isolados. (O'hara C, 2005; Jorgensen et al., 2015). Várias espécies que compõem essa família são importantes patógenos causadores de infecções nosocomiais (Murray et al., 2015). Estudos epidemiológicos relatam que as principais espécies de importância clínica são *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., *Serratia* spp.,

*Morganella* spp., *Providencia* spp., *Shigella* spp, *Proteus* spp. e *Salmonella* spp. (Goldman e Green, 2015; Jorgensen et al., 2015; Murray et al., 2015; Singh et al., 2015).

*Klebsiella* spp., *Escherichia* spp e *Enterobacter* spp. compreendem os gêneros de enterobactérias mais comumente associadas a infecções hospitalares, estando relacionadas a diversas infecções extra-intestinais, como pneumonias e endocardites (Boucher et al., 2009; Murray et al., 2015). *Klebsiella pneumoniae* possui uma vasta gama de mecanismos de virulência intrínsecos, incluindo a produção de adesinas fimbriais e fatores antifagocíticos que facilitam sua invasão nas células do hospedeiro (Paczosa e Mecsas, 2016; Navon-Venezia et al., 2017). *Escherichia coli* contém numerosos sorotipos, alguns dos quais estão associados a doenças específicas. Essa espécie pode ainda produzir enterotoxinas e outros fatores de virulência associados à invasividade, como cápsulas e o antígeno K (Boucher et al., 2009; Goldman e Green, 2015). *Enterobacter* spp. estão frequentemente associadas a infecções do trato urinário e respiratórias, mas também são reconhecidas como importantes patógenos de infecções na corrente sanguínea (Pendleton et al., 2013; Davin-Regli e Pages, 2015).

Estudos globais de monitoramento de infecções hospitalares demonstraram uma maior prevalência de infecções causadas por enterobactérias na América Latina em comparação com América do Norte e Europa. As espécies dos gêneros *Escherichia* e *Klebsiella* são os principais agentes etiológicos de infecções que acometem pacientes em estado grave (Gales et al., 2012; Morrissey et al., 2013). No Brasil, dados publicados pela Agência Nacional de Vigilância Sanitária demonstraram que as enterobactérias representam a grande maioria das infecções hospitalares, incluindo infecções de corrente sanguínea em UTIs, entre os patógenos *Klebsiella pneumoniae* (18,2%), *Escherichia coli* (4,2%), *Enterobacter spp.* (4,0%), *Serratia spp.* (2,8%) e outras enterobactérias (3,2%) (Anvisa, 2017). O aumento da resistência antimicrobiana em bactérias responsáveis por IRAS é um grande desafio à Saúde Pública e a emergência de enterobactérias multirresistentes constitui um dos principais desafios aos laboratórios clínicos e as equipes de saúde (Davies e Davies, 2010; Frieri et al., 2017; Kapoor et al., 2017; Macgowan e Macnaughton, 2017).

### 2.3 Resistência bacteriana em enterobactérias

A resistência bacteriana em enterobactérias pode ocorrer pela presença de mecanismos intrínsecos ou adquiridos (Davies e Davies, 2010). A associação desses mecanismos leva a multirresistência, limitando drasticamente as opções terapêuticas para o tratamento das infecções causadas no ambiente hospitalar. A aquisição de resistência geralmente ocorre

devido ao surgimento de alterações genéticas que são expressas bioquimicamente. Diversos mecanismos podem estar envolvidos, porém a causa mais frequente é a aquisição de genes de resistência através de elementos genéticos móveis (Davies e Davies, 2010; Frieri et al., 2017; Kapoor et al., 2017; Macgowan e Macnaughton, 2017). A resistência aos β-lactâmicos constitui um problema de saúde global e emergente, uma vez que se trata de uma categoria formada por várias classes de medicamentos bastante utilizados pela clínica médica (Sampaio e Gales, 2016; Codjoe e Donkor, 2017).

Os antibióticos β-lactâmicos tem em comum na base da sua estrutura molecular um anel β-lactâmico central e tem como mecanismo de ação a inibição da síntese de parede celular bacteriana causando o rompimento da célula devido à pressão osmótica (Davies e Davies, 2010; Fernandes et al., 2013; Aslam et al., 2018). Os principais alvos dos agentes β-lactâmicos são as proteínas ligadoras de penicilina (PBPs), essas proteínas interagem com o anel β-lactâmico inibindo a síntese de peptideoglicanos, levando a lise celular. As classes de medicamentos que pertencem a esse grupo incluem penicilinas, cefalosporinas, monobactans e carbapenêmicos (Lin, J. et al., 2015; Munita e Arias, 2016; Frieri et al., 2017; Kapoor et al., 2017).

Os carbapenêmicos possuem o mais amplo espectro de atividade contra bactérias Gram-negativas, quando comparado a outros β-lactâmicos. Os principais antibióticos que compõe essa classe são: imipenem, meropenem, doripenem e ertapenem, sendo que o último é menos eficaz, pois apresenta espectro mais limitado frente a algumas bactérias Gram-negativas. Esses medicamentos podem ser utilizados através de terapia combinada com outros agentes antimicrobianos no tratamento de infecções graves e ou provocadas por microrganismos multirresistentes. São combinados com um antibiótico ativo principalmente contra bactérias Gram-positivas (Hawkey e Livermore, 2012). Porém, a combinação de carbapenêmicos com outros agentes apresenta algumas desvantagens, como efeitos adversos e principalmente o aumento da resistência a uma das drogas utilizadas na combinação (Zhanel et al., 2007; Papp-Wallace et al., 2011; Hawkey e Livermore, 2012; Codjoe e Donkor, 2017)

A resistência bacteriana aos β-lactâmicos pode ser causada por múltiplos mecanismos, incluindo alteração do sítio alvo das proteínas ligadoras de penicilinas (PBPs), falta e/ou expressão reduzida das proteínas de membrana externa, hiperexpressão de bombas de efluxo e o mais importante, a produção de β-lactamases (Figura 2) (Lin, Jun et al., 2015; Munita e Arias, 2016; Kapoor et al., 2017; Macgowan e Macnaughton, 2017; Aslam et al., 2018)

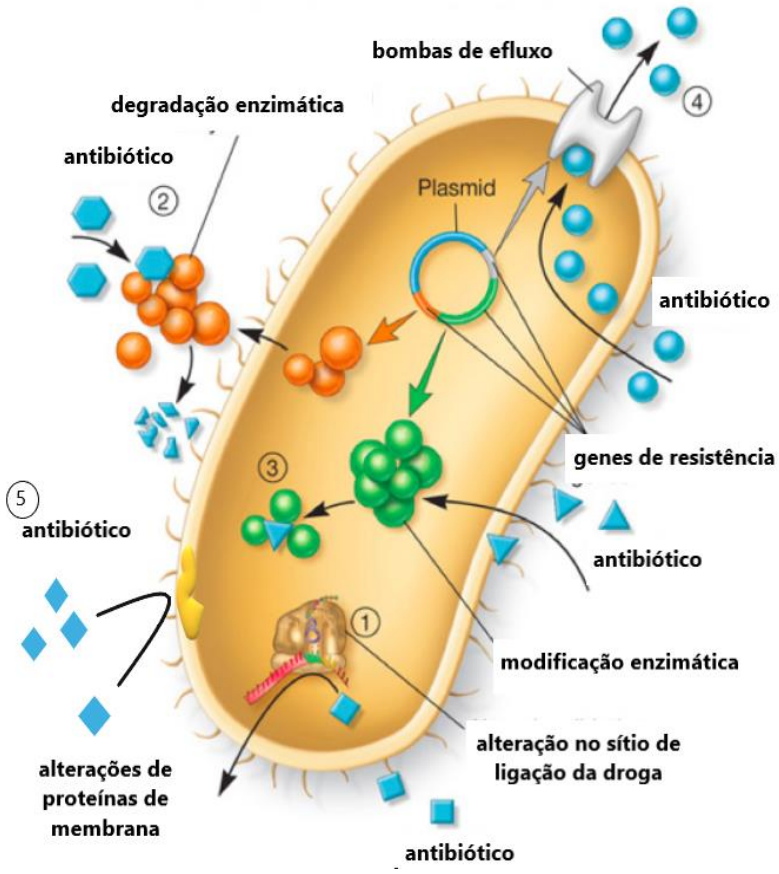


**Figura 1.** Desenho esquemático demonstrando os principais mecanismos de resistência bacteriana a antibióticos beta-lactâmicos. 1 - Alteração do sítio de ligação das drogas. 2 – Hidrólise de antimicrobianos por meio de degradação enzimática. Mecanismo mediado pela produção de betalactamases. 3 – Modificação das drogas por mecanismos enzimáticos. 4 – Hiperexpressão de bombas e sistemas de efluxo. 5 – Perda ou alterações de proteínas de membrana externa (porinas). Figura editada e obtida do site: (http://downofilebp.ga/peja/ampicillin-resistance-mechanism-in-bacteria-2257.php). Acessado em: 06 de novembro de 2018.

2.3.1 Alteração do sítio alvo das proteínas ligadoras de penicilinas (PBPs)

Os β-lactâmicos tem a capacidade de se ligar em proteínas ligadoras de penicilina (PBPs) e impedir que haja síntese da parede celular bacteriana. As PBPs são enzimas (transglicolases, transpeptidases e carboxipeptidases) que catalisam a formação de peptideoglicano, que atua como principal componente da parede celular. Modificações no sítio de ligação das drogas impedem que os antibióticos se liguem as proteínas e exerçam sua

função (Zapun et al., 2008; Hsieh et al., 2013; Blair et al., 2014; Sun, S. et al., 2014; Munita e Arias, 2016; Ealand et al., 2018).

### 2.3.2 Alterações em proteínas de membrana externa

Quando ocorre a perda ou a diminuição da expressão dos genes responsáveis pela expressão das porinas, pode haver redução da entrada de antimicrobianos na célula, diminuindo a concentração interna desses agentes, e consequentemente, contribuindo para o mecanismo de resistência aos β-lactâmicos. As mutações que afetam a expressão e/ou a função das porinas têm um impacto direto sobre a susceptibilidade aos agentes antimicrobianos. Estas mutações podem ter vários efeitos, como a diminuição ou perda total da função da porina, por meio da modificação do tamanho ou a condutância do canal de porina ou menor nível da expressão da mesma. As principais mutações responsáveis por esses efeitos são as que causam parada na tradução, exclusão ou elementos de inserção na sequência dos genes codificadores e mutações que afetam a regulação da expressão gênica. As mutações pontuais na região promotora podem causar redução da transcrição e consequentemente, perda de porinas (Fernandez e Hancock, 2012; Blair et al., 2014; Lin, J. et al., 2015; Miller, 2016; Munita e Arias, 2016).

As proteínas de membrana externa são uns dos principais mediadores da resistência aos carbapenêmicos, sendo que algumas delas interagem de maneiras diferentes com as drogas, portanto são afetadas por certos carbapenêmicos mais do que outros. Em enterobactérias as principais porinas relacionadas à resistência a carbapenêmicos são OmpC e OmpF. Em espécies do gênero *Klebsiella* alterações na expressão das porinas OmpK35 e OmpK36 são frequentemente relacionadas a resistência a carbapenêmicos (Fernandez e Hancock, 2012; Blair et al., 2014; Bajaj et al., 2016; Santajit e Indrawattana, 2016; Dam et al., 2018). A pressão seletiva exercida por carbapenêmicos também pode favorecer o surgimento de mutações em genes que regulam a expressão de porinas (Blair et al., 2014). Na maioria das vezes a perda e/ou a diminuição das porinas pode estar associada à produção de β-lactamases e a hiperexpressão de bombas de efluxo (Fernandez e Hancock, 2012; Blair et al., 2014; Bajaj et al., 2016; Balabanian et al., 2018). A Tabela 1 demonstra alguns exemplos de porinas que foram relacionadas à resistência bacteriana em bactérias Gram-negativas.

**Tabela 1.** Porinas relacionadas com a resistência em diferentes espécies de bactérias Gram-negativas.

| Espécie | Porina | Antibiótico |
|---|---|---|
| *Pseudomonas aeruginosa* | OprD | Carbapenêmicos |
| *Escherichia coli* | OmpC | β-lactâmicos |
| | OmpF | β-lactâmicos |
| *Serratia marcescens* | OmpF | β-lactâmicos |
| | OmpC | β-lactâmicos |
| *Klebsiella pneumuniae* | OmpK35 | Cefalosporinas, carbapenêmicos e quinolonas |
| | OmpK36 | Carbapenêmicos |
| *Enterobacter cloacae* | OmpF | Carbapenêmicos |
| *Enterobacter aerogenes* | OmpC | Carbapenêmicos |
| | OmpF | Carbapenêmicos |
| | Omp36 | Imipenem, cefepime |
| *Klebsiella oxytoca* | OmpK36 | Carbapenêmicos |
| *Salmonella enterica* | OmpC | β-lactâmicos |
| | OmpF | β-lactâmicos |
| *Acinetobacter baumanii* | CarO | Carbapenêmicos |

Adaptado de Fernandéz e Hancock, 2012.

2.3.3 Hiperexpressão de bombas de efluxo

A hiperexpressão dos sistemas de efluxo surgiu como um sistema evolutivo das bactérias para evitar que compostos tóxicos se acumulassem no interior das células. Este sistema tem função de bombear moléculas tóxicas do conteúdo intracelular, em um processo que não envolve a alteração ou degradação dos fármacos. As bombas de efluxo são agrupadas em várias super famílias: *Small Multidrug-resistance* (SMR) *Resistance Nodulation Division* (RND), *Major Facilitator Superfamily* (MFS), ATP-bindingcassette (ABC) e *Multidrug and toxic compound extrusion* (MATE) (Blair et al., 2014; Sun, J. et al., 2014; Aslam et al., 2018; Du et al., 2018). Algumas bombas de efluxo funcionam a partir de um substrato específico, porém a maioria é capaz de transportar uma vasta gama de substâncias, nesse caso essas bombas são classificadas como multurresistentes (Blair et al., 2014). Geralmente mutações são responsáveis pelo aumento do nível de expressão da bomba de efluxo, resultando no aumento da resistência aos compostos (Fernandez e Hancock, 2012; Blair et al., 2014; Bajaj

et al., 2016; Aslam et al., 2018; Du et al., 2018). Em bactérias Gram-negativas as bombas de efluxo mais comuns pertencem à superfamília RND, sendo que as mais estudadas e caracterizadas até o momento são AcrB enterobactérias e MexB em *P. aeruginosa* (Fernandez e Hancock, 2012; Blair et al., 2014; Santajit e Indrawattana, 2016; Yamasaki et al., 2017; Du et al., 2018).

### 2.3.4 Produção de β-lactamases

O uso indiscriminado de beta-lactâmicos associado à exposição a outros antimicrobianos e a ausência de protocolos de controle e prevenção de infecção hospitalar contribuiu para o surgimento de β-lactamases, uma família de enzimas bacterianas capazes de hidrolisar esses medicamentos. A hidrólise de antimicrobianos pelas β-lactamases é o principal mecanismo de resistência aos β-lactâmicos em bactérias Gram-negativas (Blair et al., 2014; Bonomo, 2016; Progress on antibiotic resistance, 2018; Aslam et al., 2018). O mecanismo de ação dessas enzimas é mediado pela sua capacidade de quebrar a ligação amida no anel β-lactâmico presente na estrutura dos antibióticos. Os genes que codificam a produção dessas enzimas sofrem mutações constantemente em resposta à pressão exercida pelos antimicrobianos, contribuindo para o surgimento de novas classes de enzimas com espectro de atividade cada vez maior. A síntese das β-lactamases pode ser cromossômica ou mediada por plasmídeos e transposons (Blair et al., 2014; Bonomo, 2016; Frieri et al., 2017; Bush, 2018; Morehead e Scarbrough, 2018).

Diversos tipos de β-lactamases já foram descritas e várias tentativas de classificação já foram propostas, sendo que as duas mais importantes são a de Ambler e a de Bush, Jacoby e Medeiros (Ambler, 1980; Bush et al., 1995), esta última revisada em 2010 (Bush e Jacoby, 2010). A Tabela 2 demonstra de maneira simplificada a comparação entre elas. A classificação proposta por Ambler esta relacionada com a estrutura molecular das enzimas e a sequência de aminoácidos codificados. Nessa classificação quatro classes moleculares foram descritas: I, β-lactamases de espectro estendido (ESBLs) e limitado; II, pencilinases; III, metalo-β-lactamases (MBLs) e IV, oxacilinases (Ambler, 1980). A classificação proposta por Bush relacionou propriedades inibitórias e os substratos preferenciais de cada enzima. No entanto, ao longo dos anos essa classificação foi atualizada, sendo que características estruturais e funcionais das β-lactamases foram consideradas (Bush et al., 1995; Bush e Jacoby, 2010; Bush e Fisher, 2011).

**Tabela 2**. Classificação funcional e molecular dos principais grupos de β-lactamases com importância clínica.

| BUSH-JACOBY, 2010 | | AMBLER, 1980 | Principais enzimas | Características funcionais |
|---|---|---|---|---|
| **Grupo Funcional** | **Subgrupos** | **Classe Molecular** | | |
| 1 | | C | *E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1 | Enzimas cromossômicas e plasmidiais conferem resistência a todos os β-lactâmicos, exceto carbapenêmicos. Não inibidas pelo ácido clavulânico. |
| | 1e | C | GC1, CMY-37 | Hidrólise aumentada à ceftazidima e não inibidas pelo ácido clavulânico. |
| 2 | | A, D | | Grande maioria das enzimas é inibida pelo ácido clavulânico. |
| | 2ª | A | PC1 | Penicilinases produzidas por *Staphylococcus* spp. e *Enterococcus* spp. Conferem alto grau de resistência às penicilinas. |
| | 2b | A | TEM-1, TEM-2 e SHV-1. | β-lactamases de espectro limitado de bactérias Gram-negativas. |
| | 2be | A | TEM, SHV, CTX-M, PER, VEB | β-lactamases de espectro ampliado, conferindo resistência às cefalosporinas de amplo espectro e monobactâmicos. |
| | 2br | A | β-lactamases derivadas de TEM | Resistentes aos inibidores de β-lactamases (IRT). |
| | 2ber | A | TEM-50 | Enzimas não inibidas pelo ácido clavulânico, tazobactam e sulbactam. |
| | 2c | A | PSE-1, CARB-3 | Enzimas que hidrolisam a carbenicilina com maior eficiência. |
| | 2ce | A | RTG4 | Hidrólise aumentada de carbenicilina, cefepima e cefpiroma. |
| | 2d | D | OXA-1, OXA-10 | Enzimas que hidrolisam a cloxacilina (oxacilina); fracamente inibidas pelo ácido clavulânico. |
| | 2de | D | OXA-11, OXA-15 | Enzimas que hidrolisam a cloxacilina (oxacilina) e oxinimo-β-lactâmicos. |
| | 2df | D | OXA-23, OXA-48 | Enzimas que hidrolisam a cloxacilina (oxacilina) e carbapenens. |
| | 2e | A | CepA | Cefalosporinases inibidas pelo ácido clavulânico. |
| | 2f | A | KPC, IMI, SME, GES | Enzimas que hidrolisam carbapenens com sítio ativo serina, inibidas pelo ácido clavulânico. |
| 3 | 3a, 3b, 3c | B | IMP, VIM, SIM, GIM, NDM | Metalo-β-lactamases que conferem resistência aos carbapenêmicos e todos os outros β-lactâmicos, com exceção dos monobactâmicos. Não são inibidas pelo ácido clavulânico. |
| 4 | | ND | | Enzimas não classificadas nos outros grupos. |

ND, classe excluída da classificação atual (Adaptado de Bush &Jacoby, 2010).

As β-lactamases do tipo AmpC, ou cefalosporinases cromossomais, são enzimas que tem a capacidade de hidrolisar penicilinas e cefamicinas com grande eficácia, enquanto o aztreonam e as cefalosporinas de terceira geração são hidrolisadas com menor eficácia. (Jacoby, 2009; Marsik e Nambiar, 2011; Gupta et al., 2014). As enzimas β-lactamases de Espectro Limitado e Ampliado (ESBLs) apresentam potente atividade hidrolítica contra as penicilinas e as cefalosporinas de primeira e segunda geração e o aztreonam. As enzimas classificadas nesse grupo incluem também as β-lactamases de espectro limitado, as quais são em sua maioria inibidas pelos inibidores de serino-β-lactamases. Foram descritas inicialmente em membros da família *Enterobacteriaceae,* sendo que as enzimas mais comumente descritas são: TEM, SHV e as Cefotaximases (CTX-M) (Bonomo, 2016; Kapoor et al., 2017; Bush, 2018).

As carbapenemases formam o grupo mais importante de β-lactamases. As carbapenemases da classe A, ou serino-carbapenemases hidrolisam uma ampla variedade de β-lactâmicos, incluindo penicilinas, cefalosporinas, aztreonam, carbapenêmicos, e são inibidas pelo ácido clavulânico e tazobactam (Munita e Arias, 2016; Philippon et al., 2016; Macgowan e Macnaughton, 2017). As maiores famílias de carbapenemases da classe A incluem as enzimas *Notmetalloenzyme carbapenemase (*NMC-A), Imipenemase (IMI), *Serratia marcescens* enzyme (SME) e *Klebsiella pneumoniae* carbapenemase (KPC). A KPC é a carbapenemase mais comum isolada de enterobactérias e confere resistência aos antibióticos de amplo espectro, como os carbapenêmicos, penicilinas, cefalosporinas e monobactâmicos (Stoesser et al., 2017; Palzkill, 2018).

Normalmente a KPC é codificada por genes localizados em plasmídeos e frequentemente estão relacionados com o Transposon Tn4410 com diferentes isoformas identificadas. Estas estruturas associadas a esses genes de resistência indicam a presença de um mecanismo de transposição replicativa, que permite a propagação de sequências de codificação da KPC entre diferentes unidades genéticas (Blair et al., 2014; Bonomo, 2016; Palzkill, 2018). Atualmente já foram descritas 22 variantes da enzima KPC, sendo sequencialmente classificadas como KPC-2 a KPC-23 (Naas et al., 2016). Em 2010 após vários casos de surtos de KPC no Brasil, a Agência Nacional de Vigilancia Sanitária (ANVISA) restringiu a venda de antibióticos sem prescrição médica, a fim de controlar o uso inadequado desses medicamentos.

As metalo-β-lactamases (MBL) ou carbapenemases da classe B são enzimas que apresentam potente atividade contra carbapenêmicos, mas diferem de outras carbapenemases

em três aspectos: requerem íons $Zn^{+2}$ ou outros cátions divalentes como cofator no sítio ativo; são resistentes à ação dos inibidores das serino-β-lactamases, embora sofram inibição por agentes quelantes como o ácido etilenodiamino tetra-acético (EDTA), derivados de tiol e do ácido dipicolínico; não hidrolisam monobactans como o aztreonam (Lutgring e Limbago, 2016; Salahuddin et al., 2018). Normalmente os genes que codificam MBLs estão inseridos em estruturas genéticas móveis, e as enzimas codificadas por estes genes conhecidas como MBLs móveis ou adquiridas. Atualmente, já foram descritas 10 sub-classes de MBLs adquiridas, porém as mais conhecidas são: *imipenemase* (IMP), *Verona imipenemase* (VIM), *São Paulo metallo-β-lactamase* (SPM), *German imipenemase* (GIM), *Seul imipenemase* (SIM) e *New Delhimetallo- β-lactamase* (Bonomo, 2016; Bush, 2018).

As oxacilinases (OXA) foram nomeadas assim por apresentarem uma atividade hidrolítica potente contra as penicilinas resistentes às penicilinases, dentre elas: a oxacilina, a cloxacilina e a meticilina. Uma característica marcante desse grupo de enzimas é o fato de serem fracamente inibidas pelo ácido clavulânico, mas fortemente inibidas pelo cloreto de sódio (Evans e Amyes, 2014; Munita e Arias, 2016; Santajit e Indrawattana, 2016). Essas enzimas podem ser classificadas em diferentes subgrupos: oxacilinases de espectro limitado, oxacilinases do tipo ESBLs, oxacilinases do tipo carbapenemases. Atualmente mais de 400 tipos de oxacilinases já foram descritas, sendo que uma minoria demonstrou baixos níveis de atividade hidrolítica contra antibióticos carbapenêmicos (Jeon et al., 2015; Bush, 2018).

### 2.4 Mecanismos de resistência a polimixinas

As polimixinas são medicamentos da classe das colistinas, constituindo um dos últimos recursos para o tratamento de bactérias Gram-negativas multirresistentes. Tratam-se de um peptídeo policatiônico que compartilham estruturas primárias quase idênticas, das quais a Polimixina A e E são atualmente utilizados na terapia antimicrobiana (Bialvaei e Samadi Kafil, 2015; Yu et al., 2015; Garg et al., 2017). Esses medicamentos foram originalmente descobertos em 1947 e passaram a ser utilizados no tratamento de infecções bacterianas, porém, na década de 1970 o seu uso passou a ser restringido e praticamente abandonado na década de 1980, devido suas características de nefrotoxicidade e neurotoxicidade. Entretanto em meados de 1990 a dificuldade no desenvolvimento de novos medicamentos, associada à emergência da resistência bacteriana levou à re-introdução dessas drogas na clínica médica (Dijkmans et al., 2015; Trimble et al., 2016).

A polimixina atua na membrana das bactérias Gram-negativas, ligando-se seletivamente ao lipopolissacarídeo (LPS). O LPS é composto por três domínios: lípido A, oligossacarídeo central e o antígeno O. Entre eles, o domínio mais importante é o lipídio A, tendo papel essencial na estabilidade e integridade da estrutura da membrana. Alguns cátions divalentes como $Ca^{2+}$ e $Mg^{2+}$ atuam como pontes entre as moléculas promovendo atração eletrostática e facilitando a ligação das polimixinas na estrutura do lipídio A. Essas interações levam ao enfraquecimento do lipídio A induzindo a desestabilização da membrana e consequentemente a lise e morte celular (Bialvaei e Samadi Kafil, 2015; Yu et al., 2015) (Figura 2).

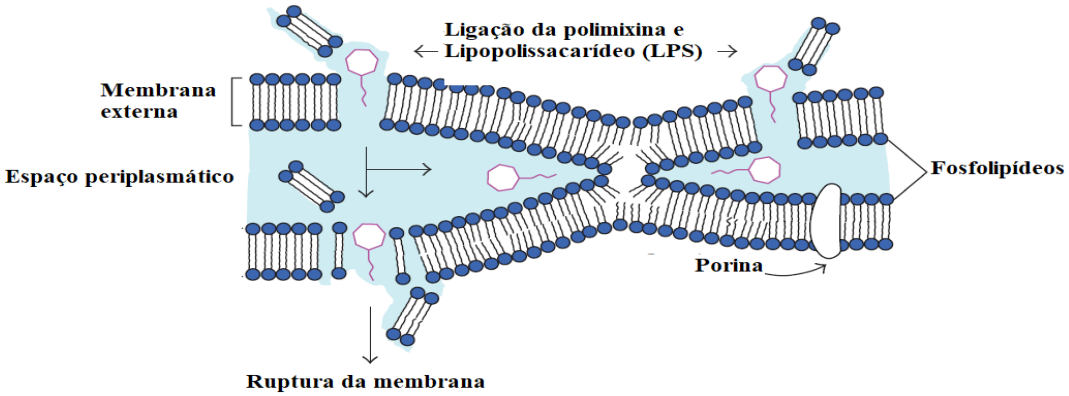


**Figura 2**. Mecanismo de ação da polimixina. Adaptado de Yu et al, 2015.

A resistência a polimixina é particularmente preocupante, uma vez que desempenha o papel de último recurso contra infecções bacterianas por Gram-negativas multidroga-resistentes (MDR), incluindo enterobactérias, *P. aeruginosa* e *A. baumannii* que são resistentes a quase todos os outros antibióticos atualmente disponíveis na clínica médica (Garg et al., 2017). A resistência à polimixina é mediada principalmente pela modificação estrutural dos LPSs da membrana, por meio de sistemas reguladores. A célula bacteriana tem a capacidade de reduzir a atração eletrostática entre os grupos fosfato do Lipídio A e a molécula de polimixina, essa mudança na carga da membrana via modificação lipídica impede que a droga se ligue e promova a lise celular, levando a resistência (Trimble et al., 2016; Watkins et al., 2016; Brown e Dawson, 2017).

O mecanismo mais comum é modificação da estrutura química do Lipídeo A através da biossíntese e adição de fosfoetanolamina (pEtN) e L-4-aminoarabinose (L-Ara4N). Essas alterações ocorrem devido a ativação de sistemas, que são regulados negativamente pelo gene *mgrB*. O sistema de dois componentes Pho-PQ age como um regulador mestre da resistência a

polimixina. Em resposta a concentrações sub-letais de polimixina, o sensor de quinase PhoQ irá fosforilar o PhoP, levando à ativação do sistema PmrA-PmrB via proteína PmrD. Consequentemente, o sistema PmrA-PmrB ativa a expressão do operon *arnBCADTEF*, necessário para a modificação covalente de grupos fosfato no lípido A. Todas essas modificações estruturais diminuem a carga negativa da membrana, evitando a interação coma polimixina (Olaitan et al., 2014; Baron et al., 2016; Jeannot et al., 2017).

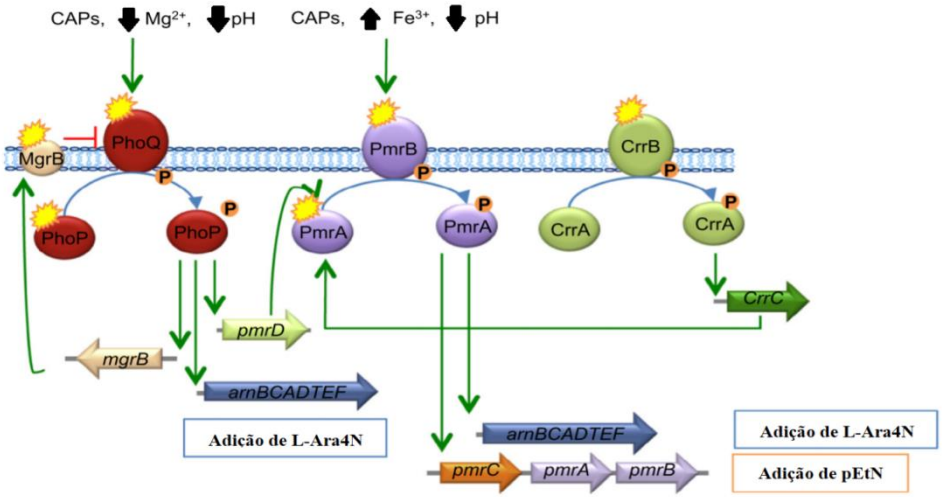


**Figura 3**. Representação esquemática da regulação e ativação de genes envolvidos na resistência a polimixina. Adaptado de Jeannot et al., 2017.

Um novo sistema regulador denominado *crrAB* foi descrito como mediador da resistência a polimixina composto por uma histidina quinase (*crB*) e seu regulador de resposta (*crA*) inativado ou ausente em algumas cepas de *K. pneumoniae*, levando a ativação do sistema *pmrCAB* (Baron et al., 2016). Numerosas mutações já foram descritas nos genes envolvidos na resistência a polimixina. A inativação do gene *mgrB* por meio de mutações nonsense, deleção de nucleotídeos e truncamento por elementos de inserção é o mecanismo mais frequente encontrado em *K. pneumoniae*. Com relação à inativação por elementos de inserção (ISs), a família IS5 é o elemento mais frequentemente encontrado, seguido pela família IS1. Esses ISs podem ser inseridos na região promotora ou codificadora do gene (Jayol et al., 2015; Aires et al., 2016; Giordano et al., 2018). O lipídio A também pode ser modificado pela adição da pEtN. Esse é o principal mecanismo de resistência encontrado em *A. baumannii* e vários genes podem estar envolvidos nesse processo incluindo *eptA* (*pmrC*), *eptB* (*pagC*) e *eptC* (*cptA*) (Baron et al., 2016).

A perda do antígeno-O através da mutação de genes envolvidos na biossíntese desse componente já foi descrita em *Yersinia enterocolitica* e em *Salmonella* spp. Em

enterobactérias outros genes reguladores que modulam a biossíntese de lipídios A, como o gene r*amA*, pode estar relacionado com a diminuição da susceptibilidade a polimixina (Olaitan et al., 2014; Gregoire et al., 2017). Bombas de efluxo também podem estar envolvidas na resistência a polimixina, sendo que vários reguladores de bombas de efluxo têm sido descritos em diferentes espécies, tais como Sap (*sensitive antimicrobial peptides*) proteins, BrlR, KpnEF ou o complexo AcrAB-TolC. Geralmente, a ativação dessas bombas leva a aumento na resistência a vários antibióticos ao mesmo tempo, incluindo a polimixina (Yu et al., 2015; Trimble et al., 2016). Acredita-se que a cápsula bacteriana também desempenhe um papel importante na resistência à polimixina, visto que o aumento da expressão de genes codificadores da síntese de cápsula foi observado em cepas de *K. pneumoniae*, *E. coli* e *P. aeruginosa* causando da resistência a polimixina (Baron et al., 2016).

A resistência à polimixina foi inicialmente descrita ligada a mecanismos cromossomomais, sem possibilidade de transferência horizontal, entretanto em 2016 foi identificado em isolados bacterianos um novo gene de resistência mediado por plasmídeo (Liu et al., 2016). O *mcr-1* é um gene que codifica uma enzima da família fosfoetanolaminatransferase responsável pela síntese e adição de pEtN ao lípido A. A primeira descrição aconteceu na China em isolados bacterianos de alimentos de origem animal (frangos e suínos). Em humanos o primeiro isolado foi detectado na América Latina em uma cepa de *E. coli* recuperada de um paciente hospitalizado (Sun et al., 2018; Wang, R. et al., 2018; Xu, F. et al., 2018). Até o momento já foram descritos oito variantes do gene *mcr-1*, sendo sequencialmente nomeadas como *mcr*-1 a *mcr-8*. Em enterobactérias os genes *mcr*-1, *mcr*-2, e *mcr*-3 foram descritos em plasmídeos e recentemente identificados em cromossomos de *Moraxella* spp. e *Aeromonas* (Liu et al., 2017; Teo et al., 2017; Sun et al., 2018; Wang, X. et al., 2018).

A disseminação de genes de resistência entre diferentes espécies bacterianas através da transferência horizontal mediada por plasmídeos contribui para uma grande variedade de fenótipos multirresistentes em bactérias capazes de causar infecção em humanos e animais. A coexistência de mcr-1 e outros genes de resistência indicam a existência de diferentes vias para a transmissão horizontal da resistência à colistina e seu alto potencial de propagação. O gene mcr-1 pode estar associado a diferentes tipos de plasmídeos, incluindo IncI2, IncHI2, IncP, IncFIP e IncX. Esses plasmídeos também estão associados a outros genes que conferem resistência a quinolonas, cefalosporinas e fosfomicina (Baron et al., 2016; Al-Tawfiq et al., 2017; Sun et al., 2018).

## 2.5 Epidemiologia molecular de Enterobactérias resistentes a polimixina

A resistência antimicrobiana é uma das mais importantes ameaças à saúde humana e animal no século XXI e a resistência a polimixina aparece como um problema ainda mais grave, agravada pelo fato de que alguns países não possuírem uma política eficiente sobre o uso de antibióticos em seres humanos e na produção animal. A prevalência mundial de resistência às polimixinas é de 10% entre as bactérias Gram-negativas, com índices maiores em países do Mediterrâneo e Sudeste da Ásia (Bialvaei e Samadi Kafil, 2015; Al-Tawfiq et al., 2017; Moawad et al., 2018). O aumento do uso de polimixina para infecções por bactérias Gram-negativas multirresistentes levou a emergência da resistência em vários países do mundo, cuja prevalência pode variar entre regiões, no entanto, alguns países como Japão e África do Sul não têm acesso a polimixina e algumas áreas do mundo têm apenas formulação de colistina, enquanto em outras áreas incluindo, EUA, Brasil, Malásia e Cingapura, os clínicos utilizam a formulação parenteral de colistina ou de polimixina B (Bergen et al., 2012; Bialvaei e Samadi Kafil, 2015; Garg et al., 2017).

A maioria dos relatórios dos EUA apresentam taxas de resistência para *P. aeruginosa* e *A. baumannii* inferiores a 5,5%. Entretanto, estudos de resistência em *K. pneumoniae* relatam taxas mais altas. Nos países africanos os relatórios são escassos, estudos realizados na Nigéria e África do Sul relataram taxas de resistência menores que 10%. Na Europa as taxas de resistência a polimixina podem variar bastante, estando relacionada principalmente com IRAS. Na região da Ásia a resistência à colistina é comum principalmente em isolados de *Enterobacter spp*. e *Klebsiella spp*., sendo observada em todos os países, exceto em Cingapura, com taxas variando de 13,8% (Índia) a 50% (Filipinas) (Bialvaei e Samadi Kafil, 2015; Al-Tawfiq et al., 2017; Garg et al., 2017; Bitrus et al., 2018).

A polimixina é frequentemente prescrita em hospitais brasileiros. As taxa de *K. pneumoniae* resistente à polimixina aumentaram de 1,8% em 2009 para 15% em 2013, 35,5% em 2015. Atualmente é considerada endêmica, estando frequentemente associadas a altas taxas de morbidade e mortalidade dos pacientes. Um estudo realizado em São Paulo em isolados de *K. pneumoniae* produtora de KPC demonstrou que o índice de resistência a polimixina variou de 0% em 2011 para 27% em 2015 (Bartolleti et al., 2016; Sampaio e Gales, 2016; Rossi et al., 2017). A exposição prolongada e esses medicamentos pode ter sido um fator determinante para o desenvolvimento e disseminação dessa resistência. Diversos estudos realizados em diferentes hospitais brasileiros identificaram cepas de enterobactérias resistentes a polimixina, cujo mecanismo responsável inclui alterações no LPS da membrana

através da inativação do gene mgrB e a presença do gene mcr-1 (Aires et al., 2016; Sampaio e Gales, 2016; Dalmolin et al., 2018).

Apesar de ter sido descrito apenas em 2016, um estudo realizado em cepas de *E. coli* obtidas de fazendas de frangos demonstrou que o mais antigo isolado carregando o gene mcr-1 foi da década de 1980, quando a colistina foi introduzida na pecuária da China. Estudos posteriores mostraram que a prevalência do gene mcr-1 foi 20% em cepas de animais e 1% em cepas humanas na China (Liu et al., 2016; Shen et al., 2016). Apenas em alguns meses após ter sido relatado pela primeira vez, o mcr-1 já foi detectado em isolados de humanos, animais e do meio ambiente em vários países da Europa, Ásia, América do Sul, América do Norte e África, sendo identificado em diversos gêneros bacterianos incluindo *Escherichia, Klebsiella, Salmonella, Shigella, Enterobacter* e principalmente de *E. coli* (Caselli, D'accolti, et al., 2018; Dalmolin et al., 2018; Kurekci et al., 2018; Lorenzoni et al., 2018; Wang, R. et al., 2018; Yamamoto et al., 2018).

A identificação de genes de resistência à polimixina em microrganismos isolados de alimentos animais reacendeu o debate sobre a contribuição do uso insdisciminado de antibióticos em animais na detecção de altos níveis de resistência em humanos. Acredita-se que a resistência a polimixina codificada pelo gene *mcr-1* tenha sido disseminada de animais para humanos, com base no fato de que foram descritos inicialmente em grupos de animais que consomem uma grande quantidade desse antibiótico durante a criação. Estima-se que aproximadamente 12.000 toneladas de colistina sejam utilizadas por ano na produção de alimentos, e até 2021 esse consumo deverá subir para 16.500 toneladas. Tendo em vista esse cenário medidas de prevenção são necessárias para reverter à dependência contínua desse antibiótico e controlar a disseminação dessa resistência (Bialvaei e Samadi Kafil, 2015; Liu et al., 2016; Shen et al., 2016; Al-Tawfiq et al., 2017; Bitrus et al., 2018).

De acordo com o Centro de Controle e Prevenção de Doenças (CDC), o conceito "*One Health*" reconhece que a saúde humana esta diretamente relacionada à saúde dos animais e do meio ambiente, portanto para que o combate a resistência bacteriana seja eficiente os programas de saúde devem ser baseados nesses três campos. Uma das principais abordagens *One Health* é diminuir o consumo de todos antibióticos. No caso da colistina seu uso em humanos deve se restringir para o tratamento baseado em testes de suscetibilidade, ou uso empírico em circunstâncias claramente definidas. Atualmente uma ação liderada e coordenada pelas Nações Unidas, definiu diversas abordagens em Saúde Única para o consumo de antibióticos, a fim de estabelecer mecanismos de monitoramento e avaliação para garantir que

os países cumpram o compromisso, o que será essencial para conter a disseminação da resistência a polimixina (Al-Tawfiq et al., 2017; Kahn, 2017; Balkhy et al., 2018; Walsh, 2018). No Brasil, a fim de diminuir o consumo de antimicrobianos na produção animal o Ministério da Agricultura, Pecuária e Abastecimento publicou a instrução normativa No - 45, de 22 de novembro de 2016, proibindo em todo o território nacional, a importação e a fabricação de sulfato de colistina, com a finalidade de melhorar o desempenho na alimentação animal.

### 2.6 Terapia antisense no combate a resistência bacteriana

Nas últimas cinco décadas, houve um declínio significativo na descoberta e desenvolvimento de novos antibióticos. O aumento acentuado da resistência bacteriana aos antibióticos disponíveis exige a elaboração de estratégias que assegure o uso apropriado de antibióticos e evidenciam a necessidade de investimentos em novas abordagens terapêuticas para o tratamento dessas infecções (Sully, Geller et al. 2016). A terapia genética consiste na introdução de DNA ou RNA em células, para tratamento de diversas patologias, podendo desta forma, ser utilizada em diversas áreas da medicina como a engenharia genética e regeneração de tecidos (Abou-El-Enein et al., 2015). O tratamento recorrendo a este tipo de terapia visa diferentes objetivos, como a substituição de um gene que se encontra mutado pelo que não possui mutação, a introdução de um novo gene na célula resultando assim na expressão de uma nova proteína com funções terapêuticas, ou ainda, a modulação da expressão de genes endógenos e exógenos.

Estas alterações ao nível molecular são importantes ferramentas no tratamento de doenças adquiridas ou hereditárias, nomeadamente, infecções virais, cancros, doenças auto-imunes, entre outras (Gehrig et al., 2014; Gould, 2014). Uma vez que o material genético é introduzido para dentro da célula-alvo e incorporado no DNA nuclear, eles podem induzir o silenciamento de genes, modular a baixa-regulação, modificação, ou reparação dos genes de células-alvo (Figura 4). Essas estruturas são agentes ideais para o desenvolvimento de novas estratégias para terapia antisense de doenças infecciosas, através do silenciamento da expressão de genes de interesse, devido à sua elevada resistência à degradação enzimática, elevada afinidade para o RNA e sua incapacidade para ativar RNAses (Abushahba et al., 2016; Rossor et al., 2018; Xu, J. Z. et al., 2018; Xue et al., 2018).

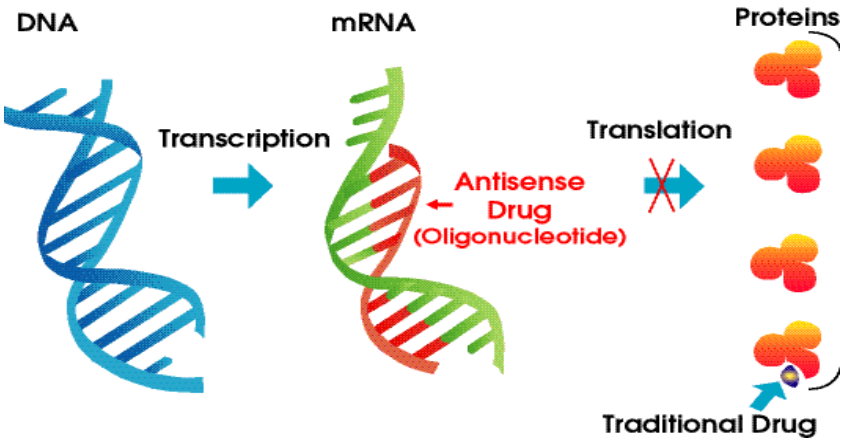


Figura 4. Representação do mecanismo de ação de drogas antisense. Adaptado de Pharmamirror, 2014.

Os peptídeos ácidos nucléicos (PNAs) anti-sentido foram aplicados a sistemas bacterianos e modificados para melhorar sua capacidade de hibridização e propriedades de estabilidade, a fim de proporcionar efeitos inibitórios mais potentes (Eriksson et al., 2002). Existem regras teóricas para a escolha da região para anelamento do PNA-alvo, sendo que a principal região considerada susceptível para inibição antisense é o códon de iniciação da replicação. Experimentos realializados *in vitro* têm demonstrado que a tradução eucariótica pode ser inibida nesta região (Brophy e Voigt, 2016; Gooding et al., 2016; Lehto et al., 2016; Sully e Geller, 2016; Rossor et al., 2018). Os resíduos de lisina de carga positiva incluídos na estrutura dos PNA podem contribuir na localização da molécula na superfície negativamente carregada de bactérias Gram-negativas, enquanto ácidos nucléicos normais seriam repelidos. Portanto, a conjugação de peptídeos aos PNAs, pode promover diversas vantagens para o desenvolvimento de agentes anti-sentido (Delcroix e Riley, 2010; Gupta et al., 2016; Lehto et al., 2016; Xue et al., 2018).

A utilização de agentes antisense para inibição de mecanismos de resistência a fim de restaurar a susceptibilidade a antimicrobianos aparece como uma estratégia inovadora no tratamento de infecções causadas por bactérias multirresistentes (Ji e Lei, 2013). A eficácia do agente antisense anti-*mecA* para inibir a expressão do gene responsável pela resistência a meticilina em isolados de *Staphylococcus* sp foi descrita em 2015 (Liang et al., 2015). Um estudo avaliou a utilização de um agente antisense na inibição do crescimento de uma cepa de *Streptococcus pyogenes* através da inibição da expressão de um gene constitutivo denominado *gyrA*, requerido para o crescimento bacteriano. Os resultados obtidos demonstraram redução

do crescimento bacteriano pela utilização do PNA empregado além da atividade sinérgica com outros antimicrobianos convencionais (Patenge et al., 2013).

O efeito de um agente antisense conjugado a um peptídeo de penetração celular foi avaliado na inibição do crescimento de bactérias gram-negativas *in vivo* e no tratamento de animais infectados. Os resultados mostraram que o tratamento de dos animais com o peptídeo conjugado reduziu significativamente a carga bacteriana no sangue e preveniu a evolução de uma infecção fatal (Tan et al., 2005). Outro estudo relatou a atividade de um peptídeo ácido nucléico na inibição de genes essenciais ao crescimento bacteriano em cepas de *Klebsiella pneumoniae* produtoras de β-lactamases (Kurupati et al., 2007). Em 2014 uma pesquisa realizada com objetivo de avaliar a eficácia de agentes antisense frente a bactérias Gram-negativas não fermentadoras demonstrou a capacidade de inibição do crescimento de cepas de *Acinetobacter baumannii* através da ação em genes constitutivos da espécie (Wang et al., 2014).

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# 4 OBJETIVOS

## 4.1 Geral

Caracterizar os mecanismos de resistência em enterobactérias resistentes a polimixina e avaliar o potencial antimicrobiano de um agente antisense frente à patógenos multirresistentes, buscando contribuir no controle da disseminação destas cepas.

## 4.2 Específicos

- Caracterizar os mecanismos genéticos envolvidos na resistência de enterobactérias resistentes a polimixina através do sequenciamento do genoma de cepas envolvidas em um surto de infecção hospitalar.
- Associar os dados genômicos e epidemiológicos, buscando identificar os fatores de risco envolvidos na aquisição desta resistência.
- Avaliar medidas de contenção da disseminação de cepas multirresistentes no ambiente hospitalar.
- Avaliar novas estratégias para inibição de mecanismos de resistência através do silenciamento da expressão de genes.
- Avaliar *in vitro* a eficácia de peptídicos sintéticos contra enterobactérias multirresistentes.

# 5 APÊNDICES

**Artigo 1: Emergence and clonal spread of polymyxin-resistant *Klebsiella pneumoniae* with multiple *mgrB* gene alterations: Molecular and epidemiological surveillance**

**Short running title:** Polymyxin-resistant *Klebsiella pneumoniae*.

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**ABSTRACT**

**Objectives:** The prevalence of polymyxin-resistant Enterobacteriaceae is increasing worldwide, limiting therapeutic options. Here we present molecular and epidemiological characteristics associated with polymyxin-resistant *Klebsiella pneumoniae*. All isolates were characterized phenotypically and genotypically, including by whole genome sequencing (WGS).

**Methods:** A total of 30 polymyxin-resistant *K. pneumoniae* isolates were collected from two Brazilian hospitals. Whole genome sequencing was performed for *in silico* analysis of multi-locus sequence typing (MLST), their resistome, virulome, plasmid typing and their core single nucleotide polymorphism (SNP) genotypes.

**Results:** Resistome analysis revealed the presence of $bla_{KPC}$ gene and less frequently $bla_{SHV}$, $bla_{TEM}$, $bla_{CTX\text{-}M}$ and $bla_{OXA}$. Other genes conferring resistance to aminoglycoside, fluoroquinolone, phenicol, sulphonamide, tetracycline, trimethoprim and macrolide-lincosamide-streptogramin were also detected. Clonal expansion of polymyxin-resistant *K. pneumoniae* isolates were observed and WGS showed that isolates were linked with three resistant mechanisms involving *mgrB* gene including inactivation by an insertion sequence (IS) and nonsense point mutations. Thus, we identified an insertion of an 80-bp sequence, resulting in 26- amino-acid duplication of MgrB in six strains. This is the first observation of this type of alteration causing polymyxin resistance.

**Conclusions:** Our findings demonstrate that mgrB alterations were shown to be the most common source of polymyxin-resistance in Brazilian clinical settings. Interestingly, distinct genetic events were identified among clonally related isolates. Thus the clinical implications and investigation of the phenotype mechanisms poses a major threat to patient safety and control of these infections. The spread of pan-resistant strains can be controlled, but may be difficult, particularly in long-term care facilities.

**Keywords:** Polymyxin-resistance, mgrB, *Klebsiella pneumoniae*, nucleotide sequencing, Intensive care unit (ICU).

## INTRODUCTION

Infections caused by multi-drug resistant (MDR) Gram-negative bacteria have become a major problem in Intensive Care Units (ICUs) worldwide [1] and are often associated with a high rate of morbidity and mortality [2, 3]. *Klebsiella pneumoniae* is a complex species of Gram-negative bacteria that can carry a variable compendium of virulence genes and cause a range of human infections [4-6]. Notably, *K. pneumoniae* has the capacity to rapidly acquire novel antimicrobial resistance mechanisms and carbapenem-resistance *K. pneumoniae* has been recognized as key agent of hospital-acquired infections. The spread of carbapenem-resistance *K. pneumoniae* is being facilitated by mobile genetic elements that carry genes conferring resistance to beta-lactams, such as OXA-48-like, NDM-type and particularly KPC-type enzymes [7-9].

Infections caused by KPC-producing *K. pneumoniae* and other MDR Gram-negative bacteria are commonly treated with regimens containing colistin/polymyxin [10, 11]. Although these antimicrobials are effective for the treatment of a variety of infections, the emergence and dissemination of polymyxin-resistance *K. pneumoniae* pose a significant threat to the treatment management of nosocomial infections and increase the potential for pan-resistant bacteria [8, 9, 12]. Resistance to polymyxins can arise through adaptive or mutational mechanisms that alter the outer membrane via modification of lipopolysaccharide (LPS) via cationic substitutions [13]. Previous studies have demonstrated that modifications in the PmrA/PmrB and PhoP/PhoQ two-component systems and the inactivation of the *mgrB*gene (a regulator of the PhoP/PhoQ system) lead to polymyxin resistance by LPS modification [14]. More recently, the plasmid-mediated transferable polymyxin resistance *mcr-1* gene, which causes resistance by modification of lipid A, was found in *Escherichia coli* and *K. pneumoniae* in China [15].

Polymyxin is commonly used in Brazilian hospitals to treat infections caused by MDR Gram-negative pathogens. This use has been associated with an increase in the prevalence of

polymyxin-resistant *K. pneumoniae*, which increased from 1.8% in 2009 to 15% in 2013and 35.5% in 2015 [9, 16, 17]. However, despite their commonality, little has been done to identify circulating clones or define the associated resistance mechanisms, which may provide some welcome insight into their prevention and treatment and also potentially reduce their transmission in Brazilian healthcare facilities. Here we aimed to investigate the molecular and epidemiological characteristics of polymyxin-resistant *Klebsiella pneumoniae* circulating in Brazilian hospitals. Specifically, we exploited whole-genome sequencing (WGS) to explore the molecular mechanisms associated with polymyxin resistance in *K. pneumoniae* found in ICUs in Brazil.

## MATERIALS AND METHODS

### Ethical standards

This study was conducted with the approval of the Research Ethics Committee from Universidade Federal da Grande Dourados (number 877.292/2014).

### Study design

Polymyxin-resistant *K. pneumoniae* were recovered from patients hospitalized in two public tertiary care hospitals located in distinct cities in Brazil: Tertiary Hospital, Dourados (Hospital A) and Regional Hospital, Campo Grande (Hospital B), between September2015 and October 2016. These locations are 187 and 352 bed facilities respectively, which are distributed among infirmaries, maternal and infant areas, IUCs adult, pediatric, neonatal and intermediate care units (IU).

Clinical records from all patients diagnosed with polymyxin-resistant strains hospitalized in Hospital A and B and were reviewed and the following data were recorded: demographics; medical history and co-morbid conditions; location prior to admission;

hospital course (duration and ward location); invasive procedures (devices use and surgery) receiving of mechanical ventilation; treatment with immunosuppressant drugs; antimicrobial exposure history; source of infection and outcome (recovery/death).

Clinical infection was defined by medical diagnosis according to clinical criteria (sepsis, fever, changes in frequency or color of secretions, or new radiological findings) associated with the decision to initiate antimicrobial therapy, as well as, isolation of one polymyxin-resistant *K. pneumoniae* organism [18]. Colonizers were defined as bacteria permanently or temporarily present in the skin or mucous membranes of the patient, dissociated from signs or symptoms of infectious disease. The endemic level of colonization and infection by polymyxin-resistance per 1,000 patient-days was calculated using a previously described method [19].

**Bacterial identification, susceptibility testing and phenotypic assays**

Bacterial species identification and antimicrobial susceptibility testing were performed using the Vitek®2 (bioMérieux, Hazelwood, MO) according to the manufacturer's instructions. After isolation, the susceptibility profile was confirmed with the evaluation of the minimal inhibitory concentrations (MICs) against various antimicrobials by broth microdilution following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines [20]. Susceptibility results were interpreted according to the most recent CLSI guidelines[20].

**Outer membrane protein analysis**

The outer membrane proteins (OMPs) of *K. pneumoniae* strains were analysed by SDS-PAGE using membrane extracts from bacteria grown overnight in nutrient broth and

gels stained with Coomassie blue. [21] Alterations of OmpK35- and OmpK36-encoding genes were also investigated by PCR and DNA sequencing. [22]

**Whole-genome sequencing and analysis**

Genomic DNA was extracted from fresh cultures of polymyxin-resistant organisms using QIAamp® DNA Mini Kit (Qiagen, Germany). The concentration and purity of DNA were determined using a Qubit® 2.0 fluorometer using the dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA). Sequencing libraries were prepared using the Nextera library kit (Illumina) and subjected to sequencing via IlluminaMiSeq Platform (Illumina, San Diego, USA), as previously described [23]. FastQC version 0.11.2 was used to preprocess the reads(http://www.bioinformatics.babraham.ac.uk/ projects/fastqc) and QC stats were exported to graph and manually assessed [24].

Reads were mapped to the reference *K. pneumoniae* MGH78578 and species identification was confirmed with Kraken [25]. Each read set was assembled using SPAdes version 3.6.1, [26] with k-mer sizes 21, 33, 55, 77, 99 and 127 with mismatch correction. These sequences were annotated using Prokka [27]. The "core" *K. pneumoniae* genome was defined as concatenation of coding sequences presenting one copy in all of the final assemblies [28].

Single nucleotide polymorphisms **(**SNPs) were identified via mapping of Illumina reads to a reference genome (*K. pneumoniae* strain NTUH-K2044, NC_006625.1) [29]. Maximum likelihood (ML) phylogenetic trees were constructed using RAxML (v 8.1.23) [30]. Analyses were performed with 100 bootstrap replicates per run, with a generalized time-reversible model and a gamma distribution to model site specific recombination (GTR+Γ substitution model; GTRGAMMA in RAxML) and was visualized using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk /software/figtree/). We selected the single tree with the highest ML as the best tree. For the larger tree containing global isolates, clades were collapsed

manually in R using the drop.tip function in the *ape* package. SRST2 [31] was used to mapping known alleles and identify MLSTs directly from reads according to the *K. pneumoniae* MLST database [29].

**Gene content analysis**

Read sets also were screened for known alleles of genes using a read mapping approach with SRST2. For acquired resistance genes we used the ARG-ANNOT database. [32] Plasmid replicon sequences were identified using ARIBA to screen reads for replicons in the PlasmidFinder database [33]. Virulence genes were identified by comparison to the gene databases for virulence and wzi alleles (*K. pneumoniae* BIGSdb) hosted by the Institut Pasteur MLST database. [29] PROVEAN software tool was used to predict whether sequence variants at the nucleotide level resulted in amino acid substitutions with an impact on the biological function of proteins. [34] Insertion Sequence (IS) elements were identified using ISFinder. [35]

**Nucleotide Sequence Accession Numbers**

The whole genome sequences described in this paper have been deposited in DDBJ/ENA/GenBank (Table S1).

**RESULTS**

**Polymyxin-resistant *Klebsiella pneumoniae***

During the period of investigation, we isolated a total of 30 polymyxin-resistant *K. pneumoniae*; 25 from hospital A and 5 from hospital B. The distribution of polymyxin-resistant *K. pneumoniae* isolates among the different hospital wards was as follows: intensive care unit (ICU) (n =18); intensive care unit neonatal (ICU neo) (n = 10) and intensive care

unit pediatric (ICU ped) (n = 2). Organisms were recovered from 30 individual patients on a median of 10 days following admission (range 2 to 40 days).

**Patients of the study and outbreak description**

The clinical characteristics of 30 patients infected or colonized by polymyxin-resistant *K. pneumoniae* are shown in Table 1.The patients ages hospitalized in the adult ICU ranged from 38 to 86 years; 12 (40%) were neonates with a median gestational age of 31 weeks and a median birth weight of 1.6 kg (range: 0.8 e 4 kg). Twenty seven patients had a history of previous hospitalization in Hospital A or in other hospitals. The remaining patients were admitted to the intensive care unit (ICU) directly from the emergency room.

Prior to isolation of polymyxin-resistant *K. pneumoniae*, all patients had received antimicrobial regimens, which included penicillins, third- or fourth-generation cephalosporins, quinolones, aminoglycosides, carbapenems, and polymyxins. However, empiric antimicrobial therapy did not provide appropriate antibacterial coverage based on the antimicrobial susceptibility testing. The analysis of data on patient outcomes revealed that polymyxin-resistant *K. pneumoniae* patients had a high mortality rate (60%).

On the basis of epidemiological data an outbreak was identified in hospital A. We constructed a timeline representing the isolation of polymyxin-resistant *K. pneumoniae* in relationship with the ward and the length of stay on each ward (Figure 1). This timeline showed that the ICU was involved continuously between September2015 and November2015, whereas the ICU neo had two different distinct periods separated by 180 days respectively (September to November/2015 and June to October/2016) (Figure 2).

Regarding the endemicity of these bacteria, rates of occurrence of infection and/or colonization with polymyxin-resistant strains per 1,000 patient-days in September/2015 and November/2015 exceeded the control limit established, above the average incidence of

colonization or infection (Figure 2). The acquisition rate of infection and/or colonization was 0.004 per 1,000 patient-days in August/2015, increasing to 0.01 per 1,000 patient-days in October/2015. Although it has not exceeded the control limit, from July/2016 to September/2016 other cases subsequently occurred and the acquisition rate of polymyxin-resistant *K. pneumoniae* strains was above the average prevalence of colonization or infection, reaching the alert limit. Furthermore, 67% (*n* = 20) of the identified strains were recovered from blood culture, 10% (*n* = 3) from tracheal aspirates, 10% (*n* = 3) from catheter, 7% (*n* = 2) from rectal swab, 3% (n = 1) from surgical wound and 3% (*n* = 1) from cerebrospinal fluid.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing was performed at the time of isolation. Polymyxin-resistant *K. pneumoniae* strains showed resistance to all the antibiotics tested by broth microdilution as follows: ceftazidime ($MIC_{50}$, >256 mg/L), cefotaxime ($MIC_{50}$, >256 mg/L), ceftriaxone ($MIC_{50}$, >256 mg/L), cefepime ($MIC_{50}$, >256 mg/L), aztreonam ($MIC_{50}$, >32 mg/L), imipenem ($MIC_{50}$, >16 mg/L), meropenem ($MIC_{50}$, >16 mg/L), ertapenem ($MIC_{50}$, >32 mg/L), amikacin ($MIC_{50}$, 64 mg/L), gentamicin ($MIC_{50}$, 32 mg/L), ciprofloxacin ($MIC_{50}$, 16 mg/L), levofloxacin ($MIC_{50}$, 16 mg/L) and polymyxin B (MICs ranging from $MIC_{50}$, 4 mg/L $MIC_{50}$, 32 mg/L).

**Outer membrane protein analysis**

Alterations in the OmpK35 and OmpK36 proteins were found in four strains. According to the SDS–PAGE results, three *K. pneumoniae* strains presented two bands, probably corresponding to OmpA and one of the main porins (either OmpK35 or OmpK36), suggesting that they have lost at least one of the main porins. PCR analysis of OMP-encoding genes showed altered amplicons of at least one main OMP-encoding gene, including a lack of

amplification (four isolates). Sequencing results showed OmpK35 was not detected in four isolates (KP32, KP34, KP37 and KP42) and OmpK36 in one isolate (KP32).

**Phylogenetic analysis**

Core genome analysis of the 30 *K. pneumoniae* whole genome sequences revealed that the core genome shared 3,792 genes among all isolates. MLST analysis of the genome sequences identified four different sequence types; ST11 was the most common (n = 24) and both non-KPC2-producing ST11 and KPC-2-producing ST11 isolates were genotipically closely related. Other STs were also represented including ST345 (n = 3), ST15 (n = 2) and ST258 (n = 1). Interesting, that is the first description of ST345 in Brazil. A maximum likelihood core tree of the 30 polymyxin-resistant *K. pneumoniae* sequences was constructed (Figure 3). An interactive version of the global phylogeny, with strains labeled by genotype, city of origin and year of isolation, is available at https://microreact.org/project/B1O5UDgIQ.

**Acquired antimicrobial resistance and virulence genes**

Resistance gene profiles varied markedly, all strains harbored several antibiotic-resistant genes including beta-lactam, aminoglycosides, fluoroquinolones and tetracycline resistance mechanisms (Figure 3). The genomic data showed that the *bla*KPC-2 gene, conferring resistance to carbapenems, was present in 29 strains and all strains carried more than one ESBL genes, including $bla_{\text{CTX-M}}$ (100%), $bla_{\text{SHV}}$ (100%) and $bla_{\text{TEM}}$ (77%). In addition, genes conferring resistance to streptomycin, rifampin, chloramphenicol, trimethoprim, macrolides and sulphonamides were also identified. The plasmid-mediated colistin resistance gene *mcr-1* was not detected. The indentified virulence genes included *clbA* (colibactin), *fyuA, irp1, irp2, ybtA, ybtE, ybtQ, ybtS, ybtT, ybtU, ybtX* (yersiniabactin), *iutA*

(aerobactin), *kfuA, kfuB, kfuC* (iron uptake), *kvgA, kvgS* (iron regulation) *mrkA, mrkB, mrkC, mrkD, mrkF, mrkH, mrkI* and *mrkJ* (fimbrial adherence determinants) (Figure 3).

We evaluated the plasmid content using PlasmidFinder. The isolates were found to harbor between one and six plasmids and a total of 16 plasmid replicon types were identified. Seventy-three percent of KPC-2-producing strains harbored IncR plasmid in addition to IncFIB or IncL. The non-KPC-2-producing strain had IncFIB, IncL and ColRNAI incompatibility groups (Figure 3).

**SNPs and occurrence of *mgrB* alterations**

To investigate potential polymyxin-resistance mechanisms the nucleotide sequences of *pmrA, pmrB, phoP, phoQ, crrB* and *mgrB* genes were analyzed and compared to sequences from a polymyxin-susceptible *K. pneumoniae* organism. None of the 30 isolates had mutations in the PmrAB/PhoP-related genes, however all organisms had a point mutation in the *phoQ* gene leading to amino acid substitution (Asp150Gly), which was predicted to have a neutral impact on the protein function. Out of 30 organisms, 22 exhibited IS mediated disruptions in the *mgrB* coding sequence (Table 2). The ISs were of five different types, IS*903* (n = 7), IS*5*-like (n = 7), IS*Kpn13* (n = 4), IS*Ecp1* (n = 3) and IS*Kpn18* (n = 1) and were found to be inserted at six different positions and in various orientations (Table 2/Fig. S1). One isolate had a point mutation (A7T) in the *mgrB* coding sequence creating a stop codon. Six strains harbored a novel 80-bp repeat sequence at nucleotide position 89, encoding a protein with 26 amino acids duplicated which altered function of the protein and predicted to be potentially deleterious (Figure 4). Finally, no mutations in the above mentioned genes were found in one polymyxin-resistant isolate (KP44 MIC 8 mg/L).

**Infection control measures**

Following the notification of the increase in the endemic levels certain number of steps had been taken to contain the spread of polymyxin-resistant strains, infection control measures have been implemented and included surveillance cultures from all patients hospitalized for more than 48 hours in various wards, especially in the case of patients with previous hospitalization. Sharing of patient equipment was limited as much as possible; hand hygiene promotion on ICU; isolation of patients colonized/infected by carbapenem-resistant strains; general environmental cleaning and disinfection of reusable medical equipment were properly executed. Cleaning of all surfaces including walls, floors, ceilings, windows, furniture, and medical equipment were intensified; exchange of antiseptic solutions; daily summary of all relevant microorganisms on ICU and communication between the departments of medical microbiology, infection prevention and other healthcare personnel was intensified through regular instruction meetings performed at the hospital wards. There was no intervention on antimicrobial administration and patients with infections were treated according to standard antimicrobial treatment policy (amikacin/polymyxin or tigeciclyne/polymyxin combined therapy). After implementing these measures, a considerably reduction in the incidence of CRAB was observed (Figure 2).

## DISCUSSION

Polymyxin-resistance is rapidly emerging worldwide in *Enterobacteriaceae*, in particular in *K. pneumoniae*. Here we aimed to further understand the genetic basis for polymyxin-resistance in *K. pneumoniae* circulating in two Brazilians hospitals, including the report of an outbreak of polymyxin-resistant and KPC-2-producing *K. pneumoniae*. Given the epidemiological timeline and location, as well as, the genetic relatedness, transmission routes could represent direct transmissions. It was observed that the index case of the outbreak

shared time and space with other patients in the ICU during almost the whole outbreak period and could be the source of polymyxin-resistant strain. There were no direct contacts between the first outbreak and the second period of isolation (four months among the last case of the first outbreak and the initial case of the second clonal expansion) in spite of persuasive genetic relatedness, likely due to transmission through health care staff, colonization of another patient in the unit, or a contaminated environmental reservoir, which was not identified in this investigation.

At the increase of polymyxin-resistant *K. pneumoniae* was observed and to prevent further spread, stringent infection control measures were introduced at ICU and ICU neonatal of hospital A. The outbreak was declared under control in November/2015 and extended infection control measures ended in December/2015. However, our data show that the infection control measures may have had a short-term effect, since gradually lost efficacy in the following months and the number of new acquisitions of MDR organisms dramatically rose in ICUs. Therefore education about CRE, strict adherence to proper hand hygiene and compliance with contact precautions were resumed decreased drastically afterwards.

In the present study, ST11 was found to be the most common clone, similar to previous report, belonged to clonal complex 258 (CC258), the most important CC associated with KPC production [17]. ST11 and ST258 types are globally disseminated high-risk clones [8, 36, 37]. ST15 type was isolated only in Hospital B and had previously been identified sporadically in South America [38]. In contrast, to our knowledge this is the first report of ST 345 in Brazil. In general, the epidemiological trend of polymyxin-resistance strains in hospital A can be divided into three stages. In the early stage of the outbreak only three ST345 strains were isolated restricted to ICU neonatal. Beginning in September/2015, ST11 strains began to be isolated in ICU adult remaining until December/2015. However, after four months, ST11 strains were recovered from ICU neonatal and pediatric. Based on this finding, we

hypothesize that the polymyxin-resistance ST11 clone was a successful clone that established itself in hospital A.

Polymyxin-resistant *K. pneumoniae* strains frequently exhibit MDR phenotypes, limiting treatment options [9, 13, 39]. This was also observed in our strain collection due to the association with a wide variety of other acquired resistance genes, including 16S rRNA methylase genes and chromosomal mutations/ insertions resulting in ciprofloxacin and colistin resistance. All the highly virulent and resistant strains carried replicons of plasmids that allow the horizontal spread of resistance genes and virulence factors, which make it easy to strains colonize or infect patients and hard to be eradicated. The co-existence of antimicrobial resistance and virulence factors is worrying as it could lead to the emergence of untreatable invasive *K. pneumoniae* infections. All these factors may have contributed to the high mortality rate of patients evaluated in this study.

Concerning about polymyxin resistance, many of the transcriptional regulation systems controlling LPS modifications are involved in the resistance [13]. We studied *pmrA, pmrB, phoP, phoQ, crrB* and *mgrB* chromosomal genes taking acolistin-susceptible isolate as reference. Although our findings show that all strains have a point mutation on *phoQ*gene leading to amino acid substitution (Asp150Gly), previous study indicated that this type of alteration seems not to be related with polymyxin resistance [40]. Several mutations in *mgrB* gene were observed and inactivation of *mgrB* by insertion sequences was most often associated with polymyxin resistance. Horizontal dissemination of plasmids harboring insertion sequences, could facilitate the acquisition of polymyxin resistance [41]. Our findings are consistent with a previous study that reported a similar rate of colistin-resistant strains with IS element insertion of *mgrB*[14, 16, 42, 43]. In addition, we also identified insertion of an 80-bp sequence, resulting in 26-amino-acid duplication of MgrB in six strains, which may interfere with the PhoQ interaction. We speculate that this is the primary mechanism of

polymyxin resistance in these strains, that to our knowledge has not been describe before. No mutations were found in the polymyxin-resistant related-genes in one isolate, indicating that other novel mechanisms were responsible for colistin resistance in this isolate.

The *bla*$_{KPC-2}$ gene was not detected in only one strain (KP32) and alterations in the OmpK35 and OmpK36 proteins were investigated. Carbapenem resistance with a lack of carbapenemases is usually explained by a combination of impaired outer membrane permeability or porin gene nonexpression (porin changes/loss) and increased efflux along with ESBL production. Although carbapenemase-encoding genes were not identified in this strain, *bla*$_{CTX-M}$, *bla*$_{TEM}$and *bla*$_{SHV}$ genes were detected and mutations in *ompK*35 and *ompK*36 were identified. Therefore, these porin alterations may have contributed to the reduction of outer membrane permeability and susceptibility to carbapenems.

In conclusion, we report a high rate of polymyxin resistance caused by three different mechanisms affecting *mgrB* gene, including a novel amino acid alteration. Clonal spread of polymyxin-resistant isolates circulating was observed and polymyxin-resistance was late detected in the outbreak and highlighting the challenges of laboratory detection. The patients involved had complex and extended inpatient stays which made understanding the origin of polymyxin-resistant isolates a challenging. This is cause for serious concern for public health, leading to high morbidity and mortality rates of hospitalized patients, as polymyxin is among the few remaining treatment options for infections by multidrug-resistant Gram-negative pathogens. Furthermore, infection control measures and use of stricter antimicrobial policies are required to control the spread of these organisms.

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## TRANSPARENCY DECLARATIONS

Authors have no conflicts of interest to declare.

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514 **Table 1.** Clinical characteristic of 30 patients infected/colonized with polymyxin-resistant *K. pneumoniae* strains.
515

| Patient | Age/Sex | Clinical specimen | Strain | Hospital admission from | Hospital unit | Length of stay (days) | Device | Outcome | Exposure to Antibiotics | Treatment/Dosage/Days of therapy |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 73/M | Blood | P | Another hospital | ICU | 84 | CVC/MV/UC | Death | Aminoglycosides/ Carbapenems/Glycopeptide/Polymyxin | AMK(500 mg)/CST-PMB(500 mg)/13 |
| 2 | 69/M | Tracheal aspirates | P | Another hospital | ICU | 56 | CVC/MV/UC | Discharge | Aminoglycosides/Carbapenems/ | TGC(50 mg)/CST-PMB(500 mg)/17 |
| 3 | 70/M | Tracheal aspirates | P | Another hospital | ICU | 44 | CVC/MV/UC | Discharge | Aminoglycosides/Carbapenems/Glycopeptide | TGC (50 mg)/CST-PMB(500 mg)/14 |
| 4 | 8days/F | Blood | P | Home | ICU neo | 75 | ETT/PVA/CVA/NGT | Death | Aminoglycosides/Cephalosporins/ Penicillins | AMK(50 mg)/CST-PMB(500 mg)/14 |
| 5 | 46/F | Blood | P | Home | ICU | 92 | CVC/MV/UC | Death | Aminoglycosides/ Carbapenems/Cephalosporins/Polymyxin | AMK(250 mg)/CST-PMB(500 mg)/17 |
| 6 | 67/M | Blood | P | Another hospital | ICU | 89 | CVC/MV/UC | Death | Aminoglycosides/Carbapenems/Fluoroquinolone | AMK(250 mg)/CST-PMB(500 mg)/14 |
| 7 | 45 days/F | Rectal swab | C | Home | UI | 42 | PVA/NGT | Discharge | Aminoglycosides/Carbapenems/ Cephalosporins/ | TGC(150 mg)/CST-PMB(500 mg)/14 |
| 8 | 30 days/M | Rectal swab | C | Another hospital | UI | 52 | PVA/NGT | Discharge | Cephalosporins/Penicillins | TGC(50 mg)/CST-PMB(500 mg)/14 |
| 9 | 80/F | Blood | P | Another hospital | ICU | 34 | CVC/MV/UC | Death | Aminoglycosides/Carbapenems/Glycopeptide | AMK(500 mg)/CST-PMB(500 mg)/13 |
| 10 | 56/M | Blood | P | Home | ICU | 40 | MV/UC | Death | Aminoglycosides/Carbapenems/Glycopeptide | TGC(150 mg)/CST-PMB(500 mg)/18 |
| 11 | 41/M | Blood | P | Another hospital | ICU | 32 | CVC/MV/UC | Death | Aminoglycosides/ Carbapenems/Glycycycline/Polymyxin | AMK(500 mg)/CST-PMB(500 mg)/20 |
| 12 | 58/F | Blood | P | Another hospital | ICU | 38 | CVC/MV/UC | Death | Aminoglycosides/ Carbapenems/Glycopeptide/Polymyxin | AMK(500 mg)/CST-PMB(500 mg)/12 |
| 13 | 72/F | Blood | P | Home | ICU | 28 | CVC/MV/UC | Death | Carbapenems/Penicillins | TGC(50 mg)/CST-PMB(500 mg)/18 |
| 14 | 56/M | Blood | P | Another hospital | ICU | 35 | CVC/MV/UC | Death | Aminoglycosides/ Carbapenems/Cephalosporins /Polymyxin | AMK(500 mg)/CST-PMB(500 mg)/10 |
| 15 | 34/F | Blood | P | Another hospital | IUC | 85 | MV | Death | Carbapenems /Polymyxin | AMK(250 mg)/CST-PMB(500 mg)/14 |
| 16 | 61/F | Blood | P | Another hospital | ICU | 35 | MV/UC | Death | Aminoglycosides/ Carbapenems/ Fluoroquinolone | AMK(500 mg)/CST-PMB(500 mg)/18 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 45 days/F | Surgical wond | C | Home | ICU neo | 90 | ETT/CVA/NEC | Discharge | Aminoglycosides/Penicillins | TGC(50 mg)/CST-PMB(500 mg)/20 |
| 18 | 29 days/M | Blood | P | Home | ICU neo | 38 | ETT/CVA/NGT | Death | Aminoglycosides/ Carbapenems/Penicillins | TGC(50 mg)/CST-PMB(500 mg)/17 |
| 19 | 15 days/M | Blood | P | Home | ICU neo | 28 | ETT/CVA/PVA/NGT | Death | Aminoglycosides/Cephalosporins/Penicillins | AMK(50 mg)/CST- PMB(500 mg)/17 |
| 20 | 8days/F | Catheter | C | Home | ICU neo | 35 | AVP/CVA/NGT | Discharge | Aminoglycosides/Cephalosporins/Penicillins | AMK(250 mg)/CST- PMB(500 mg)/15 |
| 21 | 13 days/M | Blood | P | Home | ICU neo | 59 | ETT/CVA/PVA/NGT | Death | Aminoglycosides/Penicillins | AMK(50 mg)/CST- PMB(500 mg)/17 |
| 22 | 2/M | Cerebrospinal fluid | C | Another hospital | ICU ped | 90 | CVA/NGT | Discharge | Carbapenems/Cephalosporins | TGC(50 mg)/CST- PMB(500 mg)/21 |
| 23 | 1/F | Blood | C | Home | ICU ped | 27 | - | Discharge | Aminoglycosides/Penicillins | TGC(50 mg)/CST- PMB(500 mg)/18 |
| 24 | 30 days/F | Catheter | P | Home | UI | 30 | PVA/NGT | Discharge | Aminoglycosides/Cephalosporins/Penicillins | AMK(50 mg)/CST- PMB(500 mg)/17 |
| 25 | 30 days/F | Blood | P | Home | UI | 28 | PVA/CVA/NGT | Discharge | Aminoglycosides/Penicillins | AMK(50 mg)/CST- PMB(500 mg)/10 |
| 26 | 76/M | Blood | P | Home | ICU | 15 | - | Discharge | Carbapenems/Cephalosporins | TGC(50 mg)/CST- PMB(500 mg)/14 |
| 27 | 79/F | Blood | P | Home | ICU | 25 | NGT | Death | Aminoglycosides/ Carbapenems/Glycycycline/Glycopeptide/Polymyxin | AMK(500 mg)/CST- PMB(500 mg)/14 |
| 28 | 81/F | Blood | P | Another hospital | ICU | 34 | PVA/NGT | Discharge | Aminoglycosides/ Carbapenems/Glycycycline | AMK(500 mg)/CST- PMB(500 mg)/18 |
| 29 | 46/M | Tracheal aspirates | P | Home | ICU | 92 | NGT | Death | Aminoglycosides/ Carbapenems/Cephalosporins/Polymyxin | AMK(500 mg)/CST- PMB(500 mg)/21 |
| 30 | 85/F | Catheter | C | Another hospital | ICU | 26 | CVA | Death | Carbapenems/ Fluoroquinolone | TGC (50 mg)/CST- PMB(500 mg)/18 |

**Abbreviations**: **M-** Male**; F-** Female**; P-** Pathogen**; C-** Colonizers**; ICU-** Intensive care unit; **ICU neo-** Intensive care unit neonatal; **ICU ped-** Intensive care unit pediatric; **CVC**- central venous catheter; **MV-** mechanical ventilation; **UC**- urinary catheter; **ETT**- endotracheal tube; **CVA**- central venous access; **PVA-** peripheral venous access; **NGT**- nasogastric tube; **NEC**- nasoenteral catheter; **AMK**- amikacin; **CST**- colistin; **PMB-** polymyxin B; **TGC**- Tigecycline.

525 **Table 2.** Features and *mgrB* gene status of the 30 polymyxin-resistant strains.
526

| Strains | Year | Hospital | Source | ST | Carbapenemase | Polymyxin MIC (mg/L) | *mgrB* status |
|---|---|---|---|---|---|---|---|
| KP03 | 2015 | A | Blood | 11 | KPC-2 | 32 | *mgrB* repeated sequence at nt 89 |
| KP05 | 2015 | A | Tracheal aspirates | 11 | KPC-2 | 16 | Insertional inactivation, IS*Ecp1*at nt 124 (FW) |
| KP07 | 2015 | A | Tracheal aspirates | 11 | KPC-2 | 16 | Insertional inactivation, IS*Ecp1*at nt 124 (FW) |
| KP08 | 2015 | A | Blood | 345 | KPC-2 | 16 | Insertional inactivation, IS*Kpn13* at nt 125 (FW) |
| KP10 | 2015 | A | Blood | 11 | KPC-2 | 32 | *mgrB* repeated sequence at nt 89 |
| KP11 | 2015 | A | Blood | 11 | KPC-2 | 32 | *mgrB* repeated sequence at nt 89 |
| KP12 | 2015 | A | Rectal swab | 345 | KPC-2 | 16 | Insertional inactivation, IS*Kpn13* at nt 75 (RW) |
| KP16 | 2015 | A | Rectal swab | 345 | KPC-2 | 16 | Insertional inactivation, IS*Kpn13* at nt 125 (FW) |
| KP18 | 2015 | A | Blood | 11 | KPC-2 | 32 | *mgrB* repeated sequence at nt 89 |
| KP20 | 2015 | A | Blood | 11 | KPC-2 | 32 | Insertional inactivation, IS*Kpn18* at nt 122 |
| KP25 | 2015 | A | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS903at nt 89 (FW) |
| KP26 | 2015 | A | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS*5*-like element at nt 89 (FW) |
| KP27 | 2015 | A | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS903at nt 89 (FW) |
| KP28 | 2015 | A | Blood | 11 | KPC-2 | 8 | Insertional inactivation, IS903at nt 89 (FW) |
| KP29 | 2015 | A | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS*5*-like element at nt 89 (RW) |
| KP30 | 2016 | A | Surgical wond | 11 | KPC-2 | 8 | Insertional inactivation, IS*5*-like element at nt 89 (RW) |
| KP31 | 2016 | A | Blood | 11 | KPC-2 | 8 | Insertional inactivation, IS*Ecp1* at nt 124 (FW) |
| KP32 | 2016 | A | Blood | 11 | None | 32 | *mgrB* repeated sequence at nt 89 |
| KP33 | 2016 | A | Catheter | 11 | KPC-2 | 16 | Insertional inactivation, IS903at nt 89 (FW) |
| KP34 | 2016 | A | Blood | 11 | KPC-2 | 8 | Insertional inactivation, IS903at nt 89 (FW) |
| KP35 | 2016 | A | Cerebrospinal fluid | 11 | KPC-2 | 16 | Insertional inactivation, IS*5*-like element at nt 89 (FW) |
| KP36 | 2016 | A | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS903at nt 88 (FW) |
| KP37 | 2016 | A | Catheter | 11 | KPC-2 | 8 | Insertional inactivation, IS*Kpn13* at nt 89 (FW) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| KP40 | 2016 | A | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS*5*-like element at nt 89 (RW) |
| KP41 | 2016 | A | Catheter | 11 | KPC-2 | 32 | *mgrB* repeated sequence at nt 89 |
| KP37-1 | 2016 | B | Blood | 258 | KPC-2 | 8 | Insertional inactivation, IS*5*-likeat nt 75 (FW) |
| KP38-1 | 2016 | B | Blood | 11 | KPC-2 | 8 | Insertional inactivation, IS*5*-like element at nt 89 (RW) |
| KP39-1 | 2016 | B | Blood | 11 | KPC-2 | 16 | Insertional inactivation, IS903at nt 89 (FW) |
| KP42 | 2016 | B | Tracheal aspirates | 15 | KPC-2 | 8 | Substitution at nt 7 (a > t) |
| KP44 | 2016 | B | Catheter | 15 | KPC-2 | 8 | WT |

**Abbreviations**: **KPC** – *Klebsiella pneumoniae carbapenemase*; **MIC** – Minimal inhibitory concentration; **nt –** nucleotide; **FW** – Forward; **RW** – Reverse; **WT-** Wild type.

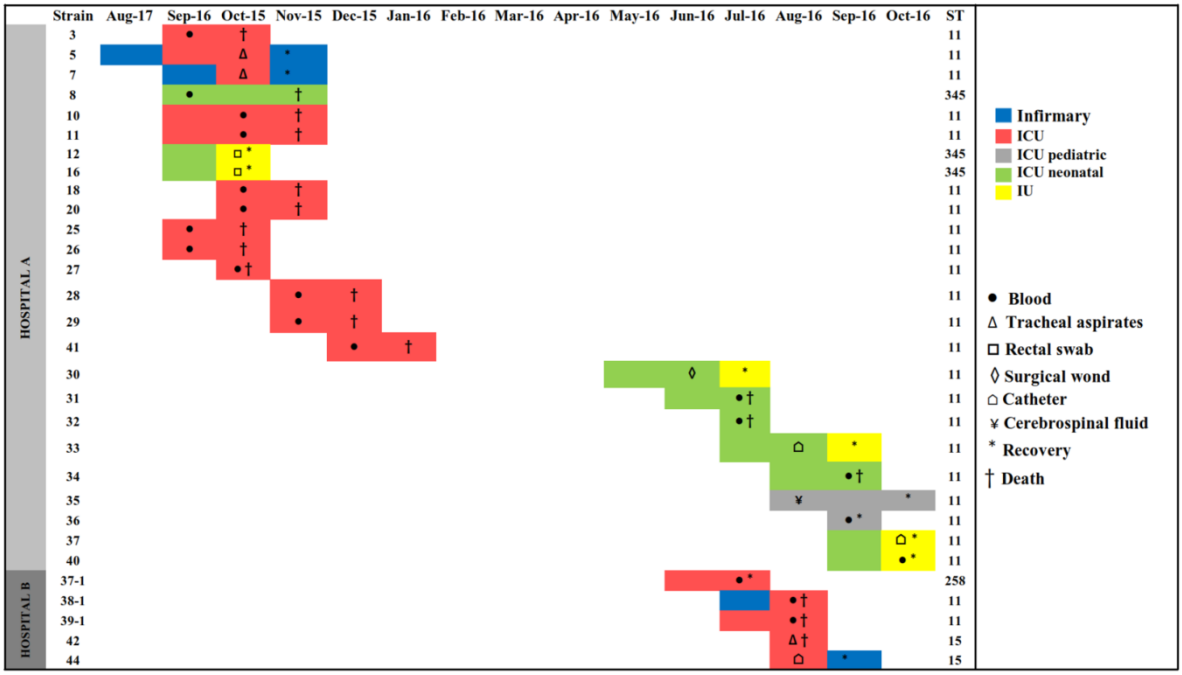


**Figure 1.** Duration and location of hospital stays of patients infected and/or colonized and clonal profile of polymyxin-resistant *K. pneumoniae* strains.

**Abbreviations**:**ICU**- General Intensive Care Unit; **ICUped**- Pediatric Intensive Care Unit; **ICUneo**- Neonatal Intensive Care Unit; **IU**- Intermediary Unit.

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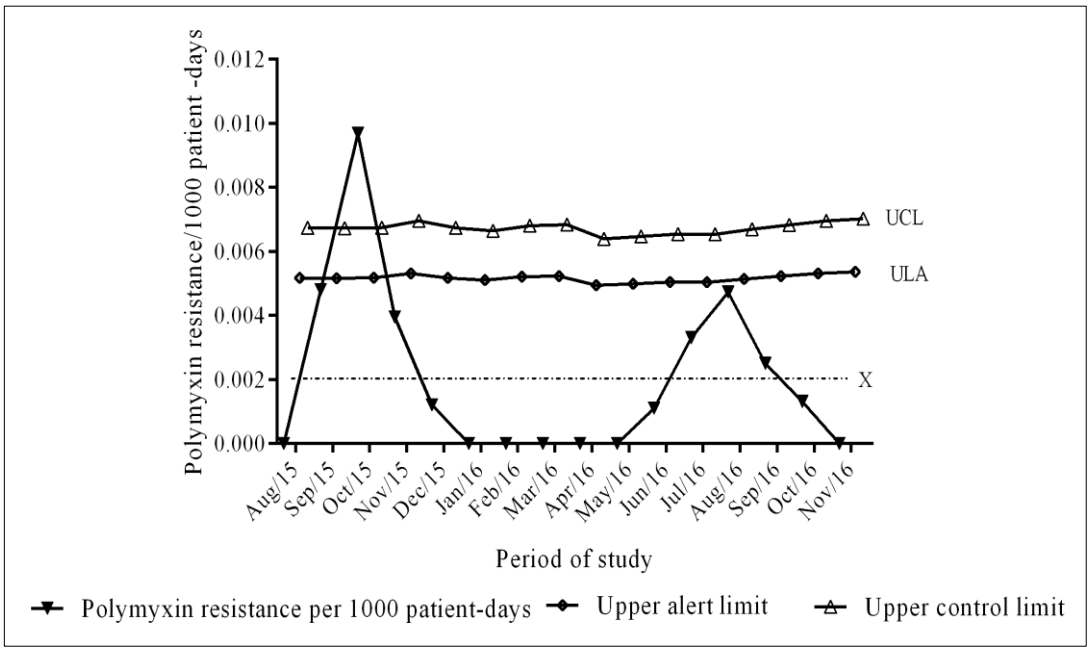
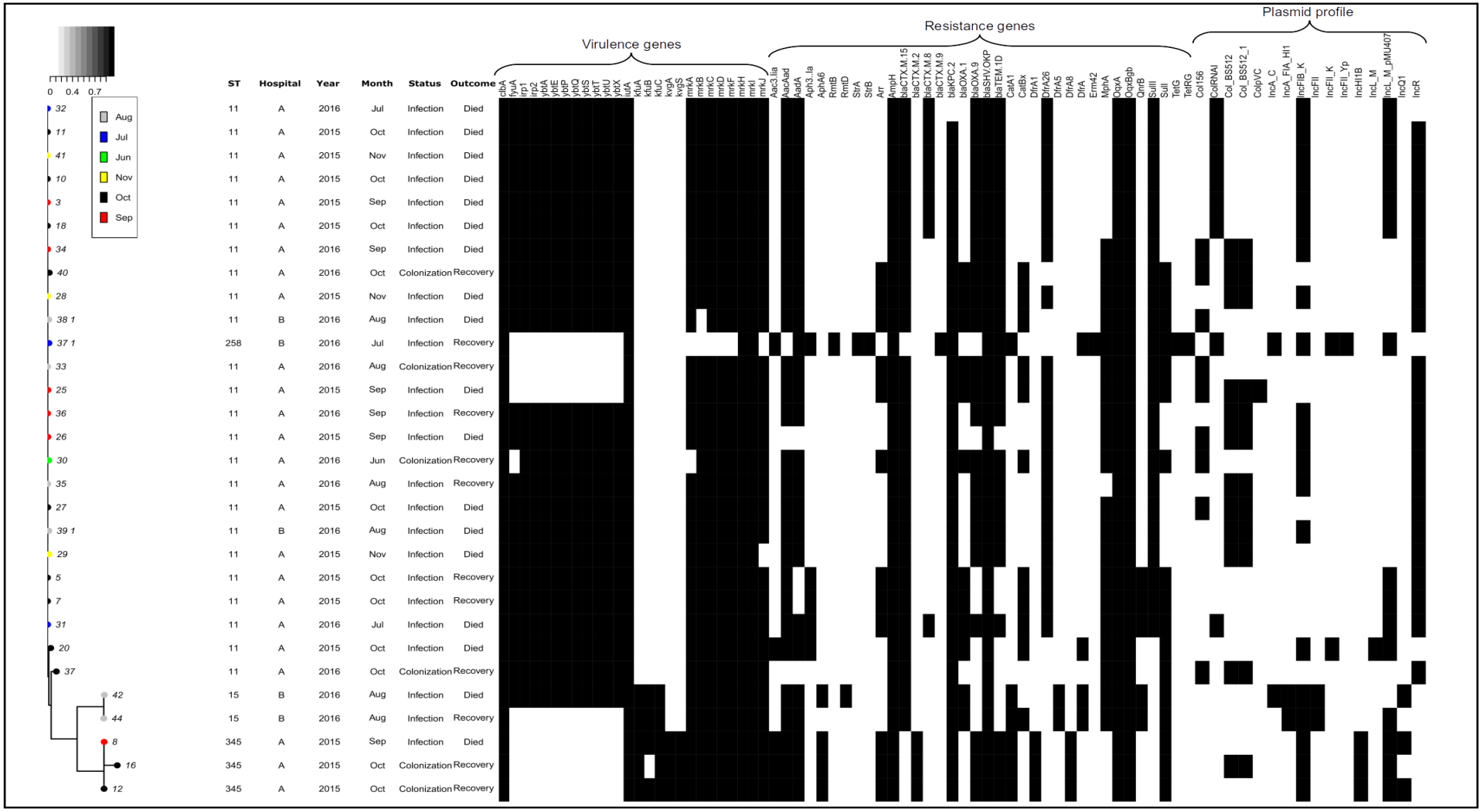
538




539

540 **Figure 2.** Endemic level of colonization/infection due polymyxin-resistant *K. pneumoniae*
541 strains per 1000 patient-days from August 2015 to November 2016. Upper control limit (3σ +
542 X); upper alert limit (2σ+ X); X: center line (average rate of polymyxin-resistance per 1000
543 patient-days).




544

545 **Figure 3.** Resistome, virulome and plasmid content analysis of 30 polymyxin-resistance *K. pneumoniae* strains correlated with the phylogenetic

546 tree. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

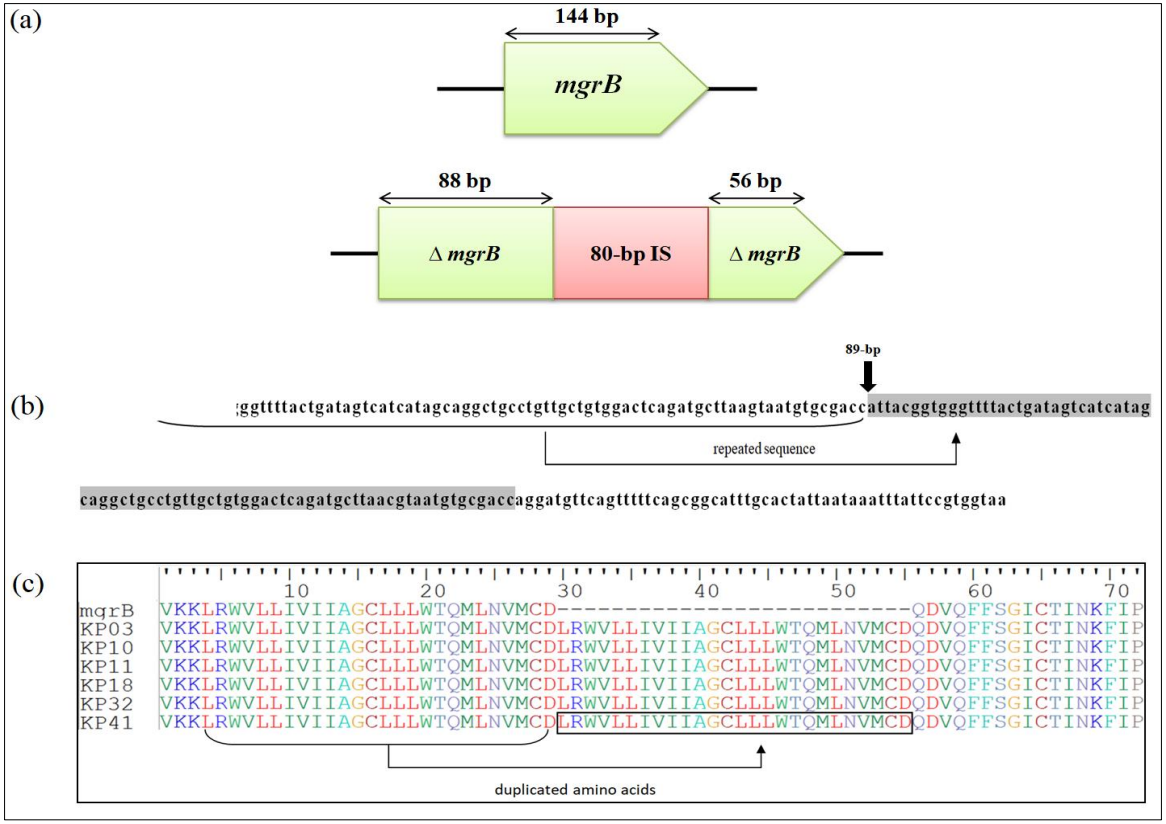
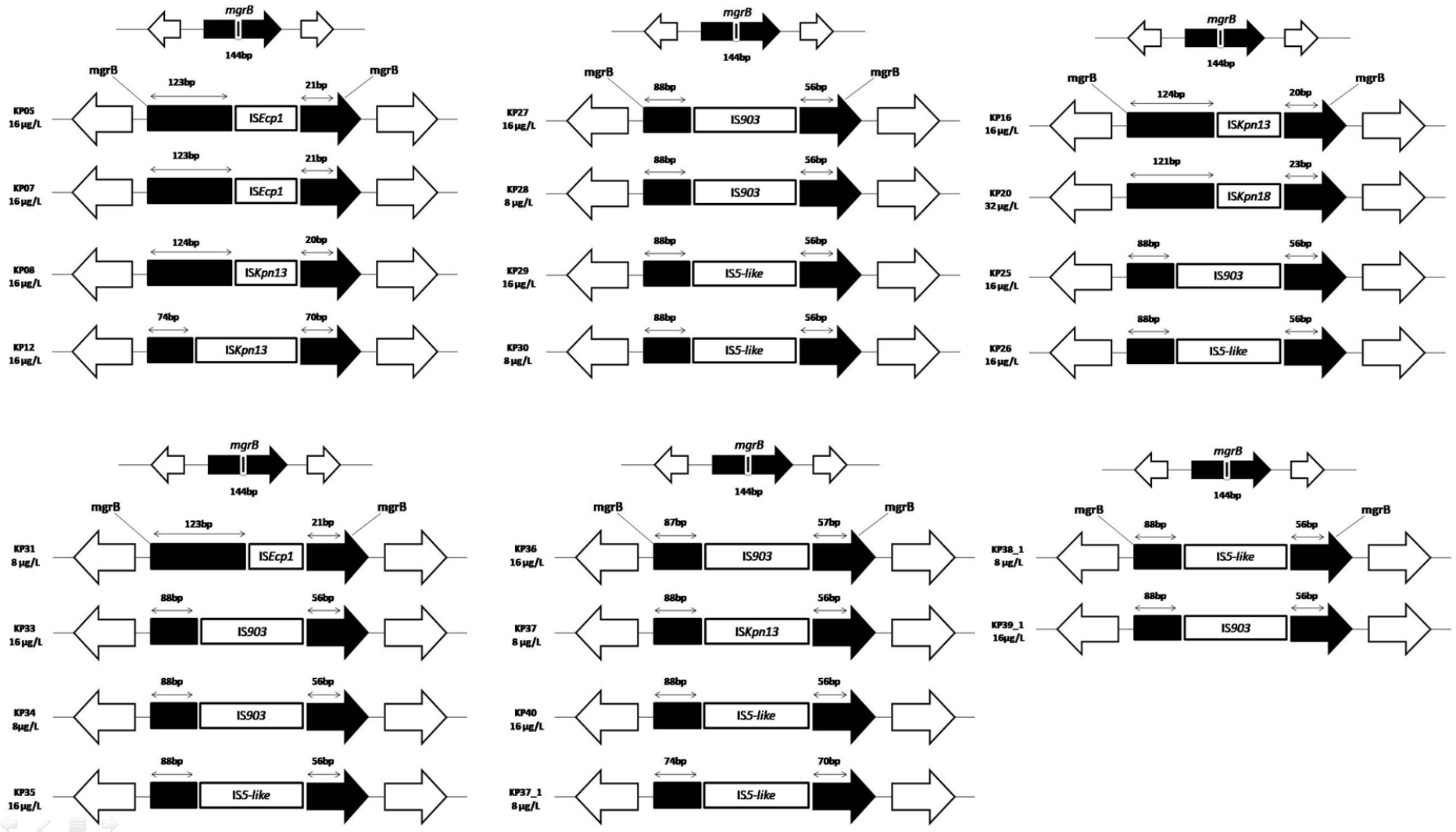



**Figure 4**. (**a**) Schematic representation of insertion event identified in the *mgrB* gene. The intact *mgrB* gene as found in wild-type isolates and in the polymyxin-resistant strain isolated in this study. (**b**) Nucleotide sequence of *mgrB* gene showing alteration mediated by insertion of a 80bp sequence at nt 89 position. (**c**) Alignment of unmutated MgrB from polymyxin-resistant *K. Pneumoniae* and mutated MgrB from polymyxin-resistant strains with 26 duplicated amino acids.

This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Supplementary Table S1. Nucleotide accession number of polymyxin-resistance strains.

| Strain | Acession number |
|---|---|
| KP03 | ERR2743730 |
| KP05 | ERR2743731 |
| KP07 | ERR2743732 |
| KP08 | ERR2743733 |
| KP10 | ERR2743734 |
| KP11 | ERR2743735 |
| KP12 | ERR2743736 |
| KP16 | ERR2743737 |
| KP18 | ERR2743670 |
| KP20 | ERR2743738 |
| KP25 | ERR2743739 |
| KP26 | ERR2743740 |
| KP27 | ERR2743741 |
| KP28 | ERR2743742 |
| KP29 | ERR2743743 |
| KP30 | ERR2743744 |
| KP31 | ERR2743745 |
| KP32 | ERR2743746 |
| KP33 | ERR2743747 |
| KP34 | ERR2743748 |
| KP35 | ERR2743749 |
| KP36 | ERR2743750 |
| KP37 | ERR2743751 |
| KP40 | ERR2743752 |
| KP41 | ERR2743753 |
| KP37-1 | ERR2743754 |
| KP38-1 | ERR2743755 |
| KP39-1 | ERR2743756 |
| KP42 | ERR2743757 |
| KP44 | ERR2743758 |




Supplementary Figure S1. *mgrB* insertion sequences identified in polymyxin-resistant *K. pneumoniae* strains.

**Artigo 2: Genetic diversity of polymyxin-resistant *Enterobacter aerogenes* isolated from Intensive care unit in Brazil**

**Short running title:** Polymyxin-resistant *Enterobacter aerogenes*.

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**ABSTRACT**

Here we aimed to further understand the scope and the genetic basis for polimixyn-resistance in *Enterobacter aerogenes* circulating in Brazil. Clinical and demographic data were evaluated. Susceptible antimicrobial profile was determined using microdilution. Polymyxin-resistant strains were subjected to whole genome sequencing to reveal their clonal background, antimicrobial resistance determinants and virulence factors. During the study period (3-month), nine polymyxin-resistant strains were isolated from patients admitted in the adult Intensive Care Unit. A high mortality rate (55.5%) was identified among the patients infected by polymyxin-resistant strains. Clonal expansion was observed and phylogeny data showed that strains were grouped in two clonal groups. Resistance gene profiles varied between strains, with some harbouring several beta-lactam, aminoglycoside, tetracycline and fluoroquinolone resistance mechanisms. In addition, virulence profile included genes encoding colibactin, yersiniabactin and aerobactin determinants. Genomic analysis showed that polymyxin resistance was mediated by three distinct mutational mechanisms including nonsynonimous alterations and amino acid substitution in the PhoP and PhoQ proteins. Thus, we identified an uncommon mutation in the *soxS* gene resulting in a truncated protein whose may affect the expression of *acrAB-tolC* efflux pump. In conclusion the spread of polymyxin resistance likely result in increased morbidity and mortality. Therefore, an active surveillance should be implemented to monitor the evolution of this resistance and its dissemination among ill critically patients.

**Keywords:** colistin-resistance, genetic mechanisms, whole genome sequencing, Enterobacteriaceae, intensive care unit.

## 1. Introduction

*Enterobacter aerogenes* represent one of the major bacterial species isolated in cases of nosocomial infections, with the ability to cause respiratory infections, urinary tract infections, endocarditis, skin/ soft-tissue infections, abdominal infections and osteomyelitis [1, 2]. These species easily acquire numerous genetic mobile elements containing resistance and virulence genes, which robustly increased pathogenicity and lethality of these bacteria. Given the presence of these concomitant important factors, this group of pathogens is progressively receiving more attention in clinical practice [3].

The emergence of polymyxin-resistant *Enterobacteriaceae* has been identified as an urgent threat to human health, since polymyxin is regarded as a drug of last resort in therapeutic management of Gram negative infections with very limited/no therapeutic options [4]. Resistance to colistin in Gram negative bacteria is typically caused by modification of LPS with 4-amino 4-dexoy arabinose or with phosphoethanolamine. These molecules, positively charged, reduce the overall negative charge of LPS, leading to a smaller electrostatic interaction with colistin, preventing cell lysis [5]. In addition to the emergence of colistin resistance due to chromosomal mutations, plasmid-borne colistin resistance has recently been reported, contributing to decreased binding of colistin to LPS [6].

Effective treatment of infections caused by these pathogens as well as implementation of adequate preventive measures to effectively contain the spread remains challenging [7]. Various studies have investigated the mechanisms associated with polymyxin-resistant in Gram negative infections, however studies on the polymyxin-resistance of *E. aerogenes* have been limited in comparison with other Enterobacteriaceae pathogens. In this study we aimed to identify the genetic mechanisms associated with polymyxin-resistance in *E. aerogenes* isolates from a tertiary hospital of the central-western region of Brazil using whole genome sequencing.

## 2. Material and methods

### 2.1 Bacterial strains

Polymyxin-resistant *E. aerogenes* strains recovery from patients hospitalized in a public tertiary care hospital from Brazil, from August/2016 to October/2016. The facilities provide 187 beds, distributed among infirmaries, UTIs adult, pediatric and neonatal. Patients' identification, clinical and demographic data were reviewed and entered into a Research Electronic Data Capture (Redcap). Infection and colonization status were defined on the basis of available clinical data associated with each strain at the time of isolation. Colonizers were defined as bacteria permanently or temporarily present in the skin or mucous membranes of the patient, dissociated from signs or symptoms of infectious disease. Clinical infection was defined by medical diagnosis according to clinical criteria (sepsis, fever, changes in frequency or color of secretions, or new radiological findings) associated with the decision to initiate antibiotic therapy, as well as, isolation of one polymyxin-resistant *E. aerogenes* strain [8].

### 2.2 Bacterial identification, susceptibility testing

The bacterial species identification and screening for antimicrobial resistance were performed by Phoenix® 86 Automated System 87 (BD Diagnostic Systems, Sparks, MD) according to the manufacturers instructions. After isolation, the susceptibility profile was confirmed and minimal inhibitory concentrations (MICs) of antimicrobials were determined by broth microdilution following the recommendations of the Clinical and Laboratory Standards Institute guidelines(CLSI) [9].

### 2.3 Whole-genome sequencing

Genomic DNA was extracted from fresh cultures using using QIAamp DNA minikit. The concentration and purity of DNA were determined with a Qubit® 2.0 fluorometer using

the dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA). Sequencing library were prepared using the Nextera library kit (Illumina). DNA samples were subjected to sequencing via Illumina MiSeq Platform (Illumina, San Diego, USA), as described previously [10]. Species identification was performed with Kraken [11]. FastQC version 0.11.2 (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc) was used to preprocess the reads. QC stats was exported to graphs and manually assessed [12]. Each read set was assembled using SPAdes version 3.6.1, [13] with k-mer sizes 21, 33, 55, 77, 99 and 127 and mismatch correction. These sequences were annotated using Prokka [14]. The "core" *E. aerogenes* genome was defined as concatenation of coding sequences presenting one copy in all of the final assemblies [15]. The whole genome sequences described in this paper have been deposited in DDBJ/ENA/GenBank. Acession number from ERR2743730 to ERR2743758.

**2.4 SNP identification and phylogenetic analysis**

SNPs were identified via mapping of Illumina reads to a reference genome. Maximum likelihood (ML) phylogenetic trees were constructed using using RAxML (v 8.1.23) [16]. Analyses were performed with 100 bootstrap replicates per run, with a generalized time-reversible model and a gamma distribution to site specific recombination (GTR+Γ substitution model; GTRGAMMA in RAxML) and were visualized using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk /software/figtree/). We selected the single tree with the highest likelihood as the best tree. For the larger tree containing global isolates, clades were collapsed manually in R. SRST2 [17] was used to mapping known alleles and identify MLSTs directly from reads according to the *E. aerogenes* MLST database. [18]

### 2.5 Gene content analysis

Read sets also were screened for known alleles of genes using a read mapping approach with SRST2. For acquired resistance genes we used the ARG-ANNOT database. [19] Plasmid replicon sequences were identified using ARIBA to screen reads for replicons in the PlasmidFinder database [20]. Virulence genes were identified by comparison to the gene databases for virulence. PROVEAN software tool was used to predict whether sequence variants at the nucleotide level resulted in amino acid substitutions with an impact on the biological function of proteins [21]. Insertion Sequence (IS) elements were identified using ISFinder [22].

### 2.6 Ethical standards

This study was conducted with the approval of the Research Ethics Committee from Universidade Federal da Grande Dourados (number 877.292/2014).

## 3. Results and discussion

### 3.1 General patient characteristics

Over the study period a total of 340 cultures were performed and out of these 124 cases of *Enterobacteriaceae* infection were identified. Twenty five cases were recovered from patients hospitalized in the adult ICU, out of those patients, polymyxin-resistant and carbapenemase-producing *E. aerogenes* strains were isolated from nine patients. The majority of the patients were female (66.7%) with a median age of 45 years, (range 31–76 years) and there were no significant differences ($P > 0.05$) among the patients with regard to baseline demographics. All the patients suffered from infectious diseases around the time of sample isolation; four of them had bloodstream infection whereas the remaining patients were diagnosed with pulmonary and urinary infections. All patients had antibiotic exposure in the

30 days prior to the initial detection of the polymyxin-resistant strain. The clinical characteristics of the patients involved in the outbreak are listed in Table 1. Outcome analysis showed that five patients infected with polymyxin-resistant *E. aerogenes* died by sepsis, giving an infection-attributable mortality rate of 55.5%.

### 3.2 WGS and phylogenetic analysis

The core genome phylogeny and MLST structure identified similar population and were used to classify the isolates into two strongly supported clonal groups. On the basis of SNPs, a maximum-likelihood phylogenetic tree was reconstructed showing that strains were closely related and partitioned into two clades. MLST analysis identified two different sequence types, ST93 (n = 5), belonged to clonal complex CC3 and ST16 (n = 3). ST93 had been previously described in Brazil, however because of the very recent release of *E. aerogenes* MLST profile, there is no sufficient information about the clonal characteristics and global distribution of this lineage. The high genetic similarity of strains suggests transfer events between patients showing that, measures must be implemented to avoid nosocomial transmission [23].

### 3.4 Antimicrobial susceptibility and gene content

Regarding the susceptibility profile all strains isolated exhibited high resistance rates to the antimicrobials tested (Table 2), including polymyxin B (MICs ranging from $MIC_{50}$, 8 mg/L to $MIC_{50}$, 32 mg/L). Comparisons of these genes among the nine genomes showed that 4,610 genes were shared by all the isolates. WGS analysis suggested distribution and dissemination of multiple resistance genes and plasmids which are key role in colonization, invasion and pathogenicity of the bacteria [18]. Resistance gene profiles varied between strains, with some harboring several beta-lactam, aminoglycoside, tetracycline and

fluoroquinolone resistance mechanisms. The *bla*$_{KPC-2}$ carbapenemase gene was identified in all isolates. In addition, the strains carried *bla*$_{CTX-M-15}$ (67%, n = 6), *bla*$_{TEM}$ (67%, n = 6) and *bla*$_{OXA-1}$ (84%, n = 21) ESBL-encoding genes. The genes selected for virulence typing were associated with virulent phenotypes in *Enterobacteriaceae* strains, including genes encoding colibactin, yersiniabactin and aerobactin determinants. By using PlasmidFinder, 10 different plasmid replicons were detected and each strain harbored three or more plasmids types. The high variability of plasmids and their spread among *Enterobacteriaceae* strains plays an important role for the transfer and the maintenance of antibiotic resistance specifying genes in clinical isolates [24].

**3.5 Colistin resistance**

To investigate mutational events related with polymyxin-resistance, the nucleotides sequences of *pmrA, pmrB, phoP, phoQ, crrB, soxS, soxR* and *mgrB* genes were analyzed and WGS data showed that the two clusters of colistin resistant isolates were linked with three separate mechanisms of mutational colistin resistance. The plasmid-encoded colistin resistance gene *mcr-1* was not detected. In opposite, mutations of selected genes associated with resistance to polymyxin were evaluated and are presented in Table 2. All strains exhibited alterations in the *phoP* gene including nonsynonymous mutations. The alterations were localized in two different amino acids positions (E218Q and Q221K). In addition, a partial deletion of the gene was identified in one isolate (EA6). A second potential mechanism of mutational colistin resistance, due to a D/G substitution at amino acid position 150 in *phoQ* was identified in a single isolate (EA39).

Recently, some studies suggest that overexpression of *acrAB-tolC* efflux pump is a marker of multidrug resistance linked to colistin in *Enterobacter* spp [2, 25, 26]. To evaluate its role as a potential polymyxin-resistance mechanism we investigate the sequences of the

198 *soxRS* genes responsible for regulate the expression of the *acrAB-tolC* efflux pump. In all
199 strains was identified an A/T substitution at amino acid position 60 in *SoxS* gene. These
200 mutations have not been identified in previous studies, however these alterations were shown
201 to be critical for polymyxin-resistance, predicted as impacting the protein function.
202 Alterations in the *mgrB*, *crrB*, *pmrA*, *pmrB* and *SoxR* genes were not detected. Although
203 MgrB and PmrAB may be responsible for most polymyxin resistance in Gram-negative
204 pathogens [7, 27, 28], our findings showed that the mechanisms of polymyxin resistance in *E.*
205 *aerogenes* appear to be highly diverse. Furthermore, additional investigations are necessary to
206 confirm the molecular mechanisms involved.
207 The emergence and spread of polymyxin-resistant strains have been reported,
208 especially in those hospitals, where $bla_{\text{KPC-2}}$ is endemic, and the increased consumption of
209 polymyxins have been proven to be major risk factors for the emergence of polymyxin-
210 resistance [29, 30]. Previous studies have reported that high levels of antimicrobials including
211 polymyxin are frequently administered in Brazilian ICUs, mainly after bacteria isolates have
212 become resistant to almost all other available antibiotics [31]. In our study, we hypothesize
213 that the polymyxin exposure triggered the transposition event that led to the modification of
214 the genes in the first isolate of each clone. This study is limited by its small sample size as
215 only includes the polymyxin-resistant *E. aerogenes* strains which were isolated during the
216 study period. However, the emergence and clonal spread of mutational colistin resistance
217 mediated by three distinct mechanisms over the course of three months is concerning
218 especially among patients admitted in ICUs.
219
220 **4. Conclusion**
221 Our findings showed that polymyxin-resistance may have occurred through mutational
222 changes in genes involved in lipopolysaccharide synthesis (*phoPQ*) and regulation of *acrAB-*

*tolC* efflux pump (*soxS)*. A careful and continued surveillance system that provides epidemiological and molecular information is important in order to follow the evolution of polymyxin-resistance in Brazil and to limit the risk of outbreaks caused by these high-risk clones**.**

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324

**Table 1.**Clinical characteristics of patients hospitalized in ICU and infected by polymyxin-resistant *E. aerogenes*.

| Strain | Age/Sex | Clinical isolates | Data of isolation | Length of stay (days) | Place prior to admission | Clinical signs of sepsis | Outcome | Exposure to antibiotics | Prior PMB (days) |
|---|---|---|---|---|---|---|---|---|---|
| EA6 | 31/F | Blood | 08/09/2016 | 29 | Home | Yes | Death | Carbapenems/ Cephalosporins/ Polymyxin B | 8 |
| EA9 | 43/F | Tracheal aspirates | 08/14/2016 | 88 | Another hospital | No | Recovery | Carbapenems/ Cephalosporins | - |
| EA13 | 48/F | Tracheal aspirates | 09/02/2016 | 87 | Another hospital | No | Recovery | Carbapenems/ Cephalosporins | - |
| EA15 | 50/M | Urine | 09/12/2016 | 84 | Another hospital | No | Recovery | Aminoglycosides/ Carbapenems | - |
| EA21 | 38/M | Tracheal aspirates | 09/18/2016 | 49 | Another hospital | Yes | Death | Aminoglycosides/ Carbapenems | - |
| EA22 | 76/M | Blood | 10/03/2016 | 19 | Another hospital | Yes | Death | Aminoglycosides/ Carbapenems | - |
| EA39 | 48/F | Blood | 10/05/2016 | 16 | Another hospital | Yes | Death | Carbapenems/ Cephalosporins/ Polymyxin B | 6 |
| EA40_1 | 36/F | Blood | 10/11/2016 | 29 | Home | Yes | Death | Carbapenems/Glycycycline /Glycopeptide | - |
| EA46 | 33/F | Urine | 10/23/2016 | 35 | Home | No | Recovery | Aminoglycosides/Cephalosporins/ Penicillins | - |

**Abbreviations**: **M-** Male**; F-** Female; **ICU –** Intensive care unit; **PMB-**polymyxin B.

**Table 2**. Phenotypic and molecular characterization of polymyxin-resistant *E. aerogenes* isolates.

| Strain | ST | MICs (mg/L) | | | | | | | | | | | | | Mutation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CAZ | CTX | CRO | FEP | ATM | IPM | MEM | ETP | AMK | GEN | CIP | LEV | PMB | PhoP | PhoQ | SoxS |
| EA6 | 93 | >256 | >256 | >256 | >256 | >32 | >16 | >16 | >32 | 64 | >32 | 16 | 8 | 16 | delATN216 | WT | A60T |
| EA9 | 93 | >256 | >256 | >256 | 128 | >32 | >16 | >16 | >32 | 64 | >32 | 16 | 8 | 16 | E218Q | WT | A60T |
| EA13 | 93 | >256 | >256 | >256 | >256 | >32 | >16 | >16 | >32 | 64 | >32 | 16 | 8 | 32 | E218Q | WT | A60T |
| EA15 | 93 | >256 | >256 | >256 | 128 | >32 | >16 | >16 | >32 | 32 | >32 | 16 | 8 | 16 | E218Q | WT | A60T |
| EA21 | 93 | >256 | >256 | 128 | >256 | >32 | >16 | >16 | >32 | 32 | >32 | 16 | 8 | 32 | E218Q | WT | A60T |
| EA22 | 93 | 128 | >256 | >256 | 128 | >32 | >16 | >16 | >32 | 32 | >32 | 16 | 8 | 32 | E218Q | WT | A60T |
| EA39 | 16 | >256 | >256 | >256 | >256 | >32 | >16 | >16 | >32 | 64 | >32 | 16 | 8 | 8 | E218Q | D150G | A60T |
| EA40_1 | 16 | >256 | >256 | 128 | >256 | >32 | >16 | >16 | >32 | 64 | >32 | 16 | 8 | 8 | Q221K | WT | A60T |
| EA46 | 16 | 128 | >256 | >256 | >256 | >32 | >16 | >16 | >32 | 64 | >32 | 16 | 8 | 8 | E218Q | WT | A60T |

**Abbreviations: MIC** - minimal inhibitory concentration; **CEF** - cephalothin; **CAZ** - ceftazidime; **CTX** - cefotaxime; **CRO** - ceftriaxone; **FEP** - cefepime; **ATM** - aztreonam; **IMP** - imipenem; **MEM** - meropenem; **ETP** - ertapenem; **AMK** - amikacin; **GEN** - gentamicin; **CIP** - ciprofloxacin; **LEV** - levofloxacin; **PMB** - polymyxin B. **WT** – Wild type; **del** – deletion;

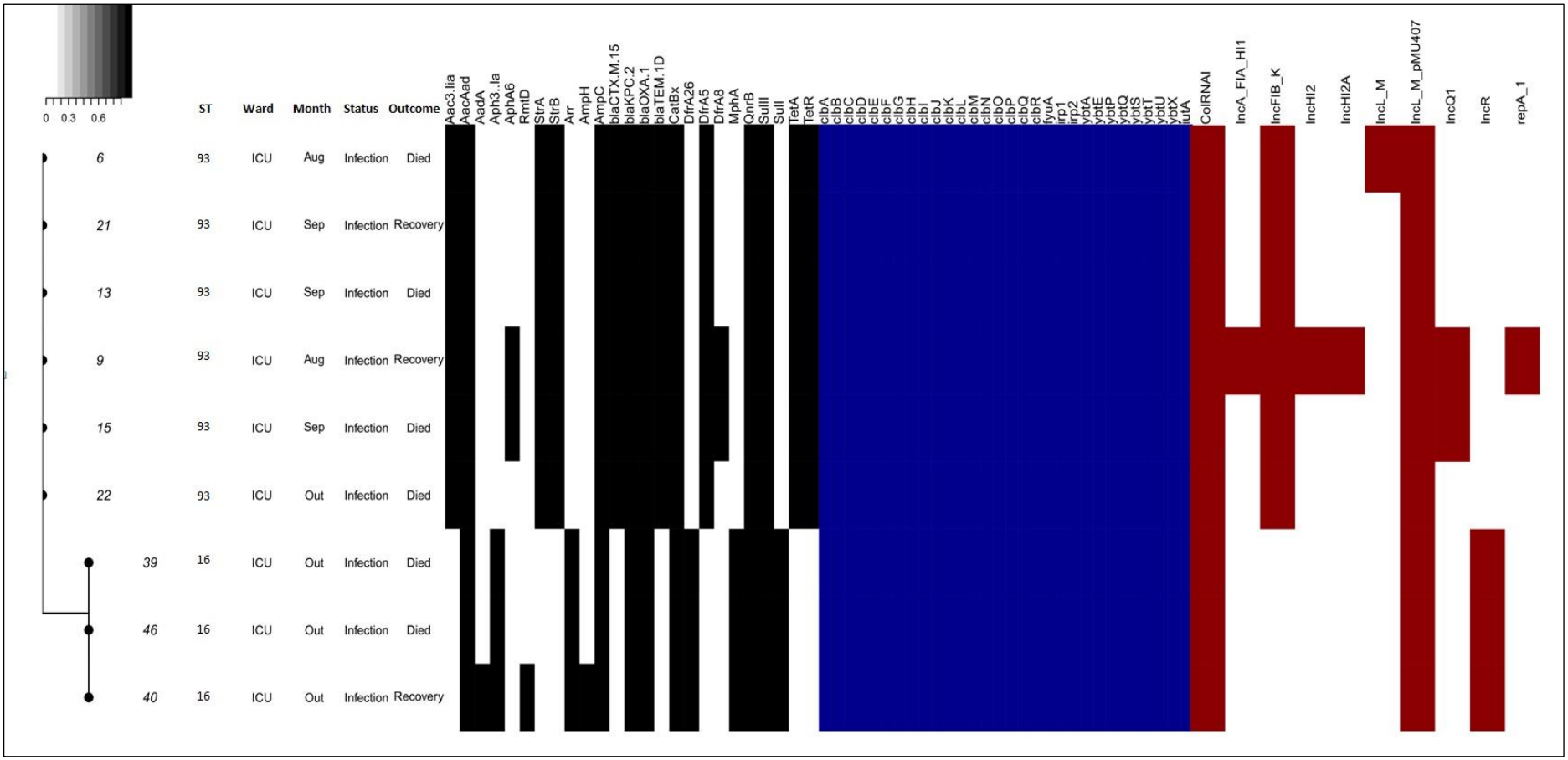


**Figure 1.** Maximum-likelihood phylogenetic tree and gene content of polymyxin-resistant *E. aerogenes* strains isolated from ICU. Black bars represent the presence of resistance genes, blue bars the virulence genes and red bars the plasmid profile.

**Artigo 3: Risk factors for polymyxin-resistant carbapenemase-producing *Enterobacteriaceae* in critically patients: An epidemiological and clinical study**

**Short running title:** Risk factors for polymyxin-resistance.

**2**

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**ABSTRACT**

**Objectives:** The aim of this study was to assess the clinical impact and potential risk factors associated with polymyxin-resistance *Enterobacteriaceae* strains isolated from patients hospitalized in adult and neonatal Intensive Care Unit.

**Methods:** We conducted a case–control study of the risk factors and clinical outcomes of hospitalized patients from September/2015 to January/2017. Antimicrobial susceptibility of isolated polymyxin-resistance *Enterobacteriaceae* strains was determined by broth microdilution. The presence of resistance genes was evaluated by PCR and DNA sequencing.

**Results:** Renal failure ($P$ = 0.02; OR 11.37; 95% CI 1.0–128.63), urinary catheter ($P$ = <0.01; OR 4.16; 95% CI 38.82–366.07), transference between hospital units ($P$ = 0.03; OR 9.98; 95% CI 1.01–98.42), carbapenem use ($P$<0.01; OR 45.49; 95% CI 6.93–298.62) and surgical procedure ($P$ = <0.01; OR 16.52; 95% CI 2.83–96.32) were risk factors for the acquisition of polymyxin-resistant strains in adult patients. For the neonatal patients, central venous catheter ($P$ = <0.01; OR 69.59; 95% CI 7.33–660.30) was the only risk factors independently associated with polymyxin-resistant. Analysis of the outcomes revealed that mortality rate was significantly higher in adults (66.6%) and newborns (23.5%) patients with polymyxin-resistant than those polymyxin-susceptible strains. In addition, polymyxin exposure ($P$<0.01; OR 4.46; 95% CI 1.63–12.22) and carbapenem exposure ($p$<0.01; OR 50.93; 95% CI 2.26–>999.999) were strongly associated with mortality. On the other hand aminoglycosides use ($p$<0.03; OR 0.06; 95% CI 0.004–0.97) was a protection factor for mortality by polymyxin-resistant strains.

**Conclusions:** Our findings showed that several risk factors were associated with polymyxin-resistance strains. Additionally the high mortality rates showed that acquisition of these strains is a predictor for unfavorable outcomes.

**Keywords:** Polymyxin-resistance, *Enterobacteriaceae*, risk factors, Intensive care units.

## Introduction

Various member of the Enterobacteriaceae are a common cause of healthcare associated infections in critically ill patients, newborns and the immunocompromised [1, 2]. Currently, the Enterobacteriaceae pose an even greater threat in healthcare associated infections as a consequence of the emergence of organisms that exhibit resistance to critical antimicrobials, such as *Klebsiella pneumoniae* that express carbapenemase (KPC) enzymes [3]. Resistance to critical antimicrobials leaves few alternatives, with the polymyxins being one of the few antimicrobial classes that retain activity carbapenemase producing organisms[4]. However, the use of polymyxins has inevitably led to polymyxin-resistant Enterobacteriaceae, which have been declared by the Centers for Disease Control and Prevention (CDC) an immediate public health threat that requires urgent and aggressive actions [5-7].

Molecular and biochemical studies have shown that the Enterobacteriaceae acquire resistance against polymyxins through intrinsic and transferable mechanisms [6]. The principal polymyxin resistance mechanisms the loss of lipopolysaccharide (LPS) mediated via a two-component regulatory system that causes the constitutive activation of LPS-modifying genes following the addition of 4-amino-4-deoxy-L-arabinose to lipid A[7-9]. Additionally, a plasmid-mediated colistin resistance gene (*mcr-1*) harbored by *Escherichia coli* and *Klebsiella pneumoniae* has also been described, demonstrating that polymyxin resistance can be horizontally transferred [10-12].

The surveillance of antimicrobial resistant organisms in healthcare facilities is critical for preventing outbreaks and improving patient management [1]. Additionally, the significance of polymyxin-resistant organisms and their association with high morbidity and mortality rates [11, 13, 14], means that such organisms should be monitored. Various studies have investigated the risk factors associated with polymyxin-resistant bacterial infections in

adults patients [13-15]; however, described risk factors for polymyxin-resistant bacterial infections in neonates are limited. In this context here, we performed a case–control study design to investigate the potential risk factors for acquisition of polymyxin-resistant carbapenemase-producing *Enterobacteriaceae* strains in adults and neonates hospitalized in Intensive Care Units (ICUs) in Brazil, as well as to describe mortality and clinical characteristics of these infections.

## Methods

*Study site and patients*

Data were collected from patients hospitalized in two public tertiary care hospitals located in distinct cities in Brazil (Hospital A and Hospital B), between September 2015 and January 2017. These respective facilities have 237 and 352 beds, distributed between infirmaries and the UTIs adult, pediatric, neonatal. Hospital A serves as a tertiary referral center for 32 cities, with an average of 9,800 annual admissions per year. Most patients admitted are resident Dourados, with the remainder resident in the surrounding cities. All data for this study originated from patients admitted onto the adult ICU (a 14-bed ward for critically ill patients), the neonatal ICU (a 10-bed ward), and the pediatric ICU (10-bed ward). The wards admit patients with a range of severe conditions and those transferred from clinical wards requiring critical care. Hospital B is a public institution that serves as a state reference center, with adult ICU (a 39-bed ward), neonatal ICU (a 10-bed ward), and pediatric ICU (8-bed ward), an average of 12,000 admissions per year and provides care for a variety of medical and surgical subspecialties.

*Study design*

The aim of the study was to identify risk factors for the acquisition of polymyxin-resistant carbapenemase-producing *Enterobacteriaceae* in critically ill patients. Consequently, we designed and performed a case-control study. A case was defined as a patient from whom a polymyxin-resistant *Enterobacteriaceae* organism was isolated from clinical cultures from any source during the study period. Controls were defined as patients from whom a polymyxin-susceptible *Enterobacteriaceae* isolated from a clinical culture from site during the study period in the first 48 hours after admission. Controls were recruited in a 2:1 ratio to cases. Case and controls were patients hospitalized on the ICUs and were selected from inpatients admitted within the study period matched for age, clinical manifestation, pathogen and hospital ward. Then the patient was removed from the selection pool for subsequent cases to avoid inclusion of the same patient more than once.

We conducted a three-part analysis: i) a case–control study in which cases were compared with controls to identify potential risk factors associated with isolation of polymyxin-resistant *Enterobacteriaceae* in patients hospitalized on the adult ICU, ii) a case–control study in which cases were compared with controls to identify potential risk factors associated with isolation of polymyxin-resistant *Enterobacteriaceae* in patients hospitalized on the neonatal ICU, and iii) a retrospective analysis to measure the mortality associated with the isolation of polymyxin-resistant *Enterobacteriaceae*.

*Clinical data*

The clinical, nursing and microbiological records of patients hospitalized were retrospectively reviewed. The following data were recorded: demographics, medical history, co-morbidities (diabetes mellitus, cardiovascular disease, renal failure, respiratory failure, chronic obstructive pulmonary disease, alcoholism, tabagism, neoplasia, neurological disease,

sepsis, substance misuse, HIV infection, decubitus ulcers, active cancer, and hypertension), location prior to admission, ward of admission, hospital course (duration and ward location), invasive procedures (devices use and surgery), mechanical ventilation, total parenteral nutrition, urinary catheter, drainage tube, nasogastric tube, tracheal intubation, treatment with immunosuppressive drugs and source of infection (blood, urinary tract, wound, respiratory source, or other). The same data were recorded for neonates with addition of type of birth, birth weight, gestational age, premorbid conditions such as preterm birth (cardiac, lung or renal dysplasia) and congenital malformations.

All antibiotics administered for ≥ 24 hours during the current hospitalization were recorded. The information collected included the drug name, start date, dose, route of administration, dosing frequency and total duration of use. Both individual and cumulative antibiotic exposures were evaluated. Data regarding the clinical outcome (recovery/death) were reviewd and death due to any cause or death attributable to infection was assessed. Septic shock was defined as sepsis associated with organ dysfunction, accompanied by persistent hypotension following volume replacement.

*Bacterial identification and antimicrobial susceptibility testing*

The definitions of the CDC were used to determine whether an isolated organism was associated with colonization or infection. Briefly, colonizers were defined as bacteria permanently or temporarily present in the skin or mucous membranes of the patient, dissociated from the signs or symptoms of infectious disease. Clinical infection was defined by medical diagnosis according to clinical criteria (sepsis, fever, changes in frequency or color of secretions, or new radiological findings) associated with the decision to initiate antimicrobial therapy, as well as isolation of a polymyxin-resistant organism [16]. Bacterial species identification and antimicrobial susceptibility testing were performed using the BD

Phoenix™ system (Franklin Lakes, NJ, USA) according to the manufacturer's recommendations.

After isolation and identification, the minimal inhibitory concentrations (MICs) of various antimicrobials were determined by broth microdilution following the recommendations of the Clinical and Laboratory Standards Institute guidelines (CLSI) [17]. Stock solutions of the tested antimicrobials were prepared by dissolving the chemical in appropriate solvents and diluents as described by the manufacturer. The following antimicrobials were tested: cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, and polymyxin B. The "American Type Culture Collection" (ATCC®) *Escherichia coli* ATCC® 25922 and *Pseudomonas aeruginosa* ATCC® 27853 strains were used as quality controls. Susceptibility results were interpreted according to CLSI [17].

*Antimicrobial resistance mechanisms*

Genomic DNA was extracted from fresh cultures; concentrations and purities were determined by spectrophotometer (BioDrop, Walnut Creek, CA, USA). The presence of β-lactamase (*bla*$_{\text{TEM-like}}$, *bla*$_{\text{SHV-like}}$, *bla*$_{\text{CTX-M-1-like}}$, *bla*$_{\text{CTX–M-2-like}}$, *bla*$_{\text{CTX-M-8-like}}$, *bla*$_{\text{CTX-M-14-like}}$, *bla*$_{\text{GES-like}}$, *bla*$_{\text{KPClike}}$, *bla*$_{\text{NDM-like}}$, *bla*$_{\text{IMP-like}}$, *bla*$_{\text{SPM-like}}$, *bla*$_{\text{VIM-like}}$, *bla*$_{\text{SIM-like}}$, *bla*$_{\text{NDM-like}}$ and *bla*$_{\text{OXA-48-like}}$) and polymyxin-resistance genes (*mcr-1*) were evaluated by PCR using specific, previously described, primers [10, 18]. To investigate chromosomal polymyxin-resistance mechanism the nucleotides sequences of *pmrA, pmrB, phoP, phoQ, crrB, soxS, soxR* and *mgrB* genes were sequenced and compared to sequence from a polymyxin-susceptible organism.

*Statistical analysis*

All clinical data were deposited in the Research Electronic Data Capture (Redcap) database and statistical analysis was performed by SAS v.9.2 (SAS Institute, Cary, NC, USA), using univariate and multivariate models. Dichotomized and categorical data were analyzed with the Chi-square test or Fisher´s exact test. For continuous variables, *t*-test or ANOVA was used. Univariate analyses were performed to verify the associations between the dependent and independent variables and those achieving a pre-specified level of significance ($p<0.2$) were included in the multivariable analysis, ($p<0.05$) was considered statistically significant. To evaluate the strength of associations, a logistic regression analysis was used to estimate the crude, adjusted odds ratios (OR) and 95% confidence intervals (CIs).

*Ethical standards*

This study was conducted with the approval of the Research Ethics Committee from the Universidade Federal da Grande Dourados (no. 877292/2014).

## Results

*Patients characteristics*

There were 1,067 episodes of infection/colonization with *Enterobaceriaceae* during the study period. A total of 695 cases were identified in patients admitted to intensive care units (adult ICU: 490, neonatal ICU: 108 and pediatric ICU: 95 patients). Throughout the study period polymyxin-resistant and carbapenemase-producing *Enterobacteriaceae* were isolated from 53 patients. Out of those 53 patients carrying polymyxin-resistant strains, 36 were admitted at the adult ICU and 17 were admitted at the neonatal ICU. We compared data from the 53 cases with 106 controls (72 adult and 34 neonatal patients) (Figure 1).

Among the adult patients (cases and controls) the median age was 56 years (range 27–81 years) and the majority was female (64; 59%). Forty-six (29%) had a history of previous hospitalization and strains were recovered from patients from 5 to 15 days following admission. There were no significant differences ($P > 0.05$) among cases and controls with regard to baseline demographics. The neonatal patients (cases and controls) included in the study, had a median gestational age of 34 weeks (range 27–40 weeks) and a median birth weight of 2.1 kg (range: 0.9 – 3.9 kg). There were no significant differences in clinical presentations between patients with polymyxin-resistant and polymyxin-susceptible organisms. The demographic and clinical data of patients included in this study are shown in Table 1 and 2.

*Coexistence of polymyxin and carbapenem resistance*

Among the polymyxin-resistant isolates, the most frequently identified species was *K. pneumoniae* (74%; 39/53), followed by *E. aerogenes* (21%; 11/53) and *E.coli* (5%; 3/53). Antimicrobial susceptibility testing results demonstrated that all organisms were resistant to all the tested antimicrobial, including imipenem ($MIC_{50}$, >8 mg/L), meropenem ($MIC_{50}$, >16 mg/L), ertapenem ($MIC_{50}$, >32 mg/L), and polymyxin B ($MIC_{50}$, >4 mg/L). Screening for carbapenem resistance genes found that 98% of organism harbored $bla_{KPC}$ and all organisms carried more than one ESBL gene, including $bla_{CTX\text{-}M}$ (98%), $bla_{SHV}$ (74%) and $bla_{TEM}$ (81%). The plasmid-mediated colistin resistance gene *mcr-1* was not detected. Regarding polymyxin-resistance several mutations in *mgrB* gene were observed in all *K. pneumoniae* strains. In the *E. aeregonenes* isolates the polymyxin-resistance occurred through mutational changes in *phoPQ* and *soxS* genes.

*Risk factors analysis for adult patients*

Adult patients (n=108) were evaluated and univariate analysis found multiple clinical factors including diabetes mellitus, hypertension, decubitus ulcers, pulmonary disease, chronic heart failure, pulmonary diseases, neurometabolic disease, neoplasia, hematologic diseases, organ transplantation, previous surgery and the use of invasive devices (mechanical ventilation, central venous catheter, urinary catheter) were associated with polymyxin-resistant organisms. Additionally, previous hospital admission, prolonged hospital stay, transfer between hospital units, and the use of antimicrobials (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones and polymyxins) were associated with polymyxin-resistant organisms (Table 1).

A multivariate analysis revealed that renal failure ($p$=0.02; OR 11.37; 95CI 1.0–128.63), urinary catheter ($p$<0.01; OR 4.16; 95CI 38.82–366.07), transfer between hospital units ($p$=0.03; OR 9.98; 95% CI 1.01–98.42), use of carbapenems ($p$<0.01; OR 45.49; 95CI 6.93–298.62) and having a surgical procedure ($p$=0.01; OR 16.52; 95CI 2.83–96.32) were all independently associated with the isolation of polymyxin-resistant organisms. Most patients infected with polymyxin-resistant organisms had various surgical procedures including tracheostomy (42.9%; 9/21), gastrointestinal surgeries (38.1%; 8/21), nefrectomy (14.3%; 3/21), and cesarean section (4.7%; 1/21).

*Risk factors analysis for neonates*

Neonatal patients (n=51) were evaluated and univariate analysis found multiple comorbidities including extremely low weight, congenital heart disease, meconium aspiration syndrome, previous hospitalization, use of invasive devices (central venous catheter, orotracheal tube, nasogastric tube, and nasoenteric tube), and previous antimicrobial exposure (aminoglycosides, penicilins/β-lactamase inhibitors andcarbapenems) were all associated with

the isolation of polymyxin-resistant organisms. A multivariate analysis showed that central venous catheter ($p$<0.01; OR 69.59; 95CI 7.33–660.30), was the only risk factor independently associated with the acquisition of polymyxin-resistant organisms (Table 2). Patients with central venous catheter had the device used for a median of 17 days (range: 5 – 31 days). Patients with central venous catheter remained using the device a median of 17 days (range: 5 – 31 days). The maternal comorbidity most frequently associated with neonates was hypertensive disease of pregnancy ($P$ = 0.02; OR 0.15; 95% CI 0.02–0.87) (Table S1).

*Outcome study*

Mortality was significantly higher in patients infected with polymyxin-resistant than in those with polymyxin-susceptible strains, 66.6% vs. 18.1%, respectively ($p$<0.01; OR 8.01; 95CI 3.63–17.69). Although mortality was higher in neonates infected with polymyxin-resistant strains (23.5%) than neonates infected with polymyxin-susceptible organisms (5.8%), this difference was not statistically significant ($p$=0.06; OR 0.20; 95CI 0.03–1.24). Overall, mortality at 30-days after polymyxin-resistant isolation was recorded in 36.1% (n=13/36) of adult patients and 11.7% (n=2/17) of neonates. A Kaplan–Meier survival analysis showed that the cumulative probability of death in the 30 days after infection was significantly higher for patients infected with polymyxin-resistance strains ($p$<0.01) (Figure 2). We additionally aimed to identify the risk factors associated with mortality in the adult patients with polymyxin-resistant strains. In a multivariable analysis carbapenem exposure ($p$<0.01; OR 50.93; 95% CI 2.26–>999.999) was strongly associated with mortality (Table 3). In addition, the multivariable analysis showed that use of aminoglycosides ($p$=0.03; OR 0.06; 95% CI 0.004–0.97) was a protection factor for mortality by polymyxin-resistant strains (Table 3). The majority of these patients were submitted to a combined therapy with polymyxin.

## Discussion

Infections caused by polymyxin-resistant strains are becoming a serious problem ICUs and are of great concern because of the limited treatment options and unfavorable impact on prognosis [6, 13]. In the present study, we described the clinical characteristics, risk factors and outcome associated with polymyxin-resistant *Enterobacteriaceae* strain in adult and neonatal population admitted in ICUs. We showed that renal failure, surgical procedure, indwelling devices (catheter urinary) and transference between hospital units were risk factors for the acquisition of polymyxin-resistant strains. Previous hospitalization, prior carbapenem-resistant infection and antibiotic exposure [4, 11, 13, 19] have been described as risk factors. There are few published reports of clinical risk factors for polymyxin resistance, and most of them reported only association [13, 15, 20]. Patients with critical illness have increased risk for infections [21]. In our study patients submitted to surgical procedures and carbapenem exposure had an impressive 16.5 and 45.5, respectively fold risk to developing polymyxin-resistance infection and this could contribute to the significantly increased mortality rate of these patients.

Regarding the neonates, our study suggested that several comorbidities, previous hospitalization, use of invasive devices and antibiotic exposure were associated with polymyxin-resistant strains, however the key find is that only central venous catheter was identified as an independent risk factor after adjustments in the multivariate model. Newborns submitted to central venous catheter had 69.59% more chances to acquiring a polymyxin-resistant strain. Although previous studies have identified risk factor associated with development of polymyxin-resistant infection, to the best of our knowledge this is the first study performed to identify risk factors and clinical outcomes in a neonatal population. Most neonates had underlying disease and were submitted to at least one invasive procedure that

put them at a higher risk. Previous studies reported these factors associated with the immunocompromised system which may favor the acquisition of multidrug-resistant infections [22-24].

The overall mortality rate of patients infected with polymyxin resistant strains was significantly higher than that reported in previous studies [4, 11, 13, 25]. On the other hand, the 30-day mortality was lower than identified in previous studies [25, 26]. Even though the acquisitions of polymyxin-resistant strains play an important role in the high mortality rates, they do not constitute the only risk factor responsible for the poor outcomes observed, considering that the patients displayed several unfavorable clinical conditions [21, 26]. In this study, the main predictors for mortality included carbapenem and polymyxin exposure. In addition the limited treatment options can contribute to the critical outcomes [6, 7, 19]. However in this study aminoglycosides use was identified as a protection factor for mortality caused by polymyxin-resistant strains, representing a good option for combination therapy with polymyxin.

Although the presence of *mcr* genes in *Enterobacteriaceae* strains has already been reported in Brazil [27-29], in this study no isolate carried the *mcr-1* gene. Concerning about polymyxin resistance we also investigated alterations in the *mgrB* chromosomal gene and insertional inactivation of *mgrB* by insertion sequences was most often associated with resistance in all *K. pneumoniae* strains. No alterations in the *mgrB* gene were found in the remaining isolates; however the *E aerogenes* presented point mutationi the *phoPQ* and *soxS* genes. Concerning about *E.coli* strains no alterations were found in the genes mentioned above, these findings indicate that the primary mechanism of polymyxin resistance in these strains is mediated by other genes involved in the LPS modifications as reported in previous studies [7, 30].

There were some limitations in this study. Firstly, it was a retrospective study, so further prospective and multicenter studies are needed. Secondly, the small number of neonates included may have impacted in the significant findings and the data in this population is very limited. Thus, more surveillance studies in newborn patients are recommended in the future. Besides the limitations, our study adds to the literature as provide insights of which patients can possible be mainly affected and how polymyxin resistance may be introduced in similar settings. In addition this is the first study in South America to evaluated risk factors for acquisition of polymyxin-resistant strains.

In conclusion, the strengths of this study are the identification of several risk factors associated with the acquisition of polymyxin-resistant strains in both adult and neonatal populations. Awareness of these patients' specific risk factors may assist providers with the selection of the most appropriate management. Our results highlight the importance of antimicrobial stewardship efforts in minimizing unnecessary use. In addition due to limited alternative therapeutic options, adherence to infection control protocols is of great importance in order to reduce dissemination of these isolates.

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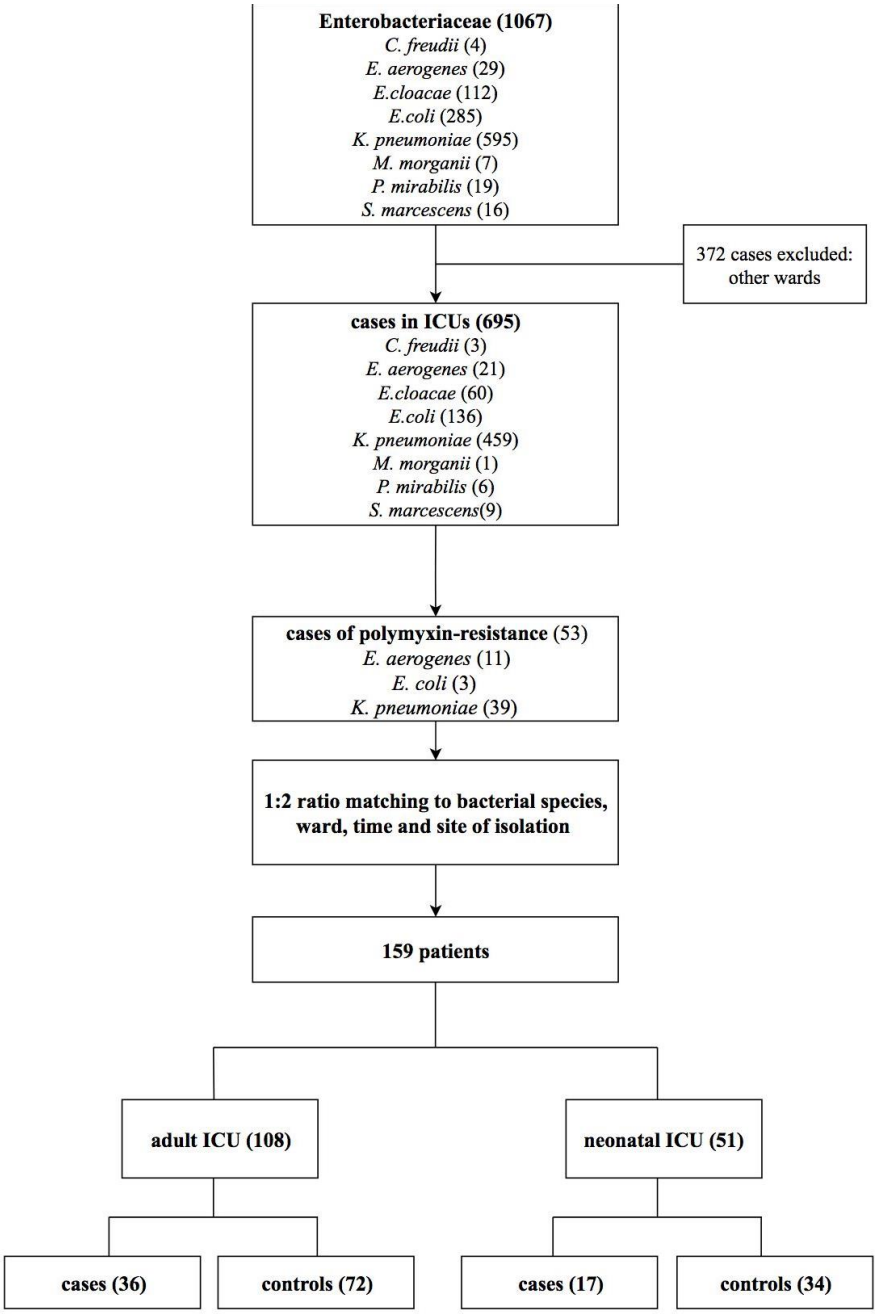


**Figure 1.** Flowchart of the definition and selection of cases and controls included in the study of risk factors for polymyxin-resistant *Enterobacteriaceae*.

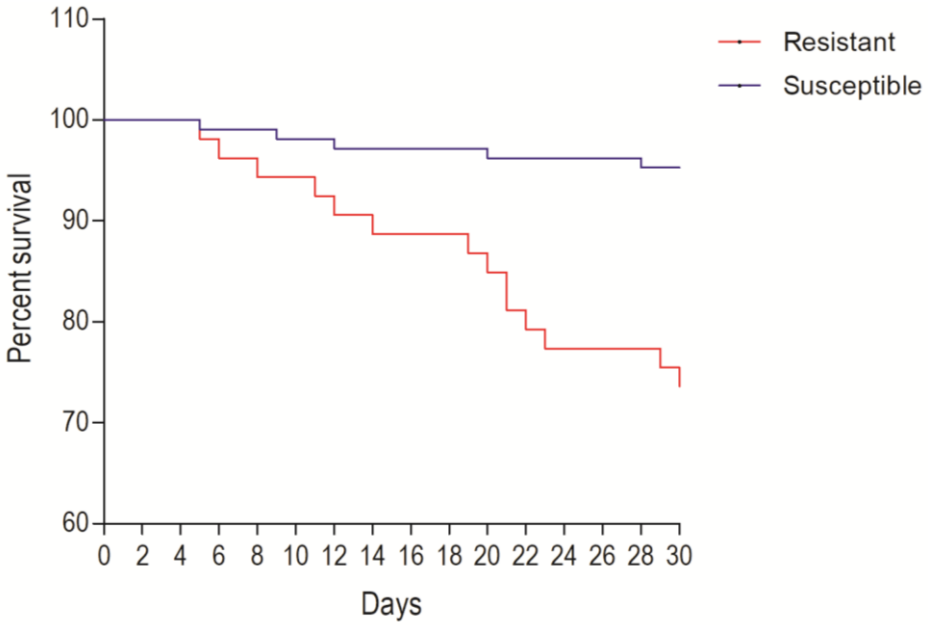


**Figure 2.** Kaplan–Meier probability estimate for the 30-day mortality of *Enterobacteriaceae* infection. The red line represents patients with infection caused by polymyxin-resistant; the blue line represents patients with infection caused by polymyxin-susceptible strains.

436 **Table 1.** Summary of risk factors associated with polymyxin-resistance *Enterobacteriaceae* strains isolated from adult patients.

437

| **Risk factors** | **Case patients n=36 (%)** | **Control patients n=72 (%)** | **Univariable analysis** | | **Multivariable analysis** | |
|---|---|---|---|---|---|---|
| | | | **OR (95% CI)** | ***P*-value** | **OR (95% CI)** | ***P*-value** |
| Age (years) | 61 σ 20.9 | 52 σ 21.9 | | 0.70 | | |
| Female | 19 (52.8) | 44 (51.1) | 1.40 (0.62-3.15) | 0.40 | | |
| **Comorbidities** | | | | | | |
| Diabetes mellitus | 16 (44.4) | 18 (25) | 0.41 (0.17-0.97) | **0.04** | | |
| Alcoholism | 3 (8.3) | 6 (8.3) | 1.0 (0.23-4.25) | 1.0 | | |
| Hypertension | 21 (58.3) | 27 (37.5) | 0.42 (0.18-0.96) | **0.04** | | |
| Decubitus ulcers | 5 (13.8) | 0 | 0.86 (0.75-0.98) | **<0.01** | | |
| Pulmonary disease | 4 (11.1) | 2 (2.7) | 0.22 (0.03-1.31) | **0.07** | | |
| Chronic heart failure | 10 (27.7) | 7 (9.7) | 0.28 (0.09-0.81) | **0.01** | | |
| Chronic renal failure | 14 (38.8) | 5 (6.9) | 0.11 (0.03-0.36) | **<0.01** | 11.37 (1.0-128.63) | **0.02** |
| Substance abuse | 2 (5.5) | 1 (1.3) | 0.23 (0.02-2.73) | 0.21 | | |
| Smoking history | 7 (19.4) | 9 (12.5) | 0.59 (0.2-1.74) | 0.33 | | |
| Neurological disease | 5 (13.8) | 1 (1.3) | 0.08 (0.01-0.77) | **<0.01** | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Neoplasia | 4 (11.1) | 2 (2.7) | 0.22 (0.03-1.31) | **0.07** | | |
| Hematologic Diseases | 3 (8.3) | 1 (1.3) | 0.15 (0.01-1.54) | **0.07** | | |
| Organ transplantation | 2 (5.5) | 0 | 0.94 (0.87-1.0) | **0.04** | | |
| Pulmonary edema | 5 (13.8) | 0 | 0.86 (0.75-0.98) | **<0.01** | | |
| HIV infection | 4 (11.1) | 5 (6.9) | 0.59 (0.15-2.37) | 0.46 | | |
| **Hospitalization** | | | | | | |
| Mechanical ventilation | 21 (58.3) | 10 (13.8) | 0.11 (0.04-0.29) | **<0.01** | | |
| Previous surgery | 21 (58.3) | 9 (12.5) | 0.12 (0.05-0.32) | **<0.01** | 16.52 (2.83-96.32) | **<0.01** |
| Central venous catheter | 23 (63.8) | 14 (19.4) | 0.13 (0.05-0.36) | **<0.01** | | |
| Urinary catheter | 17 (47.2) | 4 (5.5) | 0.06 (0.01-0.21) | **<0.01** | 4.16 (38.82-368.07) | **<0.01** |
| Use of immunosuppressive agents | 0 | 2 (2.7) | 1.02 (0.98-1.06) | 0.31 | | |
| Hemodialysis | 7 (19.4) | 2 (2.7) | 0.11 (0.02-0.60) | **<0.01** | | |
| Nasogastric tube | 17 (47.2) | 16 (22.2) | 0.31 (0.13-0.75) | **<0.01** | | |
| Chest drainage | 5 (13.8) | 2 (2.7) | 0.17 (0.03-0.96) | **0.02** | | |
| Abdominal drainage | 5 (13.8) | 0 | 0.86 (0.75-0.98) | **<0.01** | | |
| Peritoneal dialysis | 2 (5.5) | 0 | 0.94 (0.87-1.02) | **0.04** | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Previous hospital admission | 19 (52.8) | 10 (13.8) | 0.14 (0.05-0.36) | **<0.01** | | |
| Prolonged hospital stay | 18 (50) | 15 (20.7) | 6.93 (2.72-17.65) | **<0.01** | | |
| Transfer between hospital units | 15 (41.6) | 3 (4.1) | 0.06 (0.01-0.23) | **<0.01** | 9.98 (1.01-98.42) | **0.03** |
| **Use of antimicrobials** | 22 (61.1) | 20 (27.7) | 0.24 (0.10-0.57) | **<0.01** | | |
| Previous exposure | 30 (83.3) | 47 (65.2) | 2.65 (0.97-7.24) | **0.05** | | |
| Aminoglycosides | 16 (44.4) | 6 (8.3) | 0.11 (0.03-0.32) | **<0.01** | | |
| β-lactam | 17 (47.2) | 27 (37.5) | 0.67 (0.29-1.51) | 0.33 | | |
| Carbapenems | 25 (69.4) | 13 (18.1) | 0.09 (0.03-0.24) | **<0.01** | 45.49 (6.93-298.62) | **<0.01** |
| Cephalosporins | 12 (33.3) | 14 (19.4) | 0.48 (0.19-1.19) | **0.11** | | |
| Fluoroquinolones | 3 (8.3) | 2 (2.7) | 0.31 (0.05-1.97) | **0.19** | | |
| Polymyxins | 12 (33.3) | 3 (4.1) | 0.08 (0.02-0.36) | **<0.01** | | |

**Abbreviations**: **OR**- Odds Ratio; **CI**- Confidence intervals; **σ** – Standard deviation.

445 **Table 2.** Summary of risk factors associated with polymyxin-resistance *Enterobacteriaceae* strains isolated from neonatal patients.

446

| Risk factor | Case patients n=17 (%) | Control patients n=34 (%) | Univariate analysis | | Multivariable analysis | |
|---|---|---|---|---|---|---|
| | | | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| Gestational age (weeks) | 34,5 σ 4.04 | 35 σ 3.54 | | 0.86 | | |
| Female | 9 (52.9) | 14 (41.1) | 0.62 (0.19-2.0) | 0.42 | | |
| **Neonate weight** | | | | | | |
| Small for gestational age | 11 (64.7) | 14 (41.1) | 0.38 (0.11-1.27) | 0.11 | | |
| Low weight | 4 (23.5) | 7 (20.5) | 0.84 (0.20-3.40) | 0.80 | | |
| Very low weight | 3 (17.6) | 6 (17.6) | 1.0 (0.21-4.60) | 1.0 | | |
| Extreme low weight | 2 (11.7) | 0 | 0.88 (0.74-1.04) | **0.04** | | |
| **Comorbidities** | | | | | | |
| Congenital heart disease | 5 (29.5) | 1 (2.9) | 0.07 (0.01-0.68) | **<0.01** | | |
| Neonatal anoxia | 3 (17.6) | 1 (2.9) | 0.14 (0.01-1.47) | 0.06 | | |
| Hyaline membrane syndrome | 3 (17.6) | 1 (2.9) | 0.14 (0.01-1.47) | 0.06 | | |
| Meconium aspiration syndrome | 3 (17.6) | 0 | 0.82 (0.66-1.02) | **0.01** | | |
| Pulmonary Bronchodysplasia | 1 (5.8) | 0 | 0.94 (0.83-1.05) | 0.15 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Neonatal infection | 1 (5.8) | 0 | 0.94 (0.83-1.05) | 0.15 | | |
| Gastroschisis | 3 (17.6) | 1 (2.9) | 0.14 (0.01-1.47) | 0.06 | | |
| Icterus | 2 (11.7) | 2 (5.8) | 0.46 (0.06-3.65) | 0.46 | | |
| Hypoglycemia | 0 | 3 (8.8) | 1.09 (0.98-1.21) | 0.21 | | |
| **Hospitalization** | | | | | | |
| Prematurity (GA<37 weeks) | 8 (47.0) | 18 (52.9) | 1.26 (0.39-4.06) | 0.69 | | |
| Gemini | 1 (5.8) | 3 (8.2) | 1.54 (0.14-16.10) | 0.71 | | |
| Previous hospitalization | 6 (35.3) | 11 (32.4) | 1.14 (0.33-3.89) | **0.04** | | |
| Prolonged hospital stay | 7 (41.1) | 12 (35.2) | 0.98 (0.27-3.65) | 0.45 | | |
| Transfer between hospital units | 4 (23.5) | 1 (2.9) | 0.09 (0.01-0.96) | 0.01 | | |
| Surgical procedure | 3 (17.6) | 2 (5.8) | 0.29 (0.04-1.94) | 0.18 | | |
| Neonatal bed (incubator) | 5 (29.5) | 18 (52.9) | 0.87 (0.37-4.50) | 0.1 | | |
| Type of birth (normal) | 9 (52.9) | 17 (50) | 0.88 (0.27-2.85) | 0.84 | | |
| Antenatal care | 15 (88.2) | 22 (88.9) | 0.97 (0.14-6.57) | 0.98 | | |
| **Presence of device** | | | | | | |
| Peripheral access | 5 (29.4) | 5 (14.7) | 0.41 (0.10-1.69) | 0.21 | | |
| Central venous catheter | 12 (70.5) | 2 (5.8) | 0.02 (0.01-0.15) | **<0.01** | 69.59 (7.33-660.30) | **<0.01** |

| | | | | |
|---|---|---|---|---|
| Orotracheal tube | 15 (88.2) | 7 (20.5) | 0.03 (0.01-0.18) | <**0.01** |
| Nasogastric tube | 11 (64.7) | 2 (5.8) | 0.03 (0.01-0.19) | <**0.01** |
| Nasoenteric tube | 2 (11.7) | 0 | 0.88 (0.74-1.04) | **0.04** |
| Nasal CPAP | 2 (11.7) | 0 | 0.88 (0.74-1.04) | **0.04** |
| Umbilical catheter | 2 (11.7) | 8 (23.5) | 2.30 (0.43-12.31) | 0.31 |
| Urinary catheter | 1 (5.8) | 1 (2.9) | 0.48 (0.02-8.26) | 0.61 |
| **Use of antimicrobials** | | | | |
| Previous exposure | 16 (94.1) | 18 (52.9) | 14.22 (1.69-119.62) | <**0.01** |
| Aminoglycosides | 9 (52.9) | 2 (5.8) | 0.05 (0.01-0.30) | <**0.01** |
| β-lactam (penicilins/β-lactamase inhibitors) | 12 (70.5) | 9 (26.4) | 0.15 (0.04-0.54) | <**0.01** |
| Carbapenems | 5 (29.5) | 1 (2.9) | 0.07 (0.0.1-0.68) | <**0.01** |
| Cephalosporins | 7 (41.1) | 9 (26.4) | 0.51 (0.15-1.75) | 0.28 |
| Fluoroquinolones | 0 | 1 (2.9) | 1.03 (0.97-1.09) | 0.47 |
| Polymyxins | 0 | 1 (2.9) | 1.03 (0.97-1.09) | 0.47 |

**Abbreviations**: **OR**- Odds Ratio; **CI**- Confidence intervals; **σ** – Standard deviation.

**Table 3.** Risk factors associated with mortality among patients with *Enterobacteriaceae* strains isolated from adult patients.

| Risk factors | Death (n = 24) | Survivors (n = 12) | Univariable analysis | | Multivariable analysis | |
|---|---|---|---|---|---|---|
| | | | OR (95% CI) | *P*-value | OR (95% CI) | *P*-value |
| Age (years) | 62.8 σ 19.1 | 51.1 σ 22.4 | 1.18 (0.29-4.73) | 0.81 | | |
| Male | 11 (45.8) | 6 (50) | | | | |
| **Comorbidities** | | | 1.18 (0.29-4.80) | 0.81 | | |
| Diabetes mellitus | 11 (45.8) | 5 (41.6) | 1.14 (0.98-1.32) | **0.20** | | |
| Alcoholism | 3 (12.5) | 0 | 1.66 (0.41-6.76) | 0.47 | | |
| Hypertension | 15 (62.5) | 6 (50) | 1.26 (1.02-1.55) | **0.08** | | |
| Decubitus ulcers | 5 (20.8) | 0 | 1.2 (1-1.43) | **0.13** | | |
| Pulmonary disease | 4 (16.6) | 0 | 1.23 (0.25-5.97) | 0.79 | | |
| Chronic heart failure | 7 (29.1) | 3 (25) | 5 (0.89-27.81) | **0.05** | | |
| Chronic renal failure | 12 (50) | 2 (16.6) | 1.09 (0.96-1.23) | **0.30** | | |
| Substance abuse | 2 (8.3) | 0 | 1.31 (0.21-8.03) | 0.76 | | |
| Smoking history | 5 (20.8) | 2 (16.6) | 2.2 (0.21-22.19) | 0.49 | | |
| Neurological disease | 4 (16.6) | 1 (8.3) | 1.2 (1-1.43) | **0.13** | | |
| Neoplasia | 4 (16.6) | 0 | 1.14 (0.98-1.32) | 0.2 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Hematologic Diseases | 3 (12.5) | 0 | 1.09 (0.96-1.23) | 0.30 | | |
| Organ transplantation | 2 (8.3) | 0 | 0.71 (0.10-4.97) | 0.73 | | |
| Pulmonary edema | 3 (12.5) | 2 (16.6) | 1.2 (1-1.43) | **0.13** | | |
| HIV infection | 4 (16.6) | 0 | | | | |
| **Hospitalization** | | | 9. 0 (1.81-44.59) | **<0.01** | | |
| Mechanical ventilation | 18 (75) | 3 (25) | 0.33 (0.07-1.54) | 0.15 | | |
| Previous surgery | 12 (50) | 9 (75) | 7.6 (1.60-35.9) | **<0.01** | | |
| Central venous catheter | 19 (79.1) | 4 (33.3) | 1.4 (0.34-5.67) | 0.66 | | |
| Urinary catheter | 12 (50) | 4(41.6) | 1.31 (0.21-8.03) | 0.76 | | |
| Hemodialysis | 5 (20.8) | 2 (16.6) | 22 (2.39-201.76) | **<0.01** | | |
| Nasogastric tube | 16 (66.7) | 1 (8.3) | 0.71 (0.10-4.97) | 0.73 | 50.64 (3.63-705.89) | **<0.01** |
| Chest drainage | 3 (12.5) | 2 (16.6) | 0.27 (0.03-1.91) | **0.17** | | |
| Abdominal drainage | 2 (8.3) | 3 (25) | 1.09 (0.96-1.23) | 0.3 | | |
| Peritoneal dialysis | 2 (8.3) | 0 | 1.18 (0.29-4.73) | 0.81 | | |
| Previous hospital admission | 13 (54.2) | 6 (50) | | 0.28 | | |
| Prolonged hospital stay | 11 (45.8) | 7 (58.3) | 1 (0.24-4.07) | **1** | | |
| Transfer between hospital units | 10 (41.7) | 5 (41.6) | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Use of antimicrobials** | | | 0.42 (0.07-2.54) | 0.34 | | |
| Previous exposure | 21 (87.5) | 9 (75) | 0.71 (0.17-2.87) | 0.63 | | |
| Aminoglycosides | 10 (41.7) | 6 (50) | 0.84 (0.21-3.38) | 0.81 | 0.06 (0.004-0.97) | **0.03** |
| β-lactam | 11 (45.8) | 6 (50) | 7.1 (1.45-33.69) | **0.01** | | |
| Carbapenems | 20 (83.3) | 5 (41.7) | 0.33 (0.07-1.43) | **0.13** | 50.93 (2.26->999.999) | **<0.01** |
| Cephalosporins | 6 (25) | 6 (50) | 0.21 (0.01-2.68) | 0.2 | | |
| Fluoroquinolones | 1 (4.2) | 2 (16.6) | 3.57 (0.63-19.97) | **0.13** | | |
| Polymyxins | 10 (41.7) | 2 (16.7) | 1.18 (0.29-4.73) | 0.81 | 4.46 (1.63-12.22) | 0.13 |

**Abbreviations**: **OR**- Odds Ratio; **CI**- Confidence intervals; **σ** – Standard deviation.

**Table S1.** Summary of maternal risk co-morbidities associated with polymyxin-resistance *Enterobacteriaceae* strains isolated from neonatal patients.

| Risk factor | Case patients n=17 (%) | Control patients n=34 (%) | Univariate analysis | |
|---|---|---|---|---|
| | | | OR (95% CI) | *P*-value |
| Age (years) | 31 σ 5,17 | 26 σ 6.69 | | 0.40 |
| **Comorbidities** | | | | |
| Hypertensive disease of pregnancy | 5 (29.4) | 2 (5.8) | 0.15 (0.02-0.87) | **0.02** |
| Hypertension | 1 (5.8) | 0 | 0.94 (0.83-1.05) | **0.15** |
| Diabetes mellitus | 0 | 1 (2.9) | 1.03 (0.97-1.09) | 0.47 |
| Urinary tract infection | 1 (5.8) | 0 | 0.94 (0.83-1.05) | **0.15** |
| Oligohydramnio | 1 (5.8) | 0 | 0.94 (0.83-1.05) | **0.15** |
| Premature rupture of membranes | 3 (17.6) | 1 (2.9) | 0.14 (0.01-1.47) | **0.06** |
| Syphilis | 1 (5.8) | 0 | 0.94 (0.83-1.05) | **0.15** |
| Cytomegalovirus infection | 1 (5.8) | 0 | 0.94 (0.83-1.05) | **0.15** |

**Abbreviations**: **OR**- Odds Ratio; **CI**- Confidence intervals; **σ** – Standard deviation.

**Artigo 4: Antisense peptide nucleic acid inhibits growth of KPC-producing *Klebsiella pneumoniae***

**Short running title:** Antisense PNA aggainst *Klebsiella pneumoniae*.

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**ABSTRACT**

**Background:** *Klebsiella pneumoniae* causes common and severe hospital- and community-acquired infections with a high incidence of multidrug resistance (MDR) and mortality. The emergence and spread of these MDR strains limit therapeutic options and highlight the need to develop new therapeutic strategies. In this study, we investigated antisense peptide nucleic acids (PNA) conjugated to the (KFF)3K cell-penetrating peptide regarding their ability to target the *gyr*A KPC-producing *K. pneumoniae* essential gene, further inhibiting *in vitro* bacterial growth. **Methods:** Antisense PNAs were tested at final concentrations of 7.5, 12.5, 25, and 50 µM to determine the inhibitory effects on growth. In order to assess the true inhibitory potential of PNA, effects of treatments was measured using16s gene amplification. An *ex vivo* red blood cell hemolysis assay was utilized in order to determine the potential toxic and hemolytic activity. Bioinformatic analyses were performed to show PNA conformation in the aqueous environment. **Results:** PNA was capable of inhibiting *in vitro* bacterial growth at 50 µM, and we observed a 96.7% reduction in 16S gene amplification. Bioinformatics analysis demonstrated that the structure of the PNA exhibits stability in water without major changes in its secondary structure. To the best of our knowledge, this report is the first demonstration of the antibacterial efficacy of PNA against KPC-producing *K. pneumoniae*. **Conclusion:** The ability of PNA and its conjugated peptide to inhibit bacterial growth demonstrates the potential of this new class of antibacterial agents, encouraging further *in vivo* studies to confirm its therapeutic efficacy.

**Keywords**: *Klebsiella pneumoniae*, multidrug resistance, KPC-producing, PNA, antisense agent.

## Background

*Klebsiella pneumoniae* is one of the most common and clinically important pathogens, and it causes a wide spectrum of infectious diseases [1]. The emergence of carbapenem-resistant *K. pneumoniae* has become a matter of global concern as regards the control and treatment of nosocomial infections [2]. Infections caused by these microorganisms have been associated with high morbidity and mortality rates [3]. These pathogens have been characterized by multiantibiotic resistance, including resistance to penicillins, cephalosporins, monobactams, carbapenems, and even β-lactamase inhibitors [4, 5]. Resistance to carbapenems is linked to different mechanisms, mainly due to the production of carbapenemase enzymes [6].

The emergence of antibiotic-resistant bacteria reduces available therapeutic options, demonstrating the urgent need for alternative strategies to prevent MDR dissemination [7] Antisense technology is a promising tool that can decrease or inhibit the expression of specific genes through sequence-specific recognition of mRNA, and many advances such as backbone modifications, sugar moieties, and nitrogenous bases have been made to optimize the technology for therapeutic purposes [8-10].

The potential applications of antisense peptides, including peptide nucleic acids (PNAs), have attracted great attention in recent years due to a wide range of applications, and have emerged as promising therapeutic molecules. PNAs are nucleic acid analogues capable of hybridization and of forming strong and stable complexes with complementary RNA and DNA, thus allowing the inhibition of specific genes [11, 12]. Their biochemical characteristics present many of the requirements of an ideal drug candidate, including low toxicity, high specificity and prolonged stability *in vitro* [13, 14]. In addition, when targeting the start codon region, PNAs provide robust antisense activity against Gram-negative and -positive bacteria [15]. . In Gram-negative bacteria, however, the activity of PNAs is usually

reduced, as these molecules are not capable of translocating through the outer membrane to act on their intracellular targets. Nevertheless, the conjugation of PNAs with short delivery peptides, called cell-penetrating peptides (CPPs), can facilitate the delivery of nucleic acids inside the target cells, thus improving their antisense activity [16, 17]. These favorable properties, besides the inherent low toxicity, have been harnessed to silence genes critical for bacterial viability, thereby inhibiting bacterial growth [18]. In this study, we investigated the spectrum of antibacterial and hemolytic activities of antisense CPP-PNAs targeting the *gyr*A gene in KPC-producing *K. pneumoniae*, also shedding light on the structural behavior of this molecular complex through molecular dynamics simulations.

## Material and methods

### Bacterial strain

The carbapenem-resistant *K. pneumoniae*s train was obtained from urine culture of a hospitalized patient in a tertiary teaching hospital [3].

### Bacterial identification and phenotypic assays

The bacterial species was identified using the VITEK®2 automated system (bioMérieux, Hazelwood, MO, USA) and confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) using the Microflex LT spectrometer (BrukerDaltonics, Massachusetts, USA), as previously described [19]. The minimal inhibitory concentrations (MICs) of antimicrobials were determined using broth microdilution according to guidelines from the Clinical and Laboratory Standards Institute (CLSI). Preliminary screening for the presence of carbapenemases was performed by the modified Hodge test (MHT) according to CLSI guidelines [20]. Positive results obtained with

the MHT were confirmed by ertapenem hydrolysis using mass spectrometry, as previously described [21].

**PCR amplification and sequencing of β-lactamase encoding genes**

The presence of β-lactamase genes (*bla*$_{\text{TEM-like}}$, *bla*$_{\text{SHV-like}}$, *bla*$_{\text{CTX-M-1-like}}$, *bla*$_{\text{CTX–M-2-like}}$, *bla*$_{\text{CTX-M-8-like}}$, *bla*$_{\text{CTX-M-14-like}}$, *bla*$_{\text{GES-like}}$, *bla*$_{\text{KPC-like}}$, *bla*$_{\text{SME-like}}$, *bla*$_{\text{NDM-like}}$, *bla*$_{\text{IMP-like}}$, *bla*$_{\text{SPM-like}}$, *bla*$_{\text{VIM-like}}$, *bla*$_{\text{SIM-like}}$, *bla*$_{\text{GIM-like}}$, *bla*$_{\text{NDM-like}}$, and *bla*$_{\text{OXA-48-like}}$) was evaluated using PCR amplification, followed by sequencing using specific primers, as previously described [19]. The DNA sequences and their derived protein sequences were analyzed using the Lasergene Software Package (DNASTAR, Madison, USA) and compared to sequences deposited in GenBank.

**PNA synthesis**

The PNA conjugate was synthesized with manual coupling chemistry and purified by PNA BIO Inc (Thousand Oaks, CA, USA). High performance liquid chromatography (HPLC)was used to purify the synthetic PNA and, after all purification steps, the final purity was 99.9%. PNAs used were covalently conjugated with CPP (KFF)3K (where K and F are lysine and phenylalanine, respectively) at the carbonyl terminus (corresponding to the 3’ end of a conventional oligonucleotide) [13]. PNAs were diluted to 100 µM with ultrapure water and stored at -20 °C until analysis.

**Inhibition of bacterial growth**

KPC-producing *K. pneumoniae* strain bacteria were typically grown overnight in 3 mL Mueller–Hinton (MH) broth at 37°C with constant shaking at 200 rpm. Optical density was measured at 600nm the following day, and the cultures were then diluted to $10^5$ CFU.mL$^{-1}$in a

low-binding 96 well microtiter plate. Antisense PNAs were added to the wells at final concentrations of 7.5, 12.5, 25, and 50 µM. The microtiter plates were incubated at 37°C, and optical density at 600 nm ($OD_{600}$) was measured with a Sunrise microplate reader (Tecan, Zurich, Switzerland) every hour to evaluate growth rates. Samples were taken at different time points (0, 3, 6, 9, 12 and 18 h) during culture, plated on MH agar at the appropriate dilutions and grown overnight at 37°C. Viable cell counts were performed by inspection of colony-forming units (CFU) to determine the inhibitory effects of the PNA.

**Inhibitory effect on *gyr*A gene**

In order to detect 16S gene amplification in bacteria, total DNA from KPC-producing *K. pneumoniae* was extracted using PureLink Genomic DNA Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative PCR (Table 1) was performed using SYBR Premix Ex Taq II (Takara-BioInc) according to the manufacturer's instructions. Amplification was performed using a CFX96 Touch™ Real-time PCR Detection System (BioRad laboratories Inc., Hercules, CA, USA) under the following conditions: denaturation at 95°C for 3 min for the first cycle and for 30 s thereafter, annealing at 59°C for 30 s, and extension at 72°C for 40 s for 40 cycles. Final extension was at 72°C for 10 min. PCR products were also analyzed by electrophoresis on a 1% agarose gel.

**Hemolysis assay**

An *ex vivo* red blood cell hemolytic assay was conducted to determine the potential hemolytic effects of PNAs [22]. Blood from Swiss mice was collected, and erythrocytes were separated from plasma by centrifugation (1600 rpm for 2min). Plasma and the buffy layer containing white blood cells were removed and discarded. Isolated erythrocytes were prepared at 1% (v:v) and washed three times with PBS (pH 7.4). Subsequently, 50 µL of PNA

was added and gently pipetted into 96-well microplates containing 50 μL of the erythrocyte suspension. The final concentrations of PNA in the wells were 25, 50 and 100 μM. PBS and Triton X-100 1% were mixed with erythrocyte suspension1% (v:v) as negative and positive controls, respectively. Cells were incubated at room temperature for one hour with the PNA. After incubation, the microplate was centrifuged (3000 rpm for 2min) and the supernatant transferred to a new 96-well microplate. Release of hemoglobin was monitored by absorbance readings at 415 nm with a microplate photometer Multiskan FC (Thermo Scientific, Pittsburg, PA, USA) with PBS at the same pH as the blank.The results of hemolysis after treatment were calculated using the following equation:% hemolysis = 100 × [[( $absorbance_{sample}$–$absorbance_{blank}$) – ($absorbance_{negative}$–$absorbance_{blank}$)]/[ $absorbance_{positive}$–$absorbance_{negative}$–$absorbance_{blank}$ ]]. Experiments were performed in triplicate.

**Molecular modelling**

The 3D structure of CPP was built on the I-Tasser server [23-25] and validated by ProSA-web [26, 27] and PROCHECK [28, 29]. After validation, the linker was added at the C-terminal. The DNA (GCCATCTCGGACATC) was modeled on the Avogadro software [30]. The structures were joined using the tLeap tool, where connections between DNA, linker and CPP were adjusted, and then the initial structure and parameters for the MD were generated.

**Molecular Dynamics**

MD simulations were performed using Amber software version 16 [31] peptide and DNA, and GAFF to model the linker and to solvate and explicate theTIP3P water model [32]. During the simulation, PNA and solvent (counterions and water) were maintained at a

constant temperature of 310 K using the Langevin thermostat [33] with a collision frequency of 1.0 $ps^{-1}$. Van der Waals interactions were truncated at 0.8 nm with a long-range dispersion correction applied to the energy and pressure. A pressure of 1 bar was maintained using semi-isotropic pressure coupling with a Monte Carlo barostat and a time constant of 1 ps. Electrostatic interactions were treated using the smooth particle mesh Ewald (PME) [34]algorithm with a short-range cutoff of 1.0 nm. The neighbor list was updated every 25 steps during the simulations. All bonds involving hydrogen were constrained using the SHAKE [35] algorithm allowing a 2 fs time step to be applied for 100 ns of MD simulation. The system was neutralized with $Na^+$ ions. To determine the most populous conformation, the k-means clustering method was used [36]. In this method the number of groups (K) is defined by the user. Sequentially, each element *i*to be grouped is assigned to the set whose centroid is closest. The choice of the initial centroids is commonly done at random. Allocation of an element to a given group is done based on the distance from the centroid. K-Means will separate the *n* elements into K sets by minimizing the sum of the squares of distances within the set. The silhouette was used as a measure of similarity between the groups [37]. Peptide flexibility was studied by root mean square fluctuations (RMSF) and conformational stability by root mean square deviations (RMSD).

## Results

### Susceptibility and molecular testing

The KPC-producing *K. pneumoniae* strain showed resistance to the antibiotics tested by broth microdilution as follows: meropenem ($MIC_{50}$, >16 $mg.L^{-1}$), imipenem ($MIC_{50}$, >8 $mg.L^{-1}$), and ertapenem ($MIC_{50}$, >32 $mg.L^{-1}$). Carbapenemase production was detected by MHT, and carbapenem hydrolysis was detected by MALDI-TOF. PCR amplification and sequencing showed that the $bla_{KPC-2}$ gene was present. The $bla_{TEM-like}$, $bla_{SHV-like}$, $bla_{CTX-M-1-}$

like,*bla*$_{\text{CTX–M-2-like}}$, *bla*$_{\text{CTX-M-8-like}}$, *bla*$_{\text{CTX-M-14-like}}$, *bla*$_{\text{GES-like}}$, *bla*$_{\text{SME-like}}$, *bla*$_{\text{NDM-like}}$, *bla*$_{\text{IMP-like}}$, *bla*$_{\text{SPM-like}}$, *bla*$_{\text{VIM-like}}$, *bla*$_{\text{SIM-like}}$, *bla*$_{\text{GIM-like}}$, *bla*$_{\text{NDM-like}}$ and *bla*$_{\text{OXA-48-like}}$ genes were not detected (data not shown).

**Growth inhibition by targeting essential *K. pneumoniae* gene**

To test the inhibitory potential of antisense PNA, we evaluated a PNA designed to bind to the start codon region of the essential gene *gyrA*, which is involved in DNA replication. Inhibition of KPC-producing *K. pneumoniae* strain growth was evaluated by examining the effects of different PNA concentrations, and growth was inhibited by PNA anti-*gyr*A at a concentration of 50 µM. The results indicate that the inhibition effect is concentration-dependent (Figure 1a). The bacteriostatic *versus* bactericidal effects of gene inhibition were evaluated by the number of viable cells in cultures treated with antisense peptide-PNA, and aliquots were taken at different time points following treatment. The number of viable cells was indicated by counting CFUs after 18h of incubation, and thisshowed that the PNA produced a significant bacterial reduction at a concentration of 45µM (observed as a 3.50 $\log_{10}$ reduction) and at 50µM (observed as a 6.01 $\log_{10}$ reduction), respectively, confirming the bactericidal effect (Figure 1b).

**Inhibitory effect on *gyr*A gene**

Aiming to examine viable bacterial cell number reduction, a real-time polymerase chain reaction (qPCR) was used to evaluate the impact of PNA on 16S gene amplification in KPC-producing *K. pneumoniae,* and the subsequent suppression of *gyrA* expression, following antisense PNA treatment. The DNA levels were reduced in a concentration-dependent manner in treated cultures compared with the untreated control (Figure 2). Reduction of *16s* DNA amplification was observed following treatment with PNA at 45 and

50μM. Antisense PNA produced a 9.5% (at 7.25 μM), 12.1% (at 12.5 μM) 23.2% (at 25μM), 51.1% (at 45μM) and 96.7% (at 50 μM) reduction in 16S gene amplification, indicating a concentration-dependent inhibition of *gyrA* gene expression.

**Hemolysis assay**

Hemolytic assays of antisense PNA toward mice erythrocytes was evaluated at concentrations need to inhibit bacterial growth. The results indicated 3.2 and 21.1% hemolysis at 25 and 50 μM of PNA, respectively. PNA tested at the highest concentration (100μM) showed 44.9% of hemolysis (Figure S1).

**Bioinformatics analysis**

Molecular modeling and MD simulations were carried out in the present study to evaluate the trajectory of the molecular complex CPP-linker-PNA in water, for100 ns. The cluster analysis indicated the presence of a majority 3D form, around 70% of the total population during MD, which we call C1 (Figure 3D). According to the ergodic theorem [38], which defines that the mean in time is related to the spatial mean, it can be said that C1 is the most common conformation of this structure in water.

The RMSD graph (Figure 3B) is commonly used to monitor the conformational stability of the structure during MD, where it is checked whether the mean value is a threshold and does not vary significantly. In the case of Figure 6, we can see that up to ~80 ns the PNA structure presents a mean RMSD value of around 7~8 Å, not varying significantly during this time. However, from this point on, RMSD increases (around 17~18 Å), indicating significant conformational change. In addition, the higher threshold value shows that this conformation is stable, but less similar to the initial conformation. The RMSD for the separate parts of the PNA (CPP and DNA) shows that the conformational change is mainly related to that part of

the DNA that, in isolation, also changes the RMSD level after 80 ns. According to the RMSF plot (Figure 3C), the residues showed similar flexibility (average of 6 Å), except for the end of the DNA portion (residue 25), which presented greater flexibility (above 10 Å), as expected. Structurally, C1 stabilizes by the formation of 3 hydrogen bonds (HB) between NZ nitrogen from lysine 1 (LYS-1) and DNA bases DC-17 and DT-18 (Figure 3D).

These HBs, which have a length of about 3 Å, are responsible for the stabilization of the PNA in the C1 conformation in water, forming an amphipathic structure, where the DNA is naturally hydrophilic, and the part of the CPP that has a hydrophobic character is protected from the iteration with water. This form, which is similar to a spermatozoid, can potentially cross the membrane without losing its "tail".

**Discussion**

Carbapenems have been used as the drug of choice for the treatment of infections caused by multidrug resistant Gram-negative bacilli. However, the emergence and dissemination of carbapenem-resistant strains is always a public health concern [7, 39, 40]. Although *Enterobacteriaceae* can acquire carbapenem resistance through various mechanisms, including expressing multidrug efflux pumps, undergoing porin alterations, and target site modifications, the most important is the production of plasmid-mediated carbapenemases [1]. KPC-producing *K. pneumoniae* is a challenge for clinical microbiologists and clinicians, as infections caused by this bacterium are associated with a longer hospital stay, higher hospitalization costs and elevated mortality rates [2, 3]. Management of infections caused by these strains is difficult and therapeutic options become limited; consequently, the need for novel antibacterial agents against these infections has increased [7].

Antisense agents, including PNAs, a group of compounds discovered recently, represent an alternative to conventional antibiotics due to their ability to silence expression of essential genes in MDR pathogens [9, 10, 14]. One limitation of these molecules is their inability to cross cell membranes to bind their targets inside bacterial cytoplasm. This problem can be attenuated by conjugating a suitable CPP to a targeting PNA [18, 41]. The KFFKFFKFFK peptide used in this study was first reported as a potent permeabilizer in *E. coli* cells [42]. Previous studies showed that, when tested without the $(KFF)_3K$ peptide, the PNA did not present antisense effects, demonstrating that a CPP is necessary for PNA penetration through the cell membrane [43, 44].

The specific nucleotide sequence of PNA was designed to be complementary to a specific region of the mRNA, including the start codon region of the essential *gyr*A gene [13]. DNA gyrase is a specific enzyme that plays a critical role in DNA replication and bacterial growth; it is responsible for catalyzing the negative supercoiling of DNA, and is therefore essential for the maintenance of DNA topology [45]. One consequence of the irreversible inhibition of DNA gyrase function through inhibition of *gyr*A in the target microorganism is, ultimately, cell death. This supports the notion that *gyr*A is a promising target for developing novel agents based on antisense technology [46].

In the present study, the inhibitory effects of anti-*gyr*A peptides on bacterial growth were evaluated by assessing cell growth and viability of KPC-producing *K. pneumoniae* following treatment with various peptide concentrations. We demonstrated that the presence of PNA at a concentration of 50 μM successfully inhibited KPC-producing *K. pneumoniae* growth, and the effects observed were concentration-dependent. The bactericidal effect of gene inhibition was also studied by determining the number of viable cells in cultures; there was a significant reduction in CFU counts after incubation in the presence of the antisense PNA compared with the untreated culture.

In addition to ensuring that the antisense effects resulted in a significant reduction in gene expression and in order to provide a better assessment of the level of inhibition, we performed qPCR to determine the levels of 16S ribosomal DNA of the bacteria in the cultures. At the highest concentration used (50 μM), the amount of DNA was reduced by 96% compared to the untreated culture. We suggest that the decrease in the levels of 16S ribosomal DNA observed was due directly to suppression of *gyr*A expression. Peptide–PNA conjugates have been reported to reduce levels of targeted mRNAs [12, 13, 15, 44, 47-49]. However, thus far, to our knowledge, no published reports have examined the capability of inhibition of *gyr*A in KPC-producing *K. pneumoniae* using PNAs. The hemolytic activity of antimicrobial peptides against erythrocytes is often used as a measure for their cytotoxicity and to estimate their therapeutic index [33]. At the highest concentrations, PNA released small amounts of free hemoglobin, lower than that of the Triton X-100 treated samples. Treatment with PNA revealed low hemolytic side effects at concentrations needed to kill bacteria (25 and 50μM); hence, PNAs are useful as antimicrobial drugs.

In the computational part of this work, we showed that the structure of the PNA presented a stable conformation called C1, obtained by the clustering analysis, which provided relevant information about the most common PNA conformation in the aqueous medium. In addition, RMSD and RMSF graphs indicate conformational stability in the presence of water, and C1 stabilizes by forming 3 hydrogen bonds between CPP and DNA, showing that in the process of transporting the molecule to the therapeutic target, prior to gene activity, the structure remains stable. This is important for the bioavailability and resistance to molecule degradation until it reaches the therapeutic target, without undergoing significant changes in its structure while in the bloodstream.

## Conclusions

In this study, we report the first application of an antisense PNA targeting the *gyrA* gene to inhibit KPC-producing *K. pneumoniae* growth *in vitro*. Several issues still remain with regard to employing PNA antisense conjugates as a therapeutic approach for the treatment of patients infected with carbapenem-resistant strains, including delivery to the site of infection. Nevertheless, our results suggest that the anti-*gyrA* peptide can efficiently inhibit bacterial growth and has potential as a new therapeutic strategy. However, further research is required to evaluate the antisense activity in animal models of infection and toxicity to confirm the potential of the anti-*gyrA* peptide as a novel antimicrobial agent.

## Declarations


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## Availability of data and materials

All relevant data are within the paper.

## Author's contribution

Conception and design of study: KES, SS. Acquisition of laboratory and clinical data: KES, SMR, CPB, SEP. Analysis of data: KES, SEP, MHC, OF, LM, SS. Drafting of article and/or critical revision: KES, SMR, LM, SS.

## Ethical approval and Consent to participate

This study was conducted with the approval of the Research Ethics Committee from the Universidade Federal da Grande Dourados (no. 039439/2012).

## Consent for publication

N/A

## Competing interests

The authors declare that they have no competing interests.

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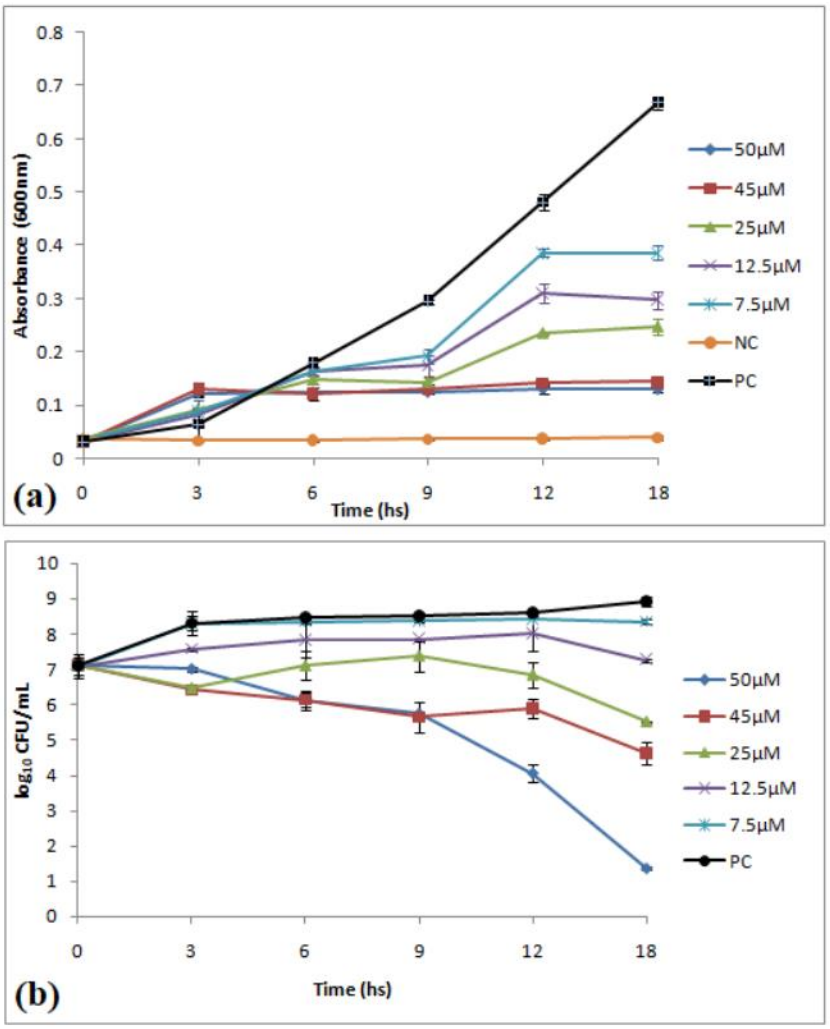


**Fig. 1.** (a) Inhibitory effect of anti-*gyr*A peptide on bacterial growth and viability of KPC-producing *K. pneumoniae*. Growth was indicated by turbidity measurements at 600 nm, and concentration-dependent growth inhibition was observed. Antisense PNA was added to cultures at 7.5 (*), 12.5 (x), 25 (▲), 45 (■) and 50 (♦) µM concentrations. NC (*) – Negative control (MH broth), PC (●) – Positive control (MH broth and bacteria). (b) The number of CFU was calculated at different time points. Antisense PNA was added to cultures at 7.5 (*), 12.5 (x), 25 (▲), 45 (■) and 50 (♦) µM concentrations. PC (●) – Positive control (MH broth and bacteria).

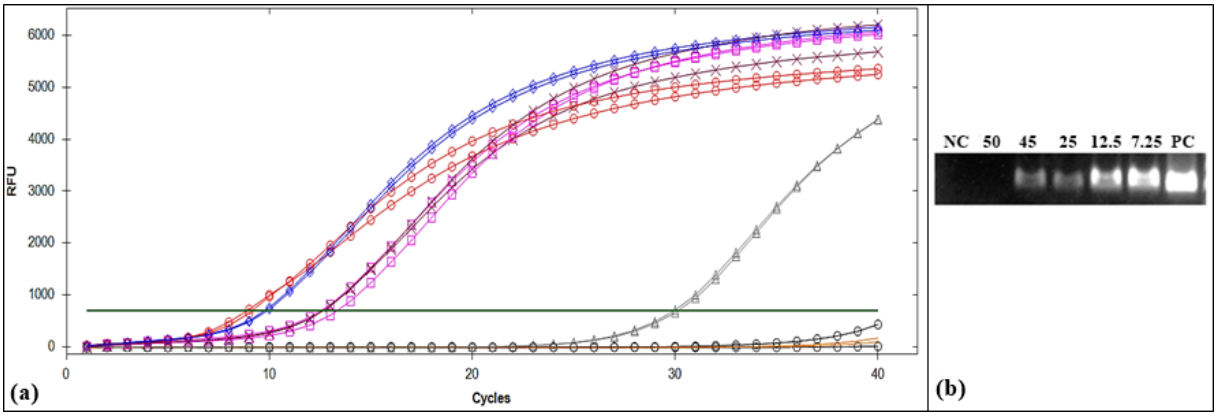


**Fig. 2.** (a) Inhibitory effect of anti-*gyr*A peptide on bacterial growth and viability of KPC-producing *K. pneumoniae.* DNA was extracted from the treated cultures at 7.25 (blue), 12.5 (pink), 25 (brown), 45 (grey), 50 (black) µM concentrations, NC (orange) – Negative control (MH broth), PC (red) – Positive control (MH broth and bacteria) and subjected to RT-PCR. Green line indicates the threshold level. Lines with the same color represent duplicates. (b). RT-PCR products were examined by 1 % agarose gel electrophoresis and ethidium bromide staining.

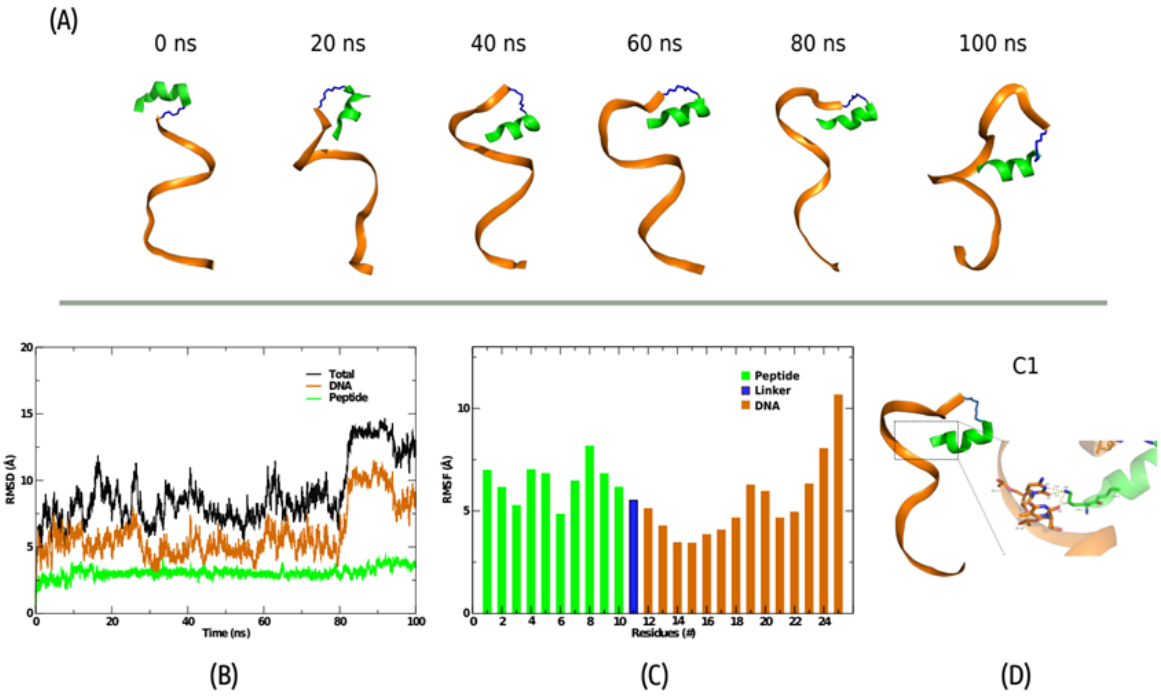


**Fig. 3.** Evaluation of the molecular dynamics of PNA. Structures demonstrated during 100 ns of trajectory in water (A). Root mean square deviations (RMSD) for the entire PNA molecule and for the peptide and DNA parts separately (B). Root mean square fluctuations (RMSF) by residue of the PNA molecule during the simulation (C). A 3D representation of the majority form C1, highlighting (dotted square) the region where there is hydrogen bonding (HB) between the nitrogen NZ of LYS-1, nitrogen N3 and oxygen O2 of cytosine 17 (DC-17) and also oxygen O2 thymine 18 (DT-18) (D). Each color represents a part of the PNA, with orange the DNA part, blue the linker ($C_5H_{13}NO_3$) and green the part of the CPP.

## 6 CONCLUSÕES

Os resultados obtidos neste estudo indicam a disseminação de enterobactérias produtoras de β-lactamases associadas a altas taxas de resistência a polimixina, com a identificação de um surto causado por *K. pneumoniae* resistente a polimixina causado por três diferentes mecanismos que levam a inativação do gene *mgr*B. Foi identificado uma sequência de inserção de 80 pb, resultando na duplicação de 26 aminoácidos do MgrB e responsável pela inativação da proteína. Esta é a primeira observação deste tipo de alteração causando resistência à polimixina. Nas cepas de *E. aerogenes* o mecanismo de resistência a polimixina foi associados a alterações nos genes *phoPQ*. Também foi identificado em todos os isolados ums mutação pontual no gene *soxS*, responsável pela hiperexpressão da bomba de efluxo *acab-tolC*, um marcador de resistência a à colistina em espécies de Enterobacter. O estudo caso-controle identificou vários fatores de risco associados à aquisição de cepas resistentes à polimixina em pacientes adultos e neonatais, incluindo insuficiência renal, procedimentos invasivos (cirurgias, uso de cateter venoso central e urinário), exposição a antibióticos e transferência entre alas hospitalares. Neste estudo, ainda relatamos a primeira aplicação de um PNA anti-sentido para inibição do crescimento de uma cepa de *K. pneumoniae* produtora de KPC, a fim de buscar novas estratégias terapêuticas para o tratamento de infecções causadas por microrganismos multirresistentes. Nossos resultados sugerem que o peptídeo antisense anti-gyrA pode inibir eficientemente o crescimento bacteriano e tem potencial como uma nova estratégia terapêutica.

# 7 ANEXOS

PLOS ONE

RESEARCH ARTICLE

# A high mortality rate associated with multidrug-resistant *Acinetobacter baumannii* ST79 and ST25 carrying OXA-23 in a Brazilian intensive care unit

**Kesia Esther da Silva[1☯], Wirlaine Glauce Maciel[1☯], Julio Croda[1,2,3], Rodrigo Cayô[4], Ana Carolina Ramos[4], Romário Oliveira de Sales[1], Mariana Neri Lucas Kurihara[1], Nathalie Gaebler Vasconcelos[1], Ana Cristina Gales[4], Simone Simionatto[1]***

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**Data Availability Statement:** All relevant data are within the paper.

## Abstract

The global spread of carbapenem-resistant *Acinetobacter baumannii* (*A. baumannii*) strains has restricted the therapeutic options available to treat infections due to this pathogen. Understanding the prevalence of such infections and the underlying genetic mechanisms of resistance may help in the implementation of adequate measures to control and prevent acquisition of nosocomial infections, especially in an intensive care unit setting. This study describes the molecular characteristics and risk factors associated with OXA-23-producing *A. baumannii* infections. A case-control study was undertaken from September/2013 to April/2015. Acquisition of OXA-23-producing *A. baumannii* was found to be associated with the use of nasogastric tubes, haemodialysis, and the use of cephalosporins. These isolates were only susceptible to amikacin, gentamicin, tigecycline, and colistin, and contained the IS*Aba1* insertion sequence upstream of $bla_{OXA-23}$ and $bla_{OXA-51}$ genes. Twenty-six OXA-23-producing *A. baumannii* strains belonged to the ST79 (CC79) clonal group, and patients infected or colonised by these isolates had a higher mortality rate (34.6%). In conclusion, this study showed a dissemination of OXA-23-producing *A. baumannii* strains that was associated with several healthcare-related risk factors and high mortality rates among intensive care unit patients.

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Brief Report

# High mortality rate associated with KPC-producing *Enterobacter cloacae* in a Brazilian hospital

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*Key Words:*
Antibiotic resistance
β-lactamases
*Enterobacteriaceae*

We describe a clonal dissemination of KPC-producing *Enterobacter cloacae* in a Brazilian hospital. Patients diagnosed with theses isolates showed high mortality rate (41.8%) and were associated with previous use of antibiotics and urinary catheterization. Therefore, infection control measures and use of stricter antibiotic policies are required to control the spread of these organisms.



*Enterobacter* spp have been recognized as nosocomial pathogens, mainly affecting patients in intensive care units (ICUs).[1] Carbapenem resistance in *Enterobacter cloacae* has been reported worldwide as a consequence of high-level extended-spectrum β-lactamase expressions combined with the loss of porins or more predominantly by the production of carbapenemases.[2,3] Although general risk factors for *Enterobacter* spp infections have been identified, few studies have reported risk factors associated with the carbapenem-resistant *E cloacae*.[1,2] This study evaluated the carbapenem-resistant mechanisms and risk factors associated with KPC-producing *E cloacae* isolated from a Brazilian hospital.

A total of 28 carbapenem-resistant *E cloacae* isolates, collected from May 2011-April 2013 from different body infection sites, were recovered from 24 patients hospitalized at a tertiary hospital located in the midwestern region of Brazil (Table S1). Patient identification and demographic data were recorded, and a case-control study was conducted to identify risk factors. A case was defined as a patient who presented KPC-producing *E cloacae* strains isolated from clinical cultures from any source. Controls were patients presenting non–carbapenemase-producing *E cloacae*. For each case, one control was selected from inpatients admitted within the study period matched for age, clinical manifestation, and hospital ward. There were no significant differences ($P > .05$) among cases and controls regarding baseline demographics. This study was conducted with the approval of the Research Ethics Committee from the Universidade Federal da Grande Dourados (no. 039439/2012).

The patients were hospitalized in different wards, and their ages ranged from 2-90 years. The frequency of isolation of KPC-producing *E cloacae* in adults was 90%, and in pediatric patients it was 10%. The medium average length of hospital stay was 45 days. Nineteen out of the 24 *E cloacae* isolates (79.16%) were recovered during ICU stay. Furthermore, most strains were isolated from urine (30%), tracheal secretions (23%), blood (17%), and surgical wounds (10%). Clinical data were analyzed by univariate and multivariate models (SAS v.9.2; SAS Institute, Cary, NC).[4] In the multivariable analysis, KPC-producing *E cloacae* isolates were associated with previous use of antibiotics and urinary catheter (Table 1). There was a significant difference in mortality between patients with KPC-producing (41.8%) and non–KPC-producing (8.7%) isolates ($P = .05$). The risk factors for pediatric patients and adults

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Conflicts of interest: None to report.

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## PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS/UFGD-MS

### PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** Epidemiologia molecular de bactérias gram negativas produtoras de carbapenemases isoladas em Hospitais de Dourados-MS.

**Pesquisador:** Simone Simionatto

**Área Temática:** Área 3. Fármacos, medicamentos, vacinas e testes diagnósticos novos (fases I, II e III) ou não registrados no país (ainda que fase IV), ou quando a pesquisa for referente a seu uso com modalidades, indicações, doses ou vias de administração diferentes daquelas estabelecidas, incluindo seu emprego em combinações.

**Versão:** 4

**CAAE:** 05666812.3.0000.5160

**Instituição Proponente:** FUNDACAO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

**Patrocinador Principal:** FUND. DE APOIO E DE DESENV. DO ENSINO,CIENCIA E TECN. DO ESTADO DO MS

**DADOS DO PARECER**

**Número do Parecer:** 877.292

**Data da Relatoria:** 09/09/2014

**Apresentação do Projeto:**

O presente projeto propõe realizar um estudo de epidemiologia molecular de cepas de Enterobactérias produtoras de KPC isoladas de pacientes atendidos no Hospital Universitário HU) da Universidade Federal da Grande Dourados (UFGD). Os resultados obtidos com as técnicas moleculares utilizadas para o diagnóstico e estudo de doenças infecciosas de origem hospitalar serão associados com a prevalência dos agentes envolvidos nestas enfermidades. Através da revisão de prontuários de pacientes internados no hospital será possível identificar os fatores de riscos associados à infecção ou colonização por microorganismos multirresistentes de interesse clínico. Também serão realizadas investigações sobre a relação entre a gravidade dos pacientes e a aquisição dos isolados resistentes, a influência do tempo de exposição ao ambiente hospitalar sobre a aquisição destes agentes infecciosos. Acredita-se que estes estudos possam contribuir para traçar medidas de contenção adequadas bem como para evitar futuros surtos de

infecção dentro do ambiente hospitalar, contribuindo desta forma com ações de vigilância em saúde e consequentemente reduzindo os gastos do Sistema Único de Saúde com internações provenientes destes problemas. ao ambiente hospitalar sobre a aquisição destes agentes

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infecciosos. Acredita-se que estes estudos possam contribuir para traçar medidas de contenção adequadas bem como para evitar futuros surtos de infecção dentro do ambiente hospitalar, contribuindo desta forma com ações de vigilância em saúde e consequentemente reduzindo os gastos do Sistema Único de Saúde com internações provenientes destes
problemas.ao ambiente hospitalar sobre a aquisição destes agentes infecciosos. Acredita-se que estes estudos possam contribuir para traçar medidas de contenção adequadas bem como para evitar futuros surtos de infecção dentro do ambiente hospitalar, contribuindo desta forma com ações de vigilância em saúde e consequentemente reduzindo os gastos do Sistema Único de Saúde com internações provenientes destes problemas.

**Objetivo da Pesquisa:**

Estudar a ocorrência de Enterobactérias produtoras de carbapenemase (KPC) isoladas de pacientes atendidos no Hospital Universitário de Dourados, visando identificar os fatores de riscos associados a aquisição de infecções causadas por estas bactérias.

**Avaliação dos Riscos e Benefícios:**

Quanto aos benefícios parece ser uma proposta que possibilitará auxiliar ações de vigilância em saúde. A avaliação dos riscos inerentes à coleta das amostras dos pacientes é inexistente. No entanto, a pesquisa é retrospectiva, uma vez que o material já foi coletado em procedimento padrão da instituição em que será realizada a pesquisa, o que torna suficiente a avaliação ora apresentada no protocolo.

**Comentários e Considerações sobre a Pesquisa:**

O tema é relevante e os resultados da pesquisa podem contribuir com ações de vigilância em saúde no HU. A pesquisadora realizou adendo no protocolo (embora sem documento de encaminhamento) que corresponde ao aumento no número de participantes na pesquisa. O aumento seria de 300 participantes mudança no n de 200 para 500 participantes).

**Considerações sobre os Termos de apresentação obrigatória:**

Descreve suficientemente o procedimento para obtenção do TCLE, além de versão reformulada do TLE (TCLE 12.11.2014).

**Recomendações:**

**Conclusões ou Pendências e Lista de Inadequações:**

Os pesquisadores descreveram detalhadamente o procedimento para obtenção dos TCLEs de forma a documentar, caso a caso, a impossibilidade da sua obtenção. No tocante a esse ponto, o

protocolo está conforme as exigências pregadas pela Res CNS 466/2012 para a dispensa do TCLE.

**Situação do Parecer:**

Aprovado

**Necessita Apreciação da CONEP:**

Não

**Considerações Finais a critério do CEP:**

DOURADOS, 19 de Novembro de 2014

**Assinado por:**
**Paulo Roberto dos Santos Ferreira**
**(Coordenador)**

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