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

Epidemiologia de bactérias Gram-negativas multirresistentes durante a pandemia de COVID-19 e avaliação de novas alternativas antimicrobianas

GLEYCE HELLEN DE ALMEIDA DE SOUZA

Dourados - MS

2023

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EPÍGRAFE

“Eu aprendi qual é o valor de um sonho alcançar
Eu entendi que, o caminho, pedras terá
Eu vi em campo aberto se erguer construção
E foi com muitas pedras e foi com muitas mãos

Eu vi o meu limite vir diante de mim
Eu enfrentei batalhas que eu não venci
Mas o troféu não é de quem não fracassou
Eu tive muitas quedas, mas não fiquei no chão

E ao olhar pra trás, tudo que passou
Venho agradecer quem comigo estava
Ergo minhas mãos pra reconhecer

E hoje eu sou quem eu sou
Pois Sua mão me acompanhava
Mas eu sei, não é o fim, é só o começo da jornada
Eu abro o meu coração pra minha nova história ...”

Trecho da Canção: Só o começo, Artista: Vocal Livre

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

%	Porcentagem
>	Maior
≥	Maior ou igual
µg/ ml	Micrograma por mililitro
µL	Microlitro
AC	Acre
AL	Alagoas
AM	Amazonas
AP	Amapá
BA	Bahia
BGN	Gram-Negativas
BGN-MR	Gram-Negativas Multirresistentes
CC	Complexo clonal
CDC	Centers for Disease Control and Prevention
CE	Ceará
CEUA	Comitê de Ética em Uso Animal
CIM	Concentração inibitória mínima
COVID-19	Doença do coronavírus 2019
DF	Distrito Federal
ES	Espírito Santo
ESBL	β-lactamase de espectro estendido
FDA	Food and Drug Administration
GO	Goiás
H	Horas
IRAS	Infecção Relacionada à Assistência à Saúde
SI	Sequências de inserção
KP-RP	*Klebsiella pneumoniae* resistente à polimixina
MA	Maranhão
MG	Minas Gerais
mg/kg	Miligramas por quilogramas
MR	Multirresistentes
MS	Mato Grosso do Sul
MT	Mato Grosso
OMS	Organização Mundial da Saúde
PA	Pará
PB	Paraíba
PE	Pernambuco
PI	Piauí
PR	Parana
RAM	Resistência antimicrobiana
RJ	Rio de Janeiro
RN	Rio Grande do Norte
RO	Rondônia
RR	Roraima

RS	Rio Grande do Sul
SC	Santa Catarina
SE	Sergipe
SP	São Paulo
ST	Sequence type
TO	Tocantins
UFGD	Universidade Federal da Grande Dourados
UNIGRAN	Centro Universitário da Grande Dourados
UTIs	Unidades de Terapia Intensiva
UE	União Européia
WHO	World Health Organization

Epidemiologia de bactérias Gram-negativas multirresistentes durante a pandemia de COVID-19 e avaliação de novas alternativas antimicrobianas

RESUMO

A resistência a antimicrobianos entre bactérias Gram-Negativas Multirresistentes (BGN-MR) representa um desafio global para a saúde, restringindo as opções terapêuticas para o tratamento das infecções. No Brasil, dados epidemiológicos sobre o impacto da ocorrência de BGN-MR em pacientes com doença do coronavírus de 2019 (COVID-19) são limitados. Desse modo, objetivou-se avaliar o desfecho clínico e os fatores associados a BGN-MR em 280 pacientes hospitalizados com ou sem COVID-19, através de um estudo de caso-controle, realizado entre de março/2020 a dezembro/2021. O grupo caso (COVID-19-BGN-MR) foi definido como pacientes positivos para COVID-19, com cultura clínica para uma BGN-MR. O controle 1 (COVID-19) foi definido como pacientes positivos para COVID-19, sem cultura para BGN. O controle 2 (BGN-MR) incluiu pacientes com cultura para BGN-MR e sem evidência clínica de COVID-19. O controle 3 (GNB) incluiu pacientes com cultura para uma BGN suscetível a carbapenêmicos e sem evidência clínica de COVID-19. O estudo envolveu controles não-probabilísticos, recrutados aleatoriamente em uma proporção de 1:1:1:1 para os casos. Estes grupos foram submetidos a análise estatística univariável e multivariável e foram identificados como fatores de risco associados à mortalidade o uso de cateter urinário e cateter venoso central; insuficiência renal; amostra clínica de secreção traqueal, uso de carbapenêmicos e polimixina. A mortalidade foi significativamente maior em pacientes do grupo caso COVID-19-BGN-MR em comparação com os três grupos controles COVID-19, BGN-MR e BGN ($p = \leq 0,02$). Os dados demonstram que a ocorrência de BGN-MR associada à COVID-19, tem um impacto expressivo no aumento da mortalidade. Com o propósito de contribuir no controle de BGN-MR foi investigado o potencial sinérgico de um composto bioativo (carvacrol) associado a um antibiótico (polimixina B), buscando contribuir no desenvolvimento de novos antimicrobianos contra *K. pneumoniae* resistente à polimixina. O composto apresentou efeito inibitório na formação de biofilme e demonstrou potencial antimicrobiano *in vitro* e *in vivo*, representando uma alternativa terapêutica a ser explorada no desenvolvimento de novos antimicrobianos. Visto que os peptídeos antimicrobianos têm se destacado no mercado farmacêutico, realizou-se uma revisão de patentes sobre peptídeos antimicrobianos desenvolvidos e testados frente a *K. pneumoniae* resistente à polimixina, a fim de investigar o progresso no desenvolvimento de novos antimicrobianos peptídicos. Os resultados indicam que, embora muitos peptídeos sejam descritos como tendo atividade contra *K. pneumoniae*, uma minoria foi testada efetivamente contra cepas resistentes à polimixina. Adicionalmente, são necessários investimentos a fim de avançar nas etapas de desenvolvimento objetivando introduzí-los no mercado de antimicrobianos. Nesse contexto, o desenvolvimento deste estudo corrobora com as preconizações da Organização Mundial da Saúde, para contenção da resistência antimicrobiana, que incentiva estudos de epidemiologia e pesquisas no desenvolvimento de novos antimicrobianos, visando controlar a disseminação de microrganismos multirresistentes e melhorar o prognóstico dos pacientes.

Palavras-chave: Fatores de risco; saúde pública; atividade antibacteriana; sinergismo; peptídeos.

Epidemiology of multidrug-resistant Gram-negative bacteria during the COVID-19 pandemic and evaluation of new antimicrobial alternatives

ABSTRACT

Antimicrobial resistance among Multidrug-resistant Gram-Negative bacteria (MDR-GNB) represents a global health challenge, restricting therapeutic options for the treatment of infections. In Brazil, epidemiological data on the impact of MDR-GNB occurrence in patients with coronavirus disease 2019 (COVID-19) are limited. Thus, the objective was to evaluate the clinical outcome and factors associated with MDR-GNB in 280 hospitalized patients with or without COVID-19, through a case-control study, carried out between March/2020 and December/2021. The case group (COVID-19-MR-GNB) was defined as patients who were positive for COVID-19, with a clinical culture for an MDR-GNB. Control 1 (COVID-19) was defined as COVID-19 positive patients with no GNB culture. Control 2 (MDR-GNB) included patients with MDR-GNB culture and no clinical evidence of COVID-19. Control 3 (GNB) included patients cultured for a carbapenem-susceptible GNB and without clinical evidence of COVID-19. The study involved non-probabilistic controls, randomly recruited in a 1:1:1:1 ratio to cases. These groups were submitted to univariate and multivariate statistical analysis and the use of urinary catheter and central venous catheter were identified as risk factors associated with mortality; renal insufficiency; clinical sample of tracheal secretion, use of carbapenems and polymyxin. Mortality was significantly higher in patients in the COVID-19-MDR-GNB case group compared to the three COVID-19, MDR-GNB and GNB control groups ($p = \leq 0.02$). The data demonstrate that the occurrence of MDR-GNB associated with COVID-19 has a significant impact on the increase in mortality. In order to contribute to the control of MDR-GNB, the synergistic potential of a bioactive compound (carvacrol) associated with an antibiotic (polymyxin B) was investigated, seeking to contribute to the development of new antimicrobials against polymyxin-resistant *K. pneumoniae*. It was observed that it had an inhibitory effect on biofilm formation and demonstrated antimicrobial potential *in vitro* and *in vivo*, representing a therapeutic alternative to be explored in the development of new antimicrobials. Since antimicrobial peptides have stood out in the pharmaceutical market, due to their antimicrobial properties, a patent review was carried out on antimicrobial peptides developed and tested against polymyxin-resistant *K. pneumoniae*, in order to investigate progress in the development of new antimicrobials peptides. The results indicate that although many peptides are reported to have activity against *K. pneumoniae*, a minority have been tested effectively against polymyxin-resistant strains. Additionally, investments are needed in order to advance in the development stages with the aim of introducing them to the antimicrobial market. In this context, the development of this study corroborates the recommendations of the World Health Organization, for the containment of antimicrobial resistance, which encourages epidemiology studies and research in the development of new antimicrobials, in order to control the spread of multidrug-resistant microorganisms and improve the prognosis of patients.

Keywords: Risk factors; public health; antibacterial activity; synergism; peptides

SUMÁRIO

1. INTRODUÇÃO

A resistência antimicrobiana constitui um problema de saúde pública global que afeta negativamente a saúde dos pacientes, aumentando o tempo de internação, os riscos de complicações e mortalidade (ARSLAN, 2022; FERNÁNDEZ-MARTÍNEZ et al., 2022; ZHEN et al., 2019). Essa problemática foi potencialmente agravada pela doença do coronavírus 2019 (COVID-19), visto que houve o uso inadequado ou excessivo de antibióticos em muitos pacientes (DAMBROSO-ALTAFINI et al., 2022; HSU, 2020; LINGAS, 2022; RAWSON et al., 2020a). Além disso, os impactos da pandemia a longo prazo são desconhecidos e particularmente preocupantes, especialmente no que se refere à propagação da resistência e os fatores de riscos subjacentes ao paciente (KNIGHT et al., 2021; PÉREZ DE LA LASTRA et al., 2022).

Nesse contexto, as BGN-MR destacam-se como patógenos prioritários, em razão da sua alta transmissibilidade e reduzidas opções terapêuticas, representando um desafio na clínica médica (GARCIA-VIDAL et al., 2021; MILLS; MARCHAIM, 2021). Somado a isso, estudos recentes revelaram que a ocorrência de infecção secundária bacteriana esteve presente em cerca de 50% dos pacientes com COVID-19 que não sobreviveram (ZHOU et al., 2020a). No entanto, os fatores relacionados à infecção secundária por BGN-MR permanecem, em grande parte, inexplorados (BAIOU et al., 2021; GARCIA-VIDAL et al., 2021; GUO et al., 2021).

No tratamento dessas infecções a polimixina é o antibiótico empregado como tratamento de último recurso (HUSSEIN et al., 2021; YANG et al., 2020). Entretanto, a utilidade clínica das polimixinas está ameaçada devido ao desenvolvimento de resistência por cepas pan-resistentes (NANG; LI; VELKOV, 2019). Dessa maneira, a busca por novas estratégias antimicrobianas é necessária e inclui otimização de dosagem de antimicrobianos a ser utilizada, desenvolvimento de peptídeos antimicrobianos e uso da polimixina em combinação com outros compostos bioativos, os quais representam alternativas terapêuticas na busca por novos antimicrobianos contra a resistência (SONG et al., 2020; TRAN et al., 2018; YANG et al., 2020).

Diante do exposto, os objetivos deste estudo foram descrever os fatores preditivos associados à ocorrência de BGN-MR em pacientes com COVID-19; avaliar a atividade sinérgica do composto bioativo carvacrol em associação com polimixina; e investigar através de uma revisão na literatura de patentes para explorar tendências de inovação no desenvolvimento de peptídeos antimicrobianos frente a bactérias Gram-negativas (BGN) resistentes à polimixina.

2. REVISÃO BIBLIOGRÁFICA

2.1 Infecções relacionadas à assistência a saúde (IRAS)

As IRAS são definidas como qualquer manifestação clínica de infecção adquirida que se desenvolva após um procedimento de assistência à saúde, esteja o paciente internado ou não (ANVISA, 2021). As IRAS representam um dos problemas de saúde pública mais relevantes globalmente, uma vez que contribuem para o aumento da morbidade e mortalidade, aumento do tempo de internação, bem como dos custos para os pacientes e para o sistema de saúde, além de que contribuem significativamente para a transmissão da resistência antimicrobiana (FACCIOLÀ et al., 2019; FRASER et al., 2021; NGUYEN; MEGIDDO; HOWICK, 2021; PROTANO; CAMMALLERI; ROMANO SPICA, 2019).

Nos Estados Unidos as IRAS são a sexta principal causa de morte, com 99.000 ocorrências anuais atribuídas (LIU; DICKTER, 2020). Na África, estima-se que a prevalência de IRAS entre todos os pacientes internados esteja entre 3% e 15% (FRASER et al., 2021). Na União Europeia e no Espaço Econômico Europeu (UE/EEE), estima-se que haja uma prevalência de 8,9 milhões de casos de IRAS ocorrendo anualmente (SUETENS et al., 2018). No Brasil, a prevalência média nacional de IRAS é de 10,8%, sendo que as porcentagens de resistência foram acima dessa taxa em todas as regiões geográficas: 13.2% no Centro Oeste, 12,5% no Norte, 11.7% no Sudeste, 9.8% no Nordeste e 8.7% no Sul (FORTALEZA et al., 2017), cenário que provavelmente foi agravado devido à pandemia de COVID-19 (ROSSATO et al., 2022).

As IRAS são mais frequentemente descritas em pacientes internados em unidade de terapia intensiva (UTI), particularmente entre imunocomprometidos, uma vez que são particularmente suscetíveis, devido a procedimentos cirúrgicos e dispositivos médicos invasivos (PELEG; HOOPER, 2010; QUAINOO et al., 2017). Essas infecções causam aumento de tempo de internação hospitalar, representando um aumento significativo dos custos hospitalares, sendo responsável por aproximadamente 90% dos custos assistenciais totais (GIRALDI; MONTESANO; SANDORFI, 2019).

Em processos infecciosos, os tratos respiratório e urinário são os sistemas mais frequentemente envolvidos, podendo haver uma evolução para um quadro de sepse (ESME et al., 2019). Além disso, os fatores de risco para a aquisição dessas infecções, incluem a imunossupressão, idade avançada, diabetes mellitus, intubação, ventilação mecânica > 48 horas, sonda nasogástrica, maior tempo de permanência no hospital, múltiplas comorbidades subjacentes, visitas frequentes a unidades de saúde,

procedimentos invasivos recentes, reoperação, exposição a cefalosporinas, dias de exposição ao cateter venoso central, admissão na unidade de terapia intensiva (UTI) e a permanência na UTI por maior tempo (DESPOTOVIC et al., 2020; RODRÍGUEZ-ACELAS et al., 2017; SYDNOR; PERL, 2011).

Logo, a infecção adquirida na UTI está associada a um maior risco de mortalidade em comparação com a infecção adquirida na comunidade (VINCENT et al., 2020). Adicionalmente, estima-se que a porcentagem de mortalidade em pacientes com infecção na UTI é 2 vezes maior, comparado a pacientes não infectados (25% vs 11%) (VINCENT, 2009). Nesse panorama, a prevenção e o controle das IRAS tornaram-se, portanto, uma prioridade para a maioria dos sistemas de saúde, a fim de garantir a segurança do paciente e reduzir os custos associados aos serviços de saúde (NGUYEN; MEGIDDO; HOWICK, 2021).

Além disso, surtos de BGN-MR representam uma ameaça frequente para populações vulneráveis de pacientes em hospitais em todo o mundo (QUAINOO et al., 2017). Na União Europeia (UE) as mortes atribuíveis a microrganismos multirresistentes foram estimadas em 33.110 por ano (CASSINI et al., 2016). Em análise global sobre a resistência, estimaram que haja anualmente cerca de 1,27 milhões de mortes atribuíveis à resistência bacteriana (MURRAY et al., 2022).

Dentre os microrganismos epidemiologicamente relevantes, os bacilos Gram-negativos aeróbios (incluindo a família de *Enterobacteriaceae*, *Pseudomonas* sp. e *Acinetobacter* sp.) são os principais causadores de IRAS (ANVISA, 2022; MEHRAD et al., 2015; TOMCZYK et al., 2019). Adicionalmente, a re-infecção em pacientes frequentemente hospitalizados, pode contribuir para as altas porcentagens de resistência a antibióticos observadas entre BGN (AGARWAL; SHIAU; LARSON, 2018).

2.2 Bactérias Gram-negativas de importância clínica

A prevalência de infecções resistentes a antibióticos entre BGN está aumentando, estando entre os mais significativos problemas de saúde pública no mundo (MIZRAHI et al., 2020; OLIVEIRA; REYGAERT, 2022). Mediante essa situação a Organização Mundial da Saúde (OMS) estabeleceu uma lista de microrganismos epidemiologicamente relevantes classificadas como patógenos prioritários, na qual *Acinetobacter baumannii*, *Pseudomonas aeruginosa* e *Enterobacteriaceae* foram listados como patógenos de alta prioridade devido à sua grande importância clínica em hospitais e à sua alta associação

com o aumento da mortalidade e morbidade (KOPOTSA; OSEI SEKYERE; MBELLE, 2019; MICHEAL et al., 2017).

No Brasil, dentre a distribuição dos microrganismos Gram-negativos notificados como agentes etiológicos de infecções em UTI adulto, as enterobactérias representam 33,2% das infecções hospitalares (destacando-se *Klebsiella pneumoniae* (19%), *Enterobacter* spp. (4,2%), *Escherichia coli* (3,7%), *Serratia* spp. (3,4%) entre outras (2,9%)), seguido por *A. baumannii* com 10,7% e *P. aeruginosa* (9,6%) (Brasil, 2017).

Enterobacteriaceae é um grupo heterogêneo de bactérias Gram-negativas, cujo habitat natural é o trato intestinal de humanos e animais (RAMOS-VIVAS et al., 2019; RICHTER et al., 2013). Refere-se a uma das famílias mais diversas taxonomicamente, suas características bioquímicas incluem fermentação da glicose, redução de nitratos a nitritos, oxidase negativos e em sua maioria anaeróbios facultativos (MARTINSON et al., 2019; MORALES-LÓPEZ et al., 2019; ROCK; DONNENBERG, 2014).

Enterobacteriaceae estão entre os patógenos mais comuns que infectam seres humanos em todo o mundo, causando diversas infecções associadas aos cuidados de saúde (PATERSON, 2002, 2006). O grupo de importância clínica inclui muitos gêneros, como *Escherichia*, *Proteus, Citrobacter Enterobacter*, *Salmonella, Shigella, Klebsiella*, *Morganela*, *Serratia*, entre outros (OLIVEIRA; REYGAERT, 2022; PROTANO; CAMMALLERI; ROMANO SPICA, 2019; ROCK; DONNENBERG, 2014).

Na clínica médica, o tratamento dessas infecções tem se tornado um desafio, uma vez que os carbapenêmicos, antibióticos β-lactâmicos usados para tratar infecções graves causadas por *Enterobacteriacea*e multirresistente, tem se tornado ineficazes (DING et al., 2019; PEREZ; VAN DUIN, 2013; SHEU et al., 2019). Desse modo, as infecções causadas por *Enterobacteriaceae* resistentes a antimicrobianos representam uma preocupação global à saúde humana (DING et al., 2019; LOGAN; WEINSTEIN, 2017; TILAHUN et al., 2021).

Na última década, a enterobactéria *K. pneumoniae* tem se destacado no cenário clínico, uma vez que este é um dos patógenos mais relevantes, responsáveis por infecções associadas à assistência à saúde, especialmente cepas multirresistentes produtoras de β-lactamases e/ou carbapenemases de espectro estendido (KHODADADIAN et al., 2018; WYRES; LAM; HOLT, 2020). Causa uma variedade de doenças infecciosas, incluindo infecções do trato urinário, bacteremia, pneumonia e abscessos hepáticos (WANG et al., 2020).

K. pneumoniae tem uma capacidade excepcional de adquirir elementos genéticos de resistência exógena (YANG et al., 2021). Desse modo, apresenta alta frequência e diversidade de genes de resistência antimicrobiana, possui uma distribuição ecológica mais ampla, composição de DNA significativamente mais variada e uma carga plasmidial maior do que outras BGN oportunistas, desempenhando um papel fundamental na disseminação da resistência entre patógenos clinicamente relevantes (WYRES; LAM; HOLT, 2020).

O aumento da resistência antimicrobiana representa um desafio para os sistemas de vigilância e levanta preocupações sobre o impacto de organismos multirresistentes na segurança do paciente (GIRALDI et al., 2019).

2.3 Resistência bacteriana

A resistência bacteriana a antimicrobianos está em constante evolução e a transferência horizontal de genes através de plasmídeos favorece a disseminação entre os microrganismos (ROZWANDOWICZ et al., 2018). Assim, a resistência a múltiplos β-lactâmicos dificulta o manejo clínico eficaz de infecções (KOPOTSA; OSEI SEKYERE; MBELLE, 2019).

Multirresistência (MR) é definida como resistência a um ou mais antimicrobianos de três ou mais categorias antimicrobianas testadas (MAGIORAKOS et al., 2012). No Brasil, *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *Enterobacter* spp, *E. coli* e *Serratia* spp. multirresistentes são frequentemente notificadas como agentes causadores de infecções em pacientes internados em UTIs. Na tabela 1, descreve-se as porcentagens de resistência entre amostras clínicas de BGN, no ano de 2020, nos estados brasileiros. No panorama nacional, esses microrganismos isolados nos estados do Amapá, Mato Grosso do Sul, Roraima, Acre, Tocantins, Pernambuco, Rio de Janeiro e Bahia apresentam elevadas porcentagens de resistência, excedendo as médias de resistência do país (Figura 1). Através da geração do mapa de calor referente às porcentagens de resistência em *K. pneumoniae* no ano de 2020, observa-se que os estados de Rondônia, Amapá, Mato Grosso do Sul, Disrito Federal e Roraima, apresentam as maiores porcentagens (> 75%) de resistência (Figura 2).

Tabela 1. Porcentagens de resistência entre isolados Gram-negativos em pacientes hospitalizados nas Unidades de Terapia Intensiva adulto, no ano de 2020 de acordo com a região geográfica. Fonte: ANVISA (2022). Elaborado pelo autor.

	Porcentagem (%) de resistência em bactérias Gram-negativas					
Estados	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*Enterobacter* spp	*E. coli*	*Serratia* spp.
RO	84,91	57,14	77,78	37,5	55,56	33,33
AP	**81,48**	**66,67**	**58,33**	**72,73**	**66,67**	**100**
MS	**78,91**	**61,11**	**52,78**	**50**	**31,25**	**66,67**
DF	78,64	74,71	43,1	21,21	16,67	31,48
RR	**78,57**	**87,5**	**80**	**-**	**100**	**-**
AC	**75**	**85,71**	**66,67**	**50**	**-**	**-**
TO	**69,57**	**75**	**33,33**	**171,43**	**33,33**	**91,67**
PE	**67,52**	**62,77**	**49,62**	**45,65**	**47,22**	**48,39**
SE	64	91,67	45	36,36	33,33	35,71
ES	62,61	74,76	34,72	53,49	14,29	16
RJ	**61,42**	**59,2**	**38,49**	**45,8**	**28,57**	**50,62**
MT	60,32	60	40,54	40	42,11	41,67
CE	59,7	45,24	33,33	22,22	19,05	53,33
BA	**59,14**	**60,49**	**29,65**	**45,67**	**33,33**	**54,81**
MG	57,96	64,38	23,35	26,09	28,48	53,76
MA	56,16	40,91	46,15	75	41,67	36,36
RS	53,51	67,93	27,78	22,33	15,66	34,09
PB	52,94	46,94	48,15	50	43,48	40
GO	51,97	51,15	37,29	21,05	37,14	30,43
SP	50,91	44	18,07	19,56	19,29	33,55
PI	50	60,98	44,83	61,54	33,33	16,67
RN	49,64	57,14	43,75	57,14	77,08	35,71
SC	49,41	34,78	28,57	30	17,95	25
PA	45,78	57,89	45,83	25	20	75
AL	43,59	50	20	23,08	25	-
AM	33,33	37,5	20,83	30	36,36	50
Brasil	**53,68**	**54,35**	**26,04**	**29,59**	**26,16**	**41,68**

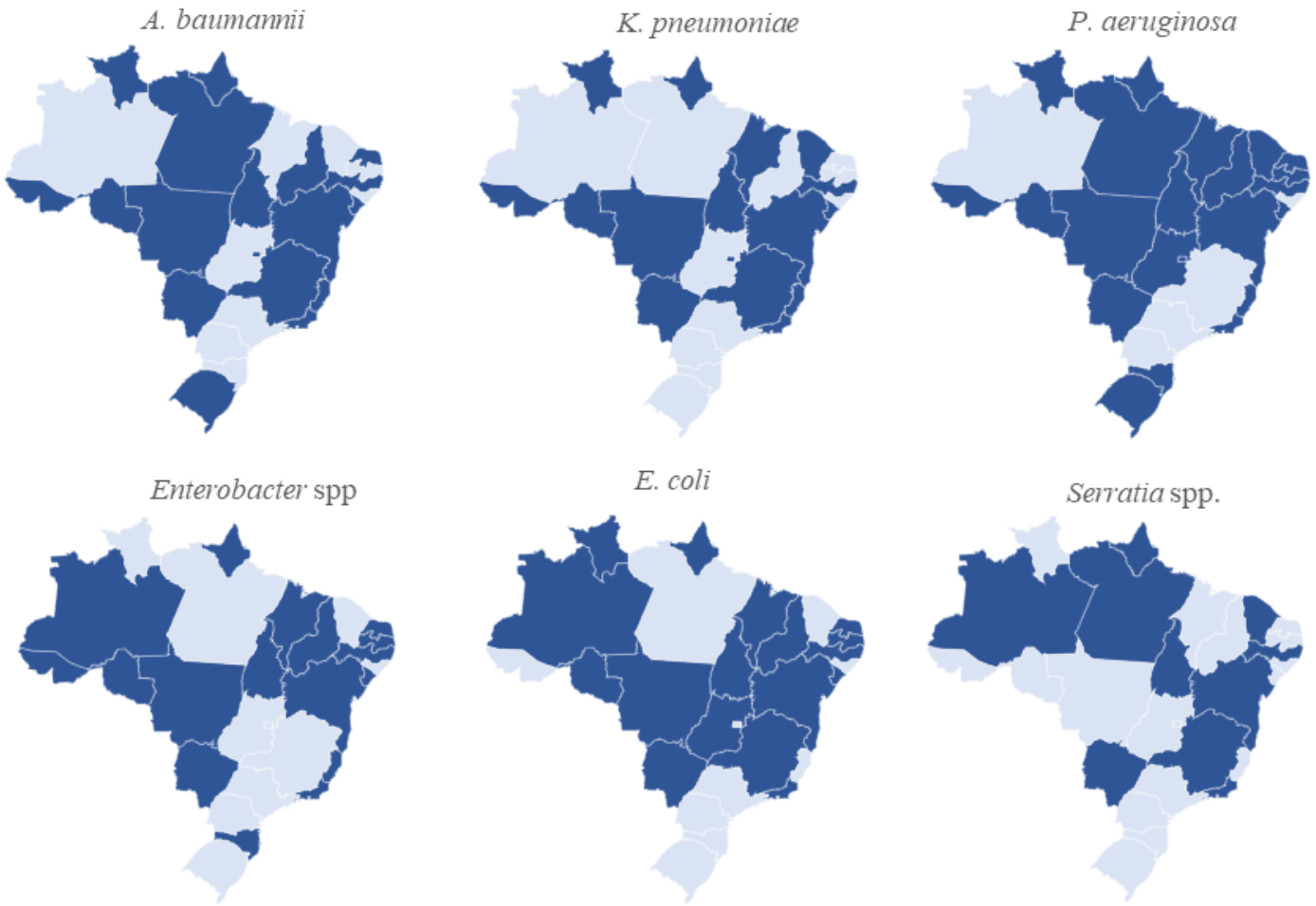

Figura 1. Estados brasileiros com porcentagens de resistência maiores que a média nacional no ano de 2020. Fonte: ANVISA (2022). Elaborado pelo autor.

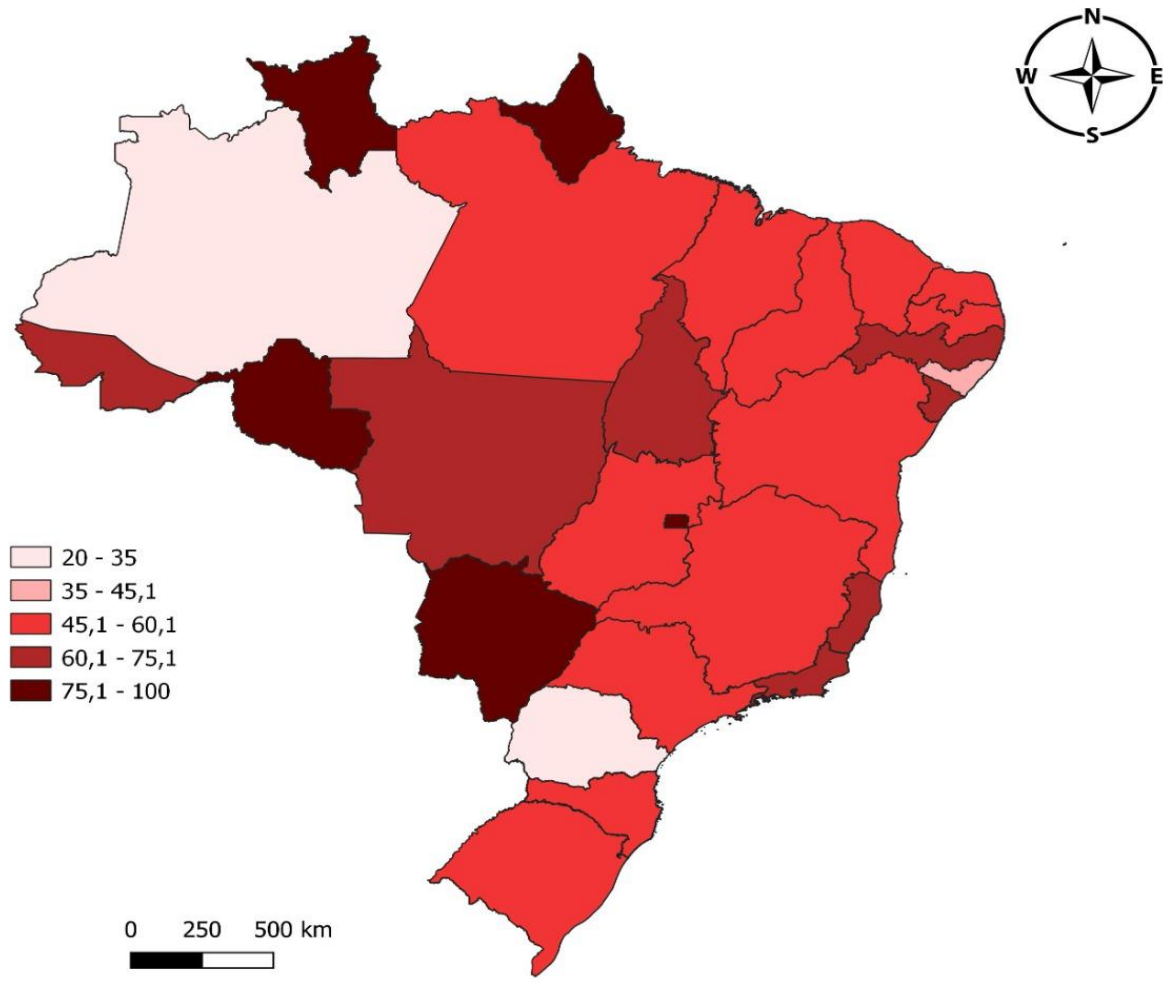

Figura 2. Mapa de calor com porcentagens de resistência em *K. pneumoniae* no ano de 2020. Fonte: ANVISA (2022). Elaborado pelo autor.

2.3.1 **Resistência a carbapenêmicos**

Enterobacteriaceae resistentes a carbapenêmicos (ERC), de acordo com o Centro de Controle e Prevenção de Doenças (CDC), são as bactérias que testam como resistentes a qualquer um dos agentes carbapenêmicos (doripenem, ertapenem, imipenem e meropenem) ou demonstram a produção de carbapenemase por meio de um ensaio fenotípico ou molecular (CDC, 2022). Infecções por ERC são reconhecidas como sendo uma ameaça à saúde pública, resultando em longos períodos de internação, aumento dos custos de saúde e maior mortalidade do que infecções por bactérias suscetíveis a carbapenêmicos (BARTSCH et al., 2017; CDC, 2013; HANSEN, 2021; LOGAN; WEINSTEIN, 2017; TAMMA et al., 2017). Entretanto, dados sobre a prevalência dessas infecções entre pacientes hospitalizados são insuficientes, devido à participação limitada do local e à disponibilidade de amostras clínicas (LODISE; YE; ZHAO, 2017).

Os relatos de resistência aos carbapenêmicos em isolados de *Enterobacteriaceae* tiveram início na década de 1990 (LUTGRING, 2019). Entretanto, a primeira publicação sobre o mecanismo de resistência a carbapenêmicos, pela presença do gene *Klebsiella pneumoniae* carbapenemase (bla_{KPC}) ocorreu somente em 2001 (YIGIT et al., 2001). Desde então, nos últimos 20 anos a resistência aos carbapenêmicos em *Enterobacteriaceae* tem sido descrita e recentemente emergiu como um problema de saúde global, uma vez que é relatada em um ritmo alarmante por todo o mundo, especialmente nas espécies *E. coli* e *K. pneumoniae* (ASLAM et al., 2020; LOGAN; WEINSTEIN, 2017).

A epidemiologia das ERC varia de acordo com o país e localidade geográfica (ASLAM et al., 2020; ZAIDAH et al., 2017). Por exemplo, um estudo retrospectivo de larga escala, demonstrou que a prevalência de infecções por ERCentre pacientes adultos nos EUA foi de 2,3% (variando de 0,9% a 5,8% por região geográfica) (LODISE; YE; ZHAO, 2017). Já a incidência de ERC foi de 2,93 por 100.000 habitantes (GUH et al., 2015).

Enterobacteriaceae estão associadas a muitas infecções graves, como infecções da corrente sanguínea, pneumonias, do trato urinário e intra-abdominais (DE ANGELIS et al., 2020; LIU et al., 2020; SHEU et al., 2019). Pacientes infectados com ERC produtores de carbapenemases apresentam maior risco de morrer dentro de 14 dias após confirmada a infecção, em comparação com pacientes com ERC não produtora de carbapenemases (TAMMA et al., 2017). A ocorrência de ERC entre pacientes internados com infecção da corrente sanguínea esteve associada ao aumento na mortalidade intra-hospitalar (35%),

no tempo de internação hospitalar (3-7 dias), diminuindo em 40% a probabilidade do paciente sobreviver (STEWARDSON et al., 2019).

Na Europa, um estudo observacional prospectivo de incidência foi realizado entre 2018-2019, mostrando cerca de 250 casos de IRAS por 100.000 leitos-dias ocupados; dentre os Gram-negativos, *E. coli* (26,64%) foi o principal causador de infecções, seguido de *K. pneumoniae* (6,15%) (STEWARDSON et al., 2019). Na UE, há uma grande heterogeneidade entre países, com proporções de resistência a carbapenêmicos variando em isolados de *A. baumannii* (0 a 92%), *K. pneumoniae (*0 a 58%*), P. aeruginosa (*0 a 55%;*)* e *E. coli* (0 a 1,6%) (ECDC, 2019). O número médio anual de mortes atribuíveis a infecções por *P. aeruginosa, A. baumanni, K. pneumoniae* e *E. coli* resistentes a carbapenêmicos foram estimados em 4155, 2363, 2118 e 141, respectivamente (CASSINI et al., 2016).

Nos EUA, em um estudo retrospectivo, usando Premiere Health Database de 2009 a 2013 as porcentagens de resistência a carbapenêmicos foram avaliadas para *E. coli, K. pneumoniae, P. aeruginosa* e *A. baumannii*, encontrando uma porcentagem geral de resistência de 4,5%. Dentre estes microrganismos, *A. baumannii* apresentou as maiores porcentagens de resistência aos carbapenêmicos em infecções da corrente sanguínea com 40,1%, seguido por *P. aeruginosa* com 10,3% de resistência e ambos representam 80% de todas as infecções resistentes a carbapenêmicos (CAI et al., 2017).

No Brasil, observa-se que a resistência média a carbapenêmicos aumentou entre isolados de *K. pneumoniae* nos anos de 2017 a 2020, com porcentagens de 44,1%, 44,3%, 51,8% e 63,2% respectivamente (Figura 3). Esse aumento também é demonstrado entre isolados de *A. baumannii*, com porcentagens de 77,7%, 79%, 79,5% e 84,3%. Notavelmente, entre os isolados de *E. coli*, nos anos de 2017-2020 a porcentagem de resistência superou o dobro de valor (7,2% vs. 15,3%) (ANVISA, 2022).

O conhecimento sobre a epidemiologia, natureza das infecções, juntamente com a susceptibilidade dos microrganismos causadores, é extremamente valioso para o tratamento empírico de infecções graves em ambientes hospitalares de terapia intensiva (PARAJULI et al., 2017). Adicionalmente, as condições de saúde dos pacientes, alterações no sistema imunológico, disfunção orgânica pré-existente e interações medicamentosas, dificultam o tratamento dessas infecções (VAN DUIN, 2017).

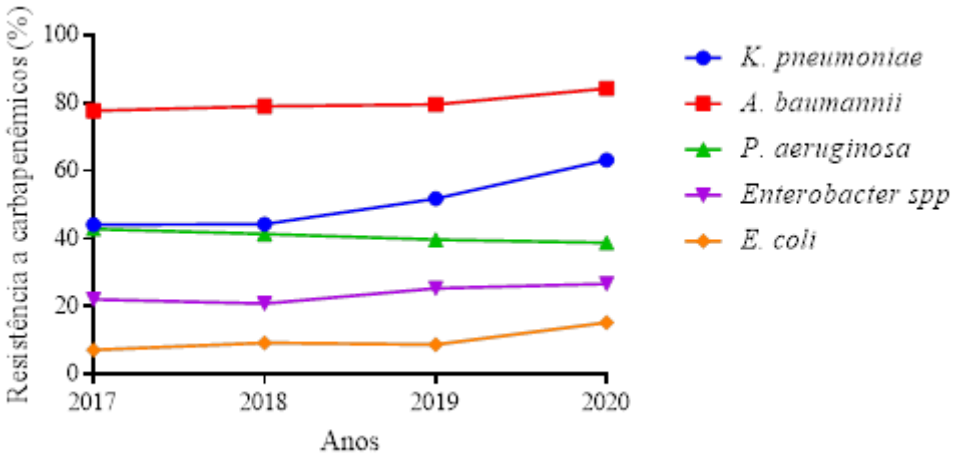

Figura 3. Percentual de Gram-negativos de importância clínica resistentes aos antimicrobianos carbapenêmicos, isolados no Brasil durante os anos de 2017-2020, em infecção de corrente sanguínea. Fonte: Anvisa.

A propagação global da resistência a antibióticos em bactérias Gram-negativas nosocomiais, por meio de genes que codificam as betalactamases, constitui um problema de saúde significativo, uma vez que inutiliza a maioria dos antibióticos comerciais (penicilinas de amplo espectro, fluoroquinolonas, aminoglicosídeos e β-lactâmicos, tais como; monobactam, cefalosporinas e carbapenêmicos), resultando no ressurgimento das polimixinas (EL-SAYED AHMED et al., 2020; MELETIS, 2016).

Atualmente, as opções de antibióticos para o tratamento de *Enterobacteriaceae* resistentes a carbapenêmicos são muito limitadas, incluindo polimixinas, tigeciclina, fosfomicina e aminoglicosídeos, sozinhos ou em combinação com outros antibióticos (ALOTAIBI, 2019; IOVLEVA; DOI, 2017; SHEU et al., 2019). Entretanto, a monoterapia com polimixina já tem sido associada ao surgimento de resistência (BERGEN et al., 2012).

2.3.2 **Resistência à polimixina**

As polimixinas (polimixina B e polimixina E - colistina) são antibióticos lipopeptídicos disponíveis comercialmente, que causam dano à membrana de bactérias Gram-negativas, devido à sua ligação seletiva ao lipopolissacarídeo (KADAR et al., 2013; NANG; LI; VELKOV, 2019). Frequentemente prescritos na década de 1950, sua utilização foi limitada na clínica médica, devido aos efeitos colaterais e seu potencial nefrotóxico (EL-SAYED AHMED et al., 2020; SILVA et al., 2022; YANG et al., 2020).

Entretanto, o surgimento de BGN-MR renovou o interesse nesses antibióticos, havendo a reintrodução das polimixinas na prática clínica, a fim de serem utilizadas como terapia de último recurso ao tratamento de patógenos multirresistentes (MOFFATT; HARPER; BOYCE, 2019; NANG et al., 2021; YANG et al., 2020). Desde então, a resistência às polimixinas tem aumentado gradualmente em vários países do mundo, estima-se que a prevalência de resistência varie de 2.7% a >40% entre isolados clínicos de *K. pneumoniae* multirresistentes (BARON et al., 2016; POIREL; JAYOL; NORDMANN, 2017).

Geralmente as porcentagens são inferiores a 10% (BIALVAEI; SAMADI KAFIL, 2015). Tal como na China, onde apesar da recente aprovação do uso das polimixinas, identificaram que 3,8% das *Enterobacteriaceae* resistentes a carbapenêmicos, apresentavam resistência à polimixina (ZHANG et al., 2021a). Em *K. pneumoniae* a porcentagem de resistência à polimixina foi de 2,2% (CHEN et al., 2022). No entanto, em estudo de coorte observacional multicêntrica, realizado em hospitais dos Estados Unidos, foi documentada resistência a polimixinas em 13% dos isolados clínicos de *K. pneumoniae* (ROJAS et al., 2016). Enquanto que nos hospitais italianos, identificaram 43% de resistência (MONACO et al., 2014). Adicionalmente, outros estudos no Nepal, Egito e Índia relataram a porcentagem de prevalência de resistência à polimixina entre isolados de *K. pneumoniae* em 10%, 18,9% e 32 %, respectivamente (ELMONIR et al., 2021; KARKI et al., 2021; MANOHAR et al., 2017).

No Brasil, a resistência antimicrobiana representa um grave problema de saúde pública (SAMPAIO; GALES, 2016). Entre 2011 a 2015, houve um crescimento alarmante nas porcentagens de resistência à polimixina B em *K. pneumoniae* (de 0% para 27,1%) (BARTOLLETI et al., 2016; SAMPAIO; GALES, 2016). Entre 2010 a 2014, em São Paulo, 7% dos isolados de *Enterobacteriaceae* foram resistentes à polimixina (ROSSI et al., 2017). Ao analisar cepas de *K. pneumoniae* oriundas de 12 estados brasileiros, identificaram 15% de resistência (PEREIRA et al., 2013). Atualmente, estudo realizado no Rio de Janeiro, mostrou uma frequência de resistência à polimixina de 29,5% em isolados de *K. pneumoniae* (CONCEIÇÃO-NETO et al., 2022).

O panorama de resistência à polimixina, de acordo com os indicadores de resistência microbiana da ANVISA (2022), para os principais patógenos Gram-negativos, está ilustrado na Figura 4. Ao avaliarmos os dados de porcentagens de resistência em isolados de *K. pneumoniae*, observa-se que houve um aumento de 127% entre 2018-2020 (ANVISA, 2022).

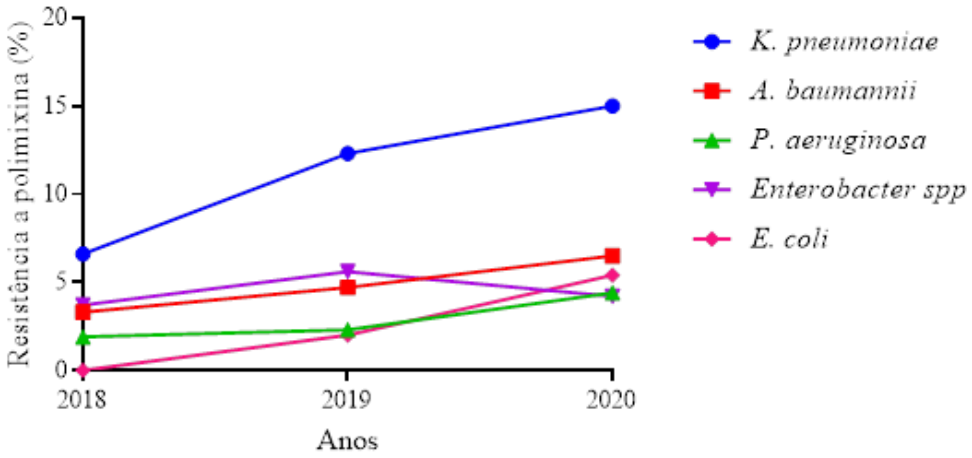

Figura 4. Percentual de Gram-negativos de importância clínica resistentes à polimixina, isolados no Brasil durante os anos de 2018-2020. Fonte: Anvisa.

A ocorrência de cepas resistentes a polimixinas estão associadas aos maiores números de letalidade hospitalar (ROJAS et al., 2016). Na Itália, identificaram que a porcentagens de mortalidade foram de 51% entre pacientes com *K. pneumoniae* resistente à polimixina (GIACOBBE et al., 2015). Enquanto que no Brasil, estavam associadas a 60% de mortalidade (DA SILVA et al., 2020b). Desse modo, programas de vigilância epidemiológica e monitoramento de resistência antimicrobiana são incentivados, para coletar dados a fim de melhorar a compreensão sobre os mecanismos de resistência e produzir evidências que norteiam as ações ao enfrentamento da resistência (DIALLO et al., 2020; SILVA et al., 2020; YU et al., 2015)

Os determinantes de resistência à polimixina estão relacionados a mecanismos intrínsecos, mutacionais e/ou adquiridos (EL-SAYED AHMED et al., 2020; MLYNARCIK; KOLAR, 2019). Os principais mecanismos descritos ocorrem através da transferência de plasmídeos, alteração do gene *mgrB*, modificação do lipídio A e a superexpressão de sistemas reguladores de dois componentes (PmrAB e PhoPQ) (HUANG et al., 2020b; MOFFATT; HARPER; BOYCE, 2019; POIREL; JAYOL; NORDMANN, 2017).

Em estudos brasileiros de caracterização molecular dessas cepas de *K. pneumoniae* resistentes à polimixina, os mecanismos determinantes de resistência foram: presença do gene *mcr-1*, interrupção do gene *mgrB* por sequências de inserção e mutações missense em genes cromossômicos (AIRES et al., 2016; CONCEIÇÃO-NETO et al.,

2022; DA SILVA et al., 2020b; ROCHA et al., 2020). Semelhantemente, na China, identificaram sequências de inserção (IS), mediando a ruptura de *mrgB*, como sendo o principal mecanismo de resistência à polimixina em isolados de *K. pneumoniae* (CHEN et al., 2022). Além disso, o complexo clonal (CC) 11 (incluindo os sequence types (ST) mais frequentes ST11, ST258 e ST437), representa o clone epidêmico de alto risco no Brasil, devido a resistência diversificada (CONCEIÇÃO-NETO et al., 2022).

Compreender sobre os determinantes envolvidos na resistência e epidemiologia desses patógenos é essencial para a investigação de novos agentes antimicrobianos, regimes de combinação, dosagem e estratégias eficazes ao tratamento (MLYNARCIK; KOLAR, 2019; SILVA et al., 2022; YANG et al., 2020). Desse modo, novas estratégias de pesquisa devem ser exploradas, a fim de acelerar o processo de descoberta de novos antibióticos (MIETHKE et al., 2021).

2.4 Estratégias terapêuticas contra *K. pneumoniae* resistentes à polimixina

A Organização Mundial da Saúde (OMS), entre suas propostas para combater a resistência antimicrobiana, destaca a necessidade de intensificar pesquisas na área do desenvolvimento de novos antimicrobianos contra patógenos listados como prioridades globais, tais como *K. pneumoniae* (BARALDI et al., 2018; DUVAL; GRARE; DEMORÉ, 2019). O desenvolvimento de novas abordagens antimicrobianas, inclui identificar compostos existentes (bioativos ou produtos farmacêuticos reaproveitados e /ou reposicionados) que possam agir sozinhos ou sinergicamente com polimixina, para produzir opções terapêuticas efetivas ao tratamento de infecções bacterianas, nos quais a monoterapia de polimixina perdeu a eficácia (VASCONCELOS et al., 2020; ZIMMERMAN et al., 2020). Dessa maneira, novas abordagens ao tratamento de infecções resistentes são essenciais para diminuir os riscos de resistência futura (ARONICA et al., 2021).

O efeito da plazomicina (novo aminoglicosídeo) foi avaliado *in vitro* contra vários isolados clínicos de enterobactérias resistentes à polimixina, incluindo *K. pneumoniae* com os seguintes mecanismos de resistência: mutações *pmrA*, mutações *pmrB phoP*, deleção de 25 nt mutação pontual *phoQ*, mutações pontuais no gene *mgrB*, gene *mgrB* truncado, sequências de inserção no gene *mgrB*, deleção parcial ou total do gene *mgrB*; *K. oxytoca* com sequências de inserção no gene *mgrB*; *E. coli* carregando o gene *mcr-1*; e *Salmonella enterica* carregando o gene *mcr-1*. *Serratia*, *Proteus*, *Morganella* e *Hafnia* eram intrinsecamente resistentes e *Enterobacter asburiae* e *E.*

cloacae apresentaram mecanismos desconhecidos. Os resultados, demonstram que as CIMs de plazomicina foram ≤ 2 mg/L para todos as cepas com gene *mcr-1* e ≤ 4 mg/L para as *Enterobacteriaceae* intrínsecamente resistentes; plazomicina ≤ 4 mg/L apresentou atividade potente, inibindo 93,7% dos isolados de enterobactérias (DENERVAUD-TENDON et al., 2017).

Ao explorar a atividade antimicrobiana *in vitro* do óleo essencial de *Zingiber officinale*, foi demonstrada a atividade frente a *K. pneumoniae* resistente à polimixina (alteração no gene *mgrB*) com CIM de 1,09 mg/ml, com morte celular após 8h de exposição ao óleo (VAZ et al., 2022). A investigação das propriedades antimicrobianas do composto bioativo monoterpenoide carvacrol, revelou atividade frente a *K. pneumoniae* resistente à polimixina (alteração no gene *mgrB*), na concentração de 130 μg/mL, erradicando as células bacterianas em 4h (DE SOUZA et al., 2021).

O nitrato de gálio III (GaN), ao ser investigado quanto a sua propriedade antimicrobiana, demonstrou atividade inibitória frente a isolados clínicos de *K. pneumoniae* resistentes à polimixina (alteração no gene *mgrB*). As CIM *in vitro* para GaN variam de 2 a 16μg/mL. O tratamento *in vivo* foi avaliado em modelo de infecção por *K. pneumoniae* resistentes à polimixina, em *Caenorhabditis elegans*, demonstrando aumento da sobrevida (>75%) (ROSSATO et al., 2022).

Desse modo, a utilização desses compostos adjuvantes, co-administrados com antibióticos, podem restaurar a eficácia de anbióticos em cepas resistentes, diminuindo a CIM (BARKER et al., 2019). A terapia combinada de compostos associadas à polimixina, tem sido documentada como uma alternativa, apresentando efetividade bactericida, e potencial em contornar a resistência à polimixina (HUSSEIN et al., 2020a, 2020b, 2017; TRAN et al., 2018; VASCONCELOS et al., 2020).

Assim, estudos de reposicionamento para fins antimicrobianos, buscando realizar triagem de fármacos de bibliotecas do FDA, representam uma estratégia para acelerar a descoberta de novas terapias contra a resistência a antibióticos (TRAN et al., 2018). A avaliação de 43 compostos bioativos em combinação com a polimixina B frente a patógenos Gram-negativos resistentes à polimixina, revelou 5 compostos (HTS03780, MGH00136, NH00518, CD04455 e JP00319) que apresentaram atividade sinérgica, reduzindo a concentração da polimixina a CIM < 2 μg/ml (ZIMMERMAN et al., 2020).

Estudo que avaliou a combinação de polimixina com mitotano (antineoplásico), mostrou que a combinação de polimixina B (2 mg/L) e mitotano (4 mg/L) teve atividade *in vitro* contra estirpes de *A. baumannii*, *P. aeruginosa e K. pneumoniae* resistentes à

polimixina, causando morte bacteriana nas primeiras 6h, com potencial ação sobre a divisão celular (TRAN et al., 2018).

A investigação da atividade antibacteriana da polimixina em combinação com sertralina (inibidor seletivo da recaptação de serotonina), mostrou ser sinérgica frente a isolados de *A. baumannii, P. aeruginosa e K. pneumoniae* resistentes à polimixina. A combinação de polimixina (4 mg/L) e sertralina (16 mg/L) mostrou morte significativa em 4h, seguida de recrescimento entre 8-24h, e o mecanismo de ação sugerido foi o dano à membrana bacteriana (HUSSEIN et al., 2020b).

Combinações sinérgicas *in vitro* da polimixina B em combinação com moduladores seletivos do receptor de estrogênio (SERMs), tais como tamoxifeno, raloxifeno e toremifeno (foram encontradas para bactérias multirresistentes. A combinação de polimixina B (2 mg/L) com tamoxifeno (8 mg/L) apresentou atividade sinérgica frente isolados de *P. aeruginosa, A. baumannii* e *K. pneumoniae* resistentes à polimixina. Na curva de sobrevivência exibiram diminuição ≥ 2-3 $\log_{10}$ na contagem bacteriana (UFC/ml) ao longo de 1–2h, seguida de recrescimento. Evidenciaram que a combinação causa danos à membrana externa das células bacterianas (HUSSEIN et al., 2017). Ao investigar a combinação do composto bioativo canabidiol com polimixina, identificaram atividade sinérgica contra patógenos Gram-negativos resistentes à polimixina (*A. baumannii*, *K. pneumoniae* e *P. aeruginosa*), exercendo ação sobre a perturbação nos lipídios da membrana bacteriana (HUSSEIN et al., 2022).

Esses estudos, sugerem a possibilidade de reposicionamento de fármacos, demonstrando que a combinação de antibióticos com fármacos aprovados pela *Food and Drug Administration* (FDA) representa uma estratégia para o tratamento de infecções intratáveis por Gram-negativas resistentes à polimixina intratáveis (HUSSEIN et al., 2020a, 2022; TRAN et al., 2018).

O desenvolvimento de terapias seguras e eficazes é essencial ao tratamento de infecções por patógenos resistentes à polimixina, assim, combinações entre antibióticos comerciais também representam uma estratégia a ser investigada a fim de verificar potenciais sinérgicos. Desse modo, foram investigadas combinações duplas e triplas à base de polimixina B (com concentrações variando de 1 a 128 mg/L), associadas a rifampicina (2-16 mg/L) e meropenem (10-120 mg/L) frente a isolados clínicos de *K. pneumoniae* resistentes à polimixina. Nenhuma combinação de dois medicamentos apresentou atividade, mas os regimes de três medicamentos (polimixina B 1 mg/L em

combinação com meropenem 30 mg/L e rifampicina 2, 5 ou 16 mg/L) resultaram em atividade bactericida (DIEP et al., 2017).

Adicionalmente, existe uma classe de moléculas que tem motivado o desenvolvimento de antibióticos, estes são os peptídeos antimicrobianos (AMPs), que consistem em peptídeos catiônicos curtos que podem apresentar uma diversidade de estruturas e alvos (ARONICA et al., 2021; BIN HAFEEZ et al., 2021). Além disso, existem ferramentas computacionais de simulação de dinâmica molecular usadas para prever a atividade e os mecanismos de ação desses AMPs (BIN HAFEEZ et al., 2021; PALMER et al., 2021). Os AMPs representam uma abordagem promissora, apresentam baixa toxicidade, diminuição da interação medicamentosa, alta especificidade, propriedades de ataque direto com maior eficácia e menor tendência a induzir resistência (BOPARAI; SHARMA, 2019; PALMER et al., 2021).

Um novo lipopeptídeo sintético, estrutural e farmacologicamente distinto da polimixina B e colistina, denominado F365 (QPX9003), mostrou atividade antimicrobiana frente a *P. aeruginosa*, *A. baumannii* e *K. pneumoniae* resistentes à polimixina (ROBERTS et al., 2022). Estudo *in vitro* com 17 análogos de Paenipeptina, que são novos lipopeptídeos lineares sintéticos, mostrou que apenas um apresentou atividade contra cepas de *E. coli* e *K. pneumoniae* resistentes à polimixina. Os resultados revelam que a atividade bactericida das paenipeptinas está ligada à ruptura e dano das membranas citoplasmáticas e pela despolarização do potencial de membrana (MOON; HUANG, 2018).

O peptídeo antibacteriano linear curto, designado SLAP-S25. (SLAP), testado *in vitro* apresentou CIM de 0,5–32 µg/mL. O efeito sinérgico do SLAP-S25 com fármacos antibacterianos (tetraciclina, vancomicina, ofloxacina, ampicilina, imipenem, rifampicina ou polimixina) foi avaliado contra *K. pneumoniae* resistente à polimixina *(mcr-1*, *mcr-6*) e demonstraram sinergismo (ΣFIC <0,5) para rifampicina (0,065), ofloxacina (0,127) e tetraciclina (0,129). O ensaio *in vivo*, usando modelo de infecção em camundongos, mostrou que o tratamento sinérgico reduziu significativamente o número de colônias em comparação ao grupo tratado com polimixina. No experimento de bacteremia em camundongos, o tratamento com SLAP-S25 aumentou a porcentagem de sobrevivência (SONG et al., 2020). A capacidade dos peptídeos em inibir o crescimento bacteriano demonstra seu potencial terapêutico como agentes antibacterianos contra patógenos multirresistentes (DA SILVA et al., 2021).

2.5 Resistência antimicrobiana (RAM) e a pandemia de COVID-19

A resistência antimicrobiana é reconhecida como uma das principais prioridades à saúde pública global. Recentemente, foi publicado o mais abrangente relatório sobre ocorrência de RAM, no qual estimaram que globalmente 4,95 milhões de mortes estiveram associadas à RAM bacteriana em 2019 (MURRAY et al., 2022). Entretanto, os dados acerca da mortidade por RAM podem ter sido subestimados; e tendem a aumentar nos próximos anos (FERREYRA et al., 2022). A Organização Mundial da Saúde (OMS) alerta que até 2050, o número de mortes atribuíveis à resistência se aproximará de 10 milhões de pessoas por ano (O'NEILL, 2016). Além disso, as estimativas foram feitas antes da pandemia de COVID-19 agravar essa problemática.

A COVID-19 é uma doença que se manifesta após a infecção causada por um novo coronavírus denominado SARS-CoV-2 (que causa a síndrome respiratória aguda grave), cuja disseminação resultou em um surto global sem precedentes (HUANG et al., 2020a; SOHRABI et al., 2020). Em janeiro de 2020, a Organização Mundial da Saúde (OMS) caracterizou propagação dos casos de SARS-CoV-2 como uma pandemia, representando uma emergência de saúde pública de importância internacional (WHO, 2021).

Até o momento (02 de fevereiro de 2023), 753.823.259 casos confirmados foram relatados globalmente, com 6.814.976 mortes, enquanto o Brasil ocupa o terceiro lugar no mundo em número de casos, com 36.824.580 notificações e 697.074 óbitos (WHO, 2023). A experiência durante o enfrentamento do COVID-19, enfatizou a intensa pressão que uma pandemia exerce sobre os sistemas de saúde, os quais foram levados ao seu limite de funcionamento, com a demanda por leitos, ventiladores mecânicos e suporte de oxigênio excedendo sua disponibilidade; além da limitada experiência clínica quanto às opções terapêuticas com evidências científicas a serem instituídas no tratamento (GARCIA-VIDAL et al., 2021; REMUZZI; REMUZZI, 2020; WALKER et al., 2020).

Como resultado, muitos pacientes hospitalizados com COVID-19 receberam prescrição empírica de antibióticos, resultando em um aumento global do uso desses medicamentos, principalmente em países em desenvolvimento (ARSHAD et al., 2021; KNIGHT et al., 2021; LANGFORD et al., 2021). Devido à gravidade da doença, associado à desinformação, à falta de outras opções terapêuticas, demora no diagnóstico combinado ao ímpeto de tratamento imediato e preventivo, os quais resultaram no uso contínuo desses medicamentos (ARSHAD et al., 2020; KNIGHT et al., 2021).

A maior parte dos pacientes não necessitava de tratamento antibacteriano empírico, uma vez que as porcentagens de coinfecção bacteriana e infecção bacteriana secundárias são baixas (3,5% e 14,3%, respectivamente), no geral, apenas 6,9% dos pacientes com COVID-19 apresentaram alguma infecção bacteriana (LANGFORD et al., 2020). Assim, estudos demonstram a alta prevalência de prescrição de antibióticos durante a pandemia (72%) e consequentemente, os níveis de resistência a longo prazo podem aumentar, esse fato é particularmente preocupante, visto que pode resultar no agravamento das IRAS devido a microrganismos multirresistentes mais virulentos (ALSHAIKH et al., 2022; ARSHAD et al., 2021; HSU, 2020; RAWSON et al., 2020b; ROSSATO; NEGRÃO; SIMIONATTO, 2020).

Entretanto, mais estudos são requeridos a fim de compreender a ocorrência das infecções, os principais patógenos e os fatores de risco subjacentes ao paciente (KNIGHT et al., 2021). Relatos recentes descrevem fatores associados ao aumento do risco de internação e óbito hospitalar por COVID-19, em pacientes do sexo masculino e com diversas comorbidades, como hipertensão, doenças cardiovasculares, obesidade, câncer ativo, etc (SEMENZATO et al., 2021). No entanto, poucos estudos avaliaram pacientes com COVID-19 associados a infecção secundária e coinfecção, principalmente quando causadas por bactérias Gram-negativas multirresistentes e os fatores que afetam o desfecho clínico (ZHOU et al., 2020b; ZHU et al., 2020). Em Wuhan, na China, local de origem do SARS-CoV-2, os estudos mostram que a infecção secundária estava presente em 50% dos pacientes que não sobreviveram (ZHOU et al., 2020a). Sabe-se que pacientes com infecção por bactérias Gram-negativas multirresistentes têm maior tempo de permanência em UTIs, apresentando maior risco de mortalidade (DA SILVA et al., 2018, 2020b, 2020b; JIN et al., 2021a).

Assim, estudos epidemiológicos são necessários para avaliar fatores de risco em pacientes com COVID-19 e infecção secundária, especialmente por bactérias Gram-negativas multirresistentes, visto que a identificação de fatores de risco associados a resultados adversos nesses pacientes pode ser usada para melhorar os resultados clínicos por meio de reconhecimento precoce e tratamento adequado (BAIOU et al., 2021; IOANNOU et al., 2020). Nesta conjuntura, legitima-se a realização de estudos locais que determinem a prevalência das infecções em ambientes hospitalares, visando identificar fatores de risco em grupos de pacientes com infecções críticas (LODISE; YE; ZHAO, 2017).Em muitos países os planos de ação nacional de resistência a antimicrobianos

foram afetados pela pandemia (incluindo atividades de monitoramento e coleta de dados, capacitação técnica; aumento do uso de antibióticos; regulamentos sobre o consumo e uso de antibióticos que não foram aplicados) (Figura 5) (PÉREZ DE LA LASTRA et al., 2022). Uma vez que os esforços científicos para descobrir novos antimicrobianos e para combater a resistência foram redirecionados ao gerenciamento do COVID-19 (BIRGAND et al., 2022).

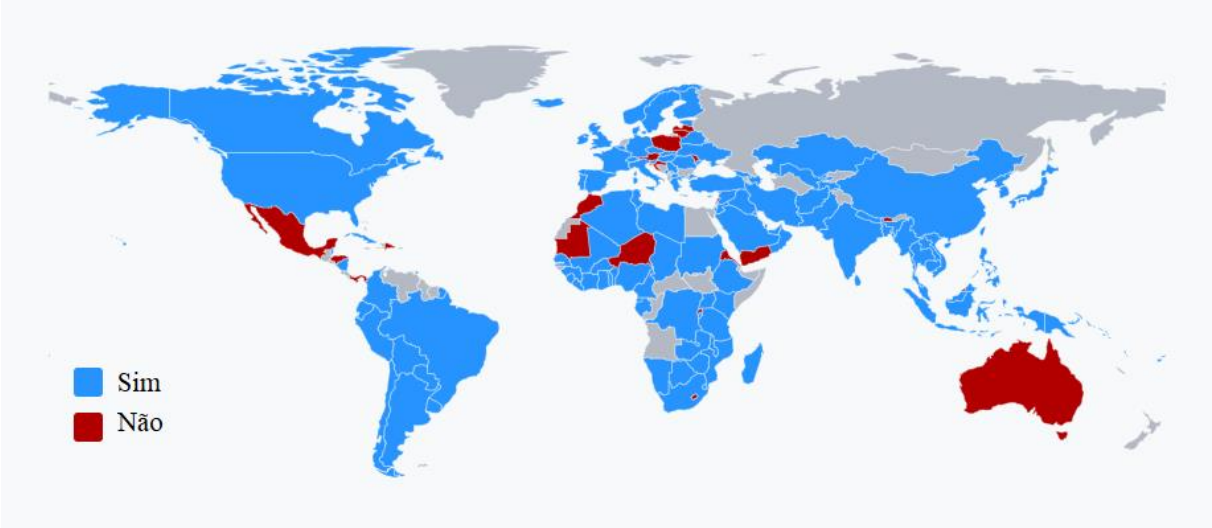

Figura 5. Mapa mundial representando os países em que o processo de desenvolvimento e implementação do Plano de Ação Nacional de RAM foram afetados pela pandemia de COVID-19. Em azul são os países que responderam "sim", à pergunta "O seu processo de desenvolvimento e implementação do Plano de Ação Nacional de RAM foram afetados pela pandemia de COVID-19 durante 2020-2021? Fonte: (TRACSS, 2021).

Nesse cenário, no qual os impactos da pandemia sobre os níveis gerais de resistência antimicrobiana ainda são desconhecidos, é imprescindível lançar planos de ação nacionais e internacionais para investimento na pesquisa e desenvolvimento de novos fármacos antimicrobianos (HSU, 2020; PÉREZ DE LA LASTRA et al., 2022; RAWSON et al., 2020b). A COVID-19 evidenciou que esforços globais, compromisso político em nível nacional e internacional e colaborações, são essenciais para enfrentar os principais desafios de saúde pública, inclusive a RAM, que mesmo não sendo caracterizada como um fenômeno pandêmico semelhante a COVID-19, requer medidas de enfrentamento na mesma magnitude, os quais têm de ser aplicados ao desenvolvimento de antimicrobianos e ao controle da resistência (FERREYRA et al., 2022; YAM, 2020).

3. OBJETIVOS

3.1 GERAIS

Descrever as características clínicas e fatores de risco associados a ocorrência de BGN-MR em pacientes críticos com ou sem COVID-19 internados em um hospital terciário de Dourados-MS, Brasil durante a pandemia de COVID-19.

Avaliar a atividade antimicrobiana do carvacrol em associação a polimixina B frente à microrganismos multirresistentes.

3.2 ESPECÍFICOS

Caracterizar o perfil epidemiológico de BGN-MR isoladas de amostras clínicas de pacientes internados entre março/2020 a dezembro/2021.

Identificar os fatores associados à infecção e/ou colonização por BGN-MR, estimar a porcentagem de mortalidade e fatores associados ao óbito em pacientes hospitalizados com ou sem COVID-19.

Investigar o potencial antimicrobiano *in vitro* e *in vivo* do carvacrol em associação a polimixina B frente à *Klebsiella pneumoniae* multirresistentes.

Realizar uma análise de revisão de patentes, a fim de identificar e explorar tendências da propriedade intelectual, referente a peptídeos antimicrobianos testados frente a *K. pneumoniae* resistentes à polimixina.

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APÊNDICE 1

Multidrug-resistant Gram-negative bacteria in patients with COVID-19: An epidemiological and clinical study

Gleyce Hellen de Almeida de Souza[1], Alexandre Ribeiro de Oliveira[1], Marcelo dos Santos Barbosa[1], Luana Rossato[1], Kerly da Silva Barbosa[2], Simone Simionatto*[1]

[1]Laboratório de Pesquisa em Ciências da Saúde, Universidade Federal da Grande Dourados - UFGD, Dourados, Mato Grosso do Sul, Brazil.

[2]Hospital Universitário da Universidade Federal da Grande Dourados – HU/UFGD/EBSERH, Dourados, Mato Grosso do Sul, Brazil.

***Corresponding author Address:** Laboratório de Pesquisa em Ciências da Saúde/Universidade Federal da Grande Dourados. Rodovia Dourados - Itahum, km 12, Cidade Universitária, 79804970, Dourados, Mato Grosso do Sul, Brasil. Phone: +55 67 3410-2225; Mobile: +55 67 99958-5355. E-mail address: simonesimionatto@ufgd.edu.br

ABSTRACT

Objectives: A case-control study was designed to determine factors associated with the occurrence of multidrug-resistant (MDR) Gram-negative bacteria (GNB) in patients with and without coronavirus disease (COVID-19) and describe the mortality rates.

Methods: The data were collected from patients hospitalized in a public tertiary care hospital in Dourados, Brazil, between March 2020 and December 2021.

Results: Of the 871 patients positive for COVID-19, 73 had secondary MDR-GNB infection, which represented 8.38% of documented community-acquired GNB-MDR

infections. The factors associated with patients COVID-19-MDR-GNB infections were obesity, heart failure, use of mechanical ventilation, urinary catheter, and previous use of β-lactams. Several factors associated with mortality were identified among patients with COVID-19 infected with MDR-GNB, including the use of a urinary catheter; renal failure; and the exposure to carbapenem antibiotics and polymyxin. Mortality was significantly higher in patients with COVID-19-MDR-GNB (68.6%) compared to three control groups, where COVID-19 was 35.7% (p = 0.000, odds ratio [OR] 392, 95% confidence interval [CI] 1.94–7.92), MDR-GNB was 50% (p = 0.002, OR 0.33, 95% CI 0.16–0.67) and GNB was 21.4% (p = 0.000, OR 8.00, 95% CI 3.73–17.14).

Conclusions: We demonstrate that MDR-GNB infection associated with COVID-19 has an expressive impact on increasing the case fatality rate, reinforcing the importance of minimizing the use of invasive devices and exposure to antimicrobials to control the bacterial spread in hospital to improve the prognosis among patients.

Keywords: Healthcare-associated infections, antimicrobial resistance, public health, SARS-CoV-2, Multi-drug resistance.

INTRODUCTION

Antimicrobial resistance (AMR) is an emerging global public health challenge hampering the health of mainly critically ill patients [1,2]. An estimated annual 4.95 million deaths are associated with bacterial resistance [3]. It is projected that by 2050, approximately 10 million individuals could die annually owing to ineffectiveness in controlling and combating AMR [4]. Long hospitalization, previous use of antimicrobials, and invasive and surgical procedures are some factors associated with the occurrence of AMR [5,6].

These factors were likely exacerbated by the coronavirus disease (COVID-19), as increased rates of hospitalization and admission in intensive care units (ICUs), inappropriate or overuse of antibiotics in many patients, and growing global antibiotic use are observed. The long-term impact of the pandemic are still unknown. However, there is a particular concern, especially with regard to the spread of resistance and underlying patient risk factors [7–9].

Secondary infection was identified in 50% of deaths in patients with COVID-19 [9]. Thereby, surveillance of patients with multidrug-resistant Gram-negative microorganisms (MDR-GNB) is essential in the healthcare environment to improve the prognosis of patients [5]. Therefore, containment and dispersion of MDR-GNB bacteria in critically ill patients, especially those with COVID-19, can be achieved by knowing the predicted factors related to their occurrence. Thus, these contribute to reducing unfavorable outcomes [10,11].

MDR-GNB are listed as resistant pathogens of the highest priority by the World Health Organization (WHO) owing to their dissemination in the hospital environment, causing a variety of infections associated with increased morbidity and mortality [7,12]. Nevertheless, investigating prediction factors associated with MDR-GNB infections in patients with COVID-19 is limited [10,13]. Thus, this case-control study aimed to investigate the factors associated with the occurrence of MDR-GNB in adults with and without COVID-19 admitted into Brazilian ICUs and to describe mortality rates and clinical characteristics of these infections.

MATERIAL AND METHODS

Study site and patients

The data were collected from patients hospitalized in a public tertiary care hospital in Dourados, Brazil, between March 2020 and December 2021. This hospital has 237 beds, including infirmaries and the ICUs (adult, pediatric, and neonatal), with an average of 9,800 annual admissions. Patients with MDR-GNB isolated from clinical cultures from any source, such as tracheal aspirates, catheter tips, swabs (nasal and rectal), and blood and urine cultures, were included in this study. Patients under 18 years with incomplete records, those transferred to other hospitals before discharge from the ICU, or those who were not monitored for loss of data or incorrect records were excluded. The records of the same patient with different clinical sources of infection were also excluded, and only the first record was considered.

Definitions

MDR was defined as resistance to one or more antimicrobials from three or more tested categories [14]. Nosocomial infection was defined by the clinical diagnosis based on the clinical criteria (sepsis, fever, changes in the frequency or color of secretions, or new radiological findings), initiated >48 h after hospital admission or within 48 h after hospital discharge, associated with the occurrence of a carbapenem-resistant microorganism [15]. In contrast, other infections were considered community-acquired [13]. COVID-19 was defined as a positive real-time reverse transcriptase polymerase chain reaction (RT-PCR) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a nasopharyngeal swab associated with suggestive clinical signs, symptoms, and/or radiological findings. The time from COVID-19 detection and initial presentation to culture time was used to assess likely community (<120 h from admission) or

healthcare-associated infection (>120 h from admission). This time point was agreed upon by the study team to define the pathogens associated with health in this study [16].

Study design

A case-control study was performed to identify factors associated with the occurrence of MDR-GNB in patients with or without COVID-19. A case (COVID-19-MDR-GNB) was defined as a patient positive for COVID-19 from whom an MDR-GNB was isolated from clinical cultures from any source during the study. Control 1 was defined as patients positive for COVID-19 admitted in the same study period. Control 2 included patients admitted in the same study period from whom an MDR-GNB was isolated from a clinical culture at least 48 h after admission and no clinical or microbiological evidence of COVID-19. Control 3 included patients admitted in the same study period from whom a susceptible GNB was isolated from a clinical culture at least 48 h after admission and there was no clinical or microbiological evidence of COVID-19. Non-probabilistic controls, randomly recruited in a 1:1:1:1 ratio to cases. Case and controls were matched for age, clinical manifestations, pathogens, and hospital wards. The inclusion of a case or control was only possible once. A two-part analysis was performed as follows: i) a case-control study in which cases were compared with controls to identify potential factors associated with the isolation of MDR-GNB in adult ICU, and ii) a retrospective analysis to measure mortality associated with the isolation of MDR-GNB.

Clinical data

The clinical, nursing, and microbiological records of hospitalized patients were reviewed retrospectively. The following data were recorded: demographics; medical

history; comorbidities; location before admission; ward of admission; hospital course (duration and ward location); invasive procedures; surgery; use of invasive medical devices (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). All antibiotics administered for ≥24 h 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. Data regarding the clinical outcome (recovery/death) were reviewed, and death owing to any cause or death attributable to infection was assessed.

Bacterial identification and susceptibility testing

The bacterial species identification and screening for antimicrobial resistance were performed using Phoenix® Automated System (BD Diagnostic Systems, Sparks, MD) according to the manufacturer's instructions. After isolation, following the recommendations of the Clinical and Laboratory Standards Institute guidelines, the susceptibility profile was confirmed, and minimal inhibitory concentrations (MICs) of antimicrobials were determined using broth microdilution (CLSI, 2021).

Statistical analysis

The Research Electronic Data Capture (Redcap) was used for the database, and statistical analysis was performed by SAS v.19.0 (SAS Institute, Cary, NC, USA) using univariate and multivariate models. Dichotomized and categorical data were analyzed using the Chi-squared test or Fisher's exact test. For continuous variables, a t-test or analysis of variance was used. Univariate analyses were performed to verify associations

between the dependent and independent variables, and those achieving a prespecified level of significance (P <0.2) were included in the multivariable analysis. P <0.05 was considered to indicate statistical significance. To evaluate the strength of associations, a logistic regression analysis was used to estimate crude, adjusted odds ratios (OR), and 95% confidence intervals (CIs). Additionally, we performed Kaplan-Meier plot analysis to visualize survival curves, cox regression, and log-rank test to compare survival curves.

RESULTS

Patient characteristics

During the study, 906 GNB were identified, of which 422 were MDR-GNB isolates, representing a 46.58% of resistance rate. Additionally, 151 patients carrying carbapenem-resistant strains were admitted to adult ICUs, of which 70 patients were included to compose the MDR-GNB control group (Suplementary Figure 1). Furthermore, in the MDR control group, 62 strains were identified as community-acquired. In total, 280 adult patients (compared data from the 70 cases with 210 controls) were included, the median age was 56 years (range 18 to 92 years), and the majority were women (n = 143; 51%) with 22.07 days (range 1 to 102 days) of mean length of hospital stay. In the sample studied, there were no significant differences (p > 0.05) between cases and controls with respect to baseline demographics. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Acinetobacter baumannii* are the main MDR-GNB species (68%) isolated in the present hospital during the period studied. For diagnosing COVID-19, 1,953 patients were tested for SARS-CoV-2 infection in the hospital, and 871 had a positive test. Among these, 73 patients had secondary infections with MDR-GNB, which represents a rate of 8.38%. Among these, 70 patients were included to compose the COVID-19-MDR-GNB case group; 22 strains were identified as nosocomial and 48

strains as community-acquired. In patients with COVID-19-MDR-GNB and MDR-GNB, the most prevalent microorganism identified was *K. pneumoniae*, representing 31.4% and 41.4%, respectively (Figure 1).

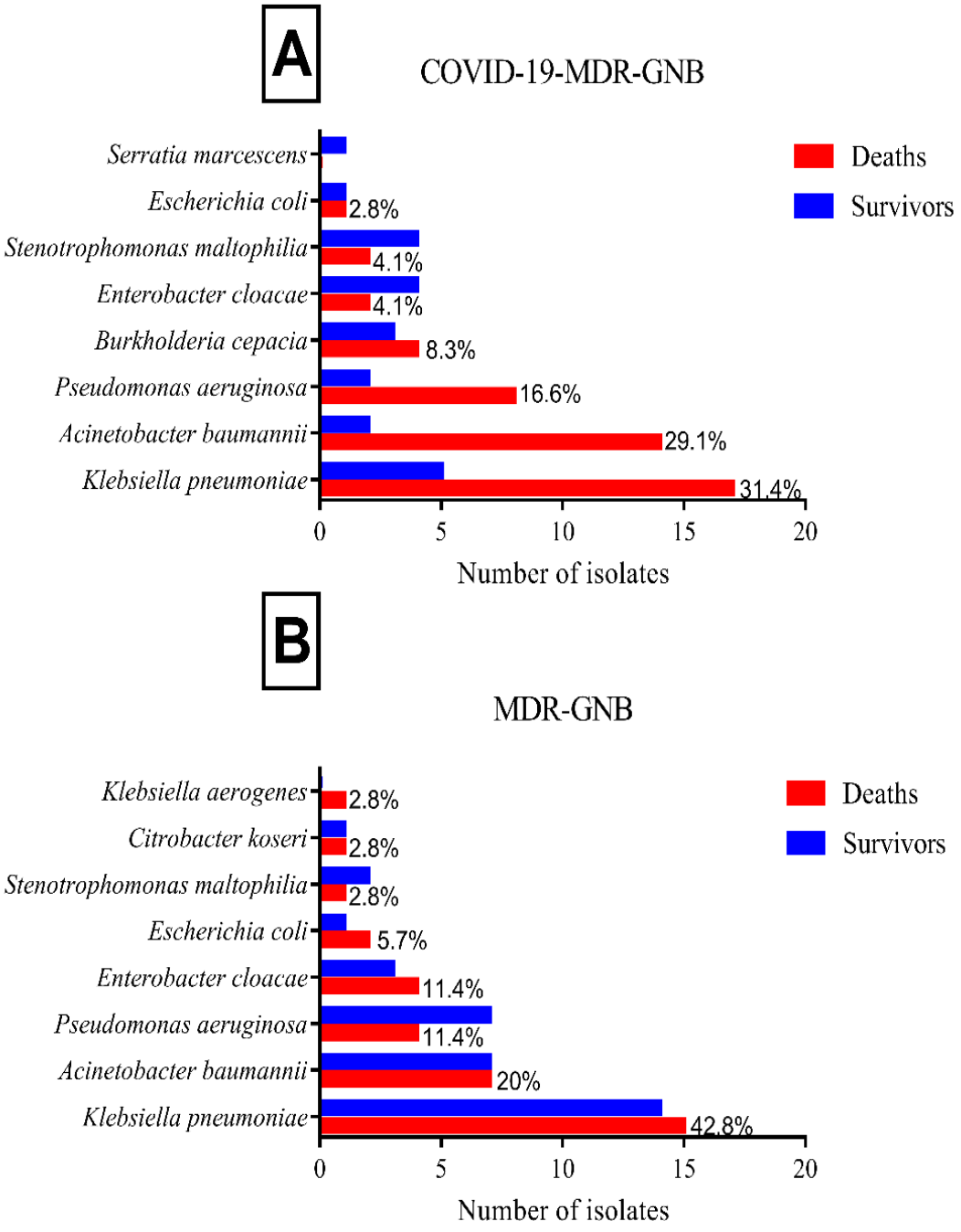

Figure 1. Total number of MDR-GNB species isolated from study patients: A) COVID-19-MDR-GNB case group (n = 70); B) MDR-GNB control group (n = 70).

Factors associated with MDR-GNB

Case patients (COVID-19-MDR-GNB) were compared to the control group (COVID-19), aiming to analyze the factors associated with the occurrence of MDR-GNB in hospitalized patients with confirmed COVID-19 diagnosis. Renal failure, use of mechanical ventilation, central venous catheter, nasoenteral tube, and length of stay up to 30 days were identified as factors associated with the acquisition of MDR-GNB among patients with COVID-19 in a univariate analysis. Among COVID-19-MDR-GNB, the majority of patients were men; and use of antimicrobials (β-lactams, aminoglycosides, carbapenems, cephalosporins, quinolones, and polymyxins) was associated with the occurrence of MDR-GNB in patients with COVID-19. Multivariate analysis revealed that renal failure, hospital stay longer than 30 days, and use of carbapenems and β-lactams were all independently associated with MDR-GNB isolation. Additionally, the outcome of death was independently associated with an increased risk of death by 3.29 in patients with MDR-GNB (Table 1).

To analyze the factors associated with COVID-19 among patients with MDR-GNB, case patients (COVID-19-MDR-GNB) were compared to control group 2 (MDR-GNB). Univariate analysis demonstrated that obesity, cardiac insufficiency, mechanical ventilation, central venous catheter, age between 23 and 60 years, age over 60 years, β-lactam and polymyxins antimicrobials use were identified as factors associated with MDR-GNB in patients with COVID-19 (Table 2). Additionally, in the multivariate analysis cardiac insufficiency and mechanical ventilation use were associated with MDR-GNB and COVID-19-MDR-GNB patients, respectively. The mortality rates of COVID-19-MDR-GNB patients were increased 2.47 when compared with MDR-GNB. Additionally, the use of polymyxin was a protective factor for the occurrence of MDR-GNB in patients with COVID-19.

Case patients (COVID-19-MDR-GNB) were compared with the control group (GNB) to analyze the associated factors to secondary MDR-GNB infection in patients with COVID-19. In the univariate analysis, several covariates showed statistically significant associations, including systemic arterial hypertension; diabetes; renal insufficiency; acute respiratory failure; smoking; mechanical ventilation; urinary catheter; central venous catheter; nasoenteral tube; use of antimicrobials (β-lactams, aminoglycosides, carbapenems, cephalosporins, and polymyxins); and age between 18 and 60 and over 60 years (Table 3). Furthermore, in the multivariate analysis, systemic arterial hypertension, renal insufficiency, mechanical ventilation, use of polymyxins and outcomes in deaths were associated with secondary MDR-GNB infection in patients with COVID-19, with an increase of 3.44 in the mortality rates. Additionally, the use of β-lactams was a protective factor for the occurrence of MDR-GNB in patients with COVID-19.

Outcome study

Mortality was significantly higher in patients with COVID-19-MDR-GNB compared with COVID-19 controls (68.6% vs. 35.7%, respectively; p = 0.000, odds ratio [OR] 392, 95% confidence interval [CI] 1.94–7.92), MDR-GNB (68.6% vs. 50%, respectively; p = 0.002, OR 0.33, 95% CI 0.16–0.67) and GNB (68.6% vs. 21.4%, respectively; p = 0.000, OR 8.00, 95% CI 3.73–17.14). Overall, mortality at 30 days for patients with COVID-19-MDR-GNB was recorded at 57.1% (n = 40/70). A Kaplan–Meier survival analysis showed the cumulative probability of death in the 30 days was significantly different among the COVID-19-MDR-GNB, MDR-GNB, GNB, and COVID-19 patient groups (P = 0.008) and higher among patients infected with COVID-19-MDR-GNB strains (Figure 2). Additionally, this study also aimed to identify factors

associated with mortality. In a multivariate analysis of patients with COVID-19-MDR-GNB, the use of a urinary catheter and exposure to polymyxin were associated with mortality. In contrast, systemic arterial hypertension and surveillance swabs were protective factors. While for patients with MDR-GNB, the use of carbapenems and the origin of the bacterial culture as tracheal secretion were factors associated with mortality. For patients with COVID-19, age over 60 years, renal failure, and central venous catheter use were associated with mortality (Table 4).

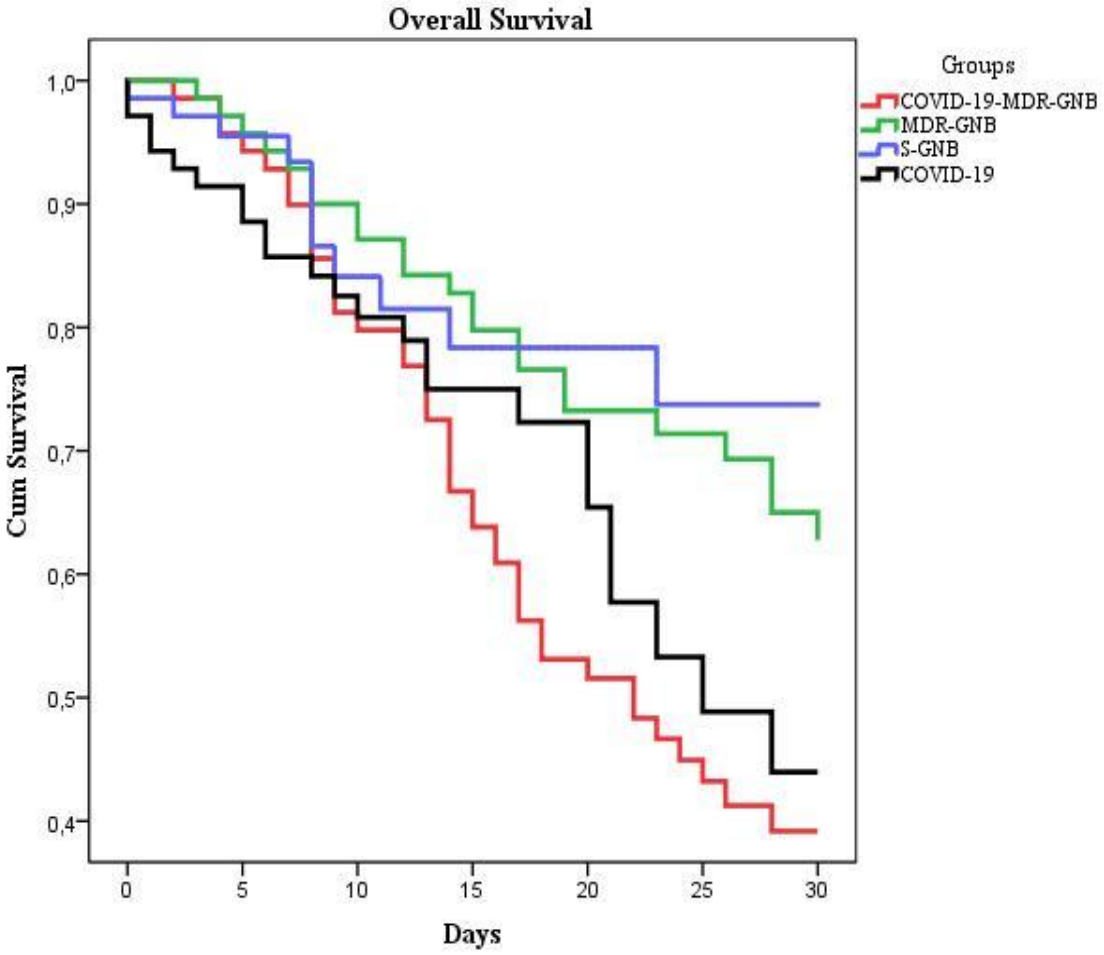

Figure 2. Kaplan–Meier cumulative (cum) survival curve for 30-day mortality of MDR-GNB infection by log-rank test ($p = 0.008$). The red line represents patients with COVID-19 and infection caused by MDR-GNB strains, the green line represents patients with infection caused by MDR-GNB strains, the blue line represents patients with infection caused by GNB strains, and the black line represents patients with COVID-19.

DISCUSSION

Even before the COVID-19 pandemic, infections caused by MDR-GNB, including *Enterobacteriaceae, P. aeruginosa,* and *A. baumannii*, represented a global public health concern owing to antimicrobial restriction and increased lethality [12,17]. This problem was probably exacerbated during the pandemic since hospitalized patients with COVID-19 were generally vulnerable, staying hospitalized for long periods in ICU, requiring a greater number of invasive procedures, mechanical ventilation: factors that contribute to the risk of acquiring MDR-GNB [9,10].

The ecology of microorganisms and their resistance patterns reflects the institutional epidemiological situation [13]. Our study showed that in the study, *P. aeruginosa* (29.4%), *K. pneumoniae* (22.7%), and *A. baumannii* (15.9%) were the main MDR-GNB species isolated in the hospital. In contrast, as per the reported data described in Taiwan, the most common isolates were *E. coli* (45,1%), *K. pneumoniae (17.3%)*, and *Acinetobacter* spp. (14.3%) [7]. The pattern of resistance distribution among pathogens varies geographically, reinforcing the need for local estimates to adapt local responses to the control of MDR-GNB [3].

Additionally, when analyzing the pathogens among patients with COVID-19-MDR-GNB, we identified *K. pneumoniae* (31.4%), *A. baumannii* (29.1%), and *P. aeruginosa* (16.6%) as the main pathogens isolated in patients who died. This same pattern of main pathogens was observed among patients with MDR-GNB, although with non-identical rates, namely, *K. pneumoniae* (42.8%), *A. baumannii* (20%), and *P. aeruginosa* (11.4%). Infections caused by resistant *Enterobacteriaceae* are associated with increased mortality [13,18]. Previous studies described that the mortality rate among patients with resistant *K. pneumoniae* infections is high, ranging from 28.6% to 66.6% [6,19].

In our study, MDR-GNB isolation in patients with COVID-19 was associated with mortality. In addition, in Qatar, mechanical ventilation (p = 0.015, OR 1.06, 95% CI 1.01–1.11) was described as a risk factor associated with the isolation of MDR-GNB in patients with COVID-19-MDR-GNB (n=78) [10]. Additionally, in Spain, a retrospective case-control study assessed patients with COVID-19 and carbapenem-resistant Enterobacteriaceae (CRE) infection (n = 30), compared to patients without COVID-19 and CRE (n = 24) and identified a 30-day mortality rate of 30% and 16.7%, respectively (p = 0.25). Additionally, *K. pneumoniae* (80.8%), *Serratia marcescens* (11%), and *Enterobacter cloacae* (4.1%) were the most frequent bacteria isolated in these groups [13].

Several risk factors have been described associated with MDR-GNB, including previous use of antibiotics, use of carbapenem, mechanical ventilation, intubation, previous hospitalization, dialysis, use of invasive devices, longer ICU stay, and presence of underlying comorbidities [20]. Additionally, in patients with COVID-19, risk factors associated with mortality have been described associated with men; smokers; age ≥60 years, and comorbidities such as hypertension, cardiovascular disease, diabetes, chronic obstructive pulmonary disease, cancer, hypercholesterolemia; and ICU admission [21,22]. Thus, the occurrence of these factors is determinant, making patients subject to risks of acquiring MDR-GNB infections and/or greater complications owing to COVID-19.

Hypertension and cardiovascular disease were described as risk factors associated with the progression of COVID-19 [23]. However, in our study systemic arterial hypertension was a protective factor against mortality in patients with COVID-19-MDR-GNB. The underlying effects of antihypertensive treatments, including angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), have been investigated and shown to increase intrinsic antiviral cellular responses and the cell-

epithelial-immune interactions, respectively [24]. Thus, further studies are needed to improve the understanding of these variables.

Furthermore, 30-day mortality rates for patients with COVID-19-MDR-GNB and COVID-19 were 57.1% and 34.2%, respectively. The worrying mortality rates identified in our study highlight the need for isolation and identification of the early susceptibility profile of these microorganisms to initiate adequate treatment against these MDR-GNB, which can be potentially fatal in patients with COVID-19. In Taiwan, the 30-day mortality rate among patients with MDR-GNB bacteremia versus patients without MDR-GNB was 27.4% and 13%, respectively [25]. Thus, in our study, the occurrence of MDR-GNB negatively impacted the prognosis of patients with COVID-19, increasing the 30-day mortality. This data is particularly important for hospital infection control services, reinforcing the need for measures to prevent and control the transmission of MDR-GNB, especially among critically ill patients, to improve outcomes and hospital survival.

Our findings demonstrate polymyxin exposure and urinary catheter were independently associated with mortality in patients with COVID-19-MDR-GNB, increasing the risks by 5.7 and 6.0-fold, respectively. In contrast, in patients with MDR-GNB, the use of carbapenems and bacterial origin of tracheal secretion are factors associated with mortality, increasing the risks by 3.9 and 7.7-fold, respectively. Additionally, in patients with COVID-19, age over 60 years, use of polymyxin, central venous catheter use, and renal failure are factors associated with mortality, increasing the risks by 4.5, 6.7, 21.3, and 24.5 times, respectively. In the United States and Taiwan, the mortality predictors in patients with MDR-GNB include longer hospital stay, surgical re-exploration, urinary catheter as a source of infection, exposure of antibiotics, and use of carbapenems and fluoroquinolones that are independent factors associated with MDR-GNB infection [7,26]. Reinforcing procedures to prevent MDR-GNB infection in patients with COVID-19 should include minimizing the use of invasive devices to improve the

prognosis [10]. Thus, despite all the control measures instituted in hospitals, patients with COVID-19 may be at increased risk of secondary bacterial infections with MDR pathogens, as they are often exposed to risk factors such as invasive devices, mechanical ventilation, prolonged ICU stay, and extensive use of broad-spectrum antimicrobials [27,28].

In a systematic review, with 42 studies and more than 400 thousand patients, the risk factors related to mortality in patients with COVID-19 were evaluated, identifying that chronic comorbidities, acute kidney disease, diabetes, hypertension, cancer, male sex, advanced age, current smoking, and obesity are factors associated with lethality [29]. In these circumstances, previous studies demonstrate that secondary bacterial infection has been associated with a negative impact on prognosis in patients with COVID-19 [30–32]. In our study, the percentage of mortality among patients with COVID-19 was 35.7, corroborating the literature [21]. Mortality among patients with MDR-GNB and GNB was 50% and 21.4%, respectively. Thus, our findings demonstrate that secondary MDR-GNB infection in patients with COVID-19 increases the case fatality rate to 68.7%, imposing a huge burden on healthcare facilities, particularly for patients with comorbidities. These results highlight the need to improve awareness and early recognition, as the occurrence of MDR-GNB infection can be potentially fatal in such patients. These findings assist in identifying patients at a higher risk to improve the prognosis, and surveillance and prevention strategies must be developed to limit the impact of these pathogens.

In contrast, our study had some limitations. First, this is a retrospective study performed in a single hospital. Thus, a prospective and multicenter studies are needed since microbiological epidemiology may vary according to geographic locations. However, we demonstrate that the occurrence of MDR-GNB increases mortality among

patients, especially those with COVID-19. To the best of our knowledge, this is the first study to describe the factors of the occurrence of MDR-GNB in patients with COVID-19 in Brazil. Our data reinforce the need to prevent the spread of these strains within the hospital environment, aiming to minimize these risks. Thus, these findings are important health indicators for hospitalized patients with COVID-19, emphasizing the need of surveillance and strategies to reduce the impact of MDR-GNB, especially in critically ill patients.

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Institutional Review Board Statement

The study was conducted with the approval of the Research Ethics Committee from Universidade Federal da Grande Dourados (4.255.410/2020).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflict of interest.

Table 1. Univariate and multi-variate analysis between COVID-19-MDR-GNB patients compared to COVID-19 control groups.

	COVID-19-MDR-GNB	COVID-19	Univariable analysis		Multi-variable analysis	
Factors	n = 70 (%)	n = 70 (%)	OR (95% CI)	P-Value	OR (95% CI)	P-Value
Age (years)	58.0 (26-86)	56.3 (36-90)				
Comorbidities						
Renal insufficiency	23 (32.9)	8 (11.4)	3.79 (1.55-9.22)	**0.002**	**2.84 (1.03-7.80)**	**0.043**
Hospitalization						
Mechanical ventilation	56 (80)	43 (61.4)	2.51 (1.17-5.36)	**0.016**		
Central venous cateter	37 (52.9)	17 (24.3)	3.49 (1.70-7.18)	**0.001**		
Nasoenteral tube	6 (8.6)	0	0.91 (0.85-0.98)	**0.028***		
Length of stay	28.0	16.0				
Up to 30 days	53 (75.7)	63 (90)	0.34 (0.13-0.89)	**0.025**	**3.69 (1.25-10.91)**	**0.018**
Use of antimicrobials						
Carbapenems	39 (55.7)	22 (31.4)	2.74 (1.37-5.47)	**0.004**	**3.48 (1.37-8.80)**	**0.008**
Cephalosporin	27 (38.5)	40 (57.1)	0.47 (0.24-0.92)	**0.028**		
Aminoglycosides	16 (22.8)	5 (7.14)	3.85 (1.32-11.19)	**0.009**		
Polymyxins	29 (41.2)	10 (14.2)	4.24 (1.86-9.64)	**0.001**		
β-lactams	33 (47.1)	48 (68.6)	0.40 (0.20-0.81)	**0.010**	**4.11 (1.63-10.33)**	**0.003**
Deaths	48 (68.6)	25 (35.7)	3.92 (1.94-7.92)	**0.000**	**3.29 (1.45-7.49)**	**0.004**

*Fisher's Exact Test.

Table 2. Univariate and multi-variate analysis between COVID-19-MDR-GNB case patients compared to MDR-GNB control groups.

	COVID-19-MDR-GNB	MDR-GNB	Univariable analysis		Multi-variable analysis	
Factors	n = 70 (%)	n= 70 (%)	OR (95 % CI)	P-Value	OR (95 % CI)	P-Value
Age (years)	58.0 (26-86)	64.7 (23-92)				
23-60	37	25	2.01 (1.02-3.97)	**0.041**		
> 61	33	45	0.45 (0.25-0.97)	**0.041**		
Comorbidities						
Obesity	10 (14.3)	2 (2.9)	5.66 (1.19-26.89)	**0.031***		
Cardiac insufficiency	7 (10)	23 (32.9)	4.40 (1.74-11.12)	**0.001**	**4.68 (1.59-13.73)**	**0.005**
Hospitalization	28.0	29.5				
Mechanical ventilation	56 (80)	38 (54.3)	3.36 (1.58-7.13)	**0.001**	**3.05 (1.28-7.26)**	**0.011**
Central venous cateter	37 (52.9)	24 (34.3)	2.14 (1.088-4.24)	**0.027**		
Use of antimicrobials						
Polymyxins	29	33	0.79 (0.40-1.54)	0.496	**0.41 (0.17-0.97)**	**0.045**
β-lactams	33	51	0.33 (0.16-0.67)	**0.002**	**4.22 (1.75-10.14)**	**0.001**
Origin of culture						
Tracheal secretion	35 (50)	12 (17.6)	4.83(2.21-10.52)	**0.000**		
Swabs retal	17 (24.6)	38 (55.9)	0.27(0.13-0.55)	**0.000**		
Microorganisms						
Burkholderia cepacia	7	0	2.11 (1.76-2.52)	**0.013***		
Deaths	48 (68.6)	35 (50)	2.18 (1.09-4.34)	**0.025**	**2.47 (1.07-5.68)**	**0.033**

*Fisher's Exact Test.

Table 3. Univariate and multi-variate analysis between COVID-19-MDR-GNB patients compared to GNB control groups.

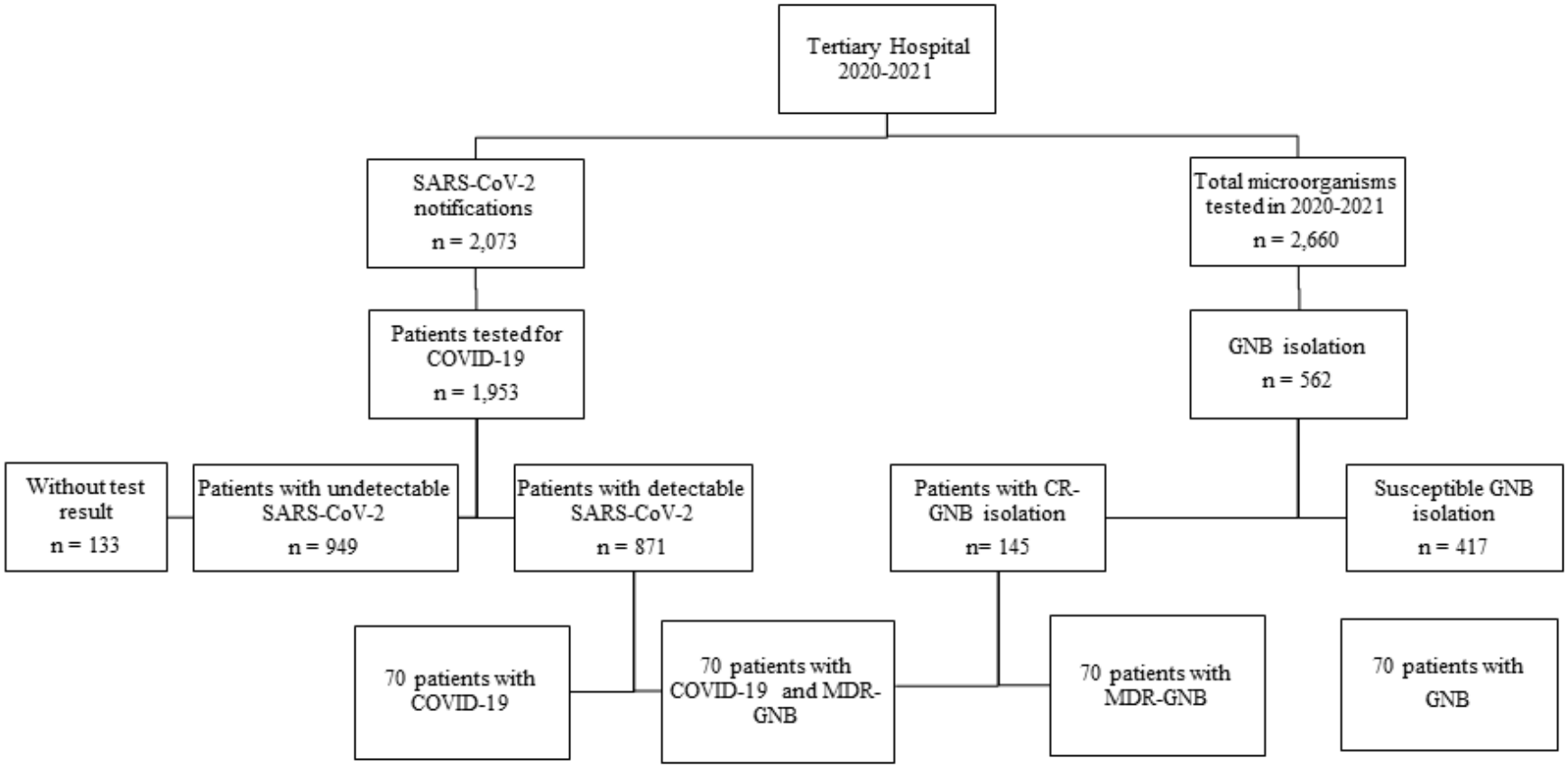
	COVID-19-MDR-GNB	GNB	Univariable analysis		Multi-variable analysis	
Factors	n = 70 (%)	n= 70 (%)	OR (95% CI)	P-Value	OR (95% CI)	P-Value
Age (years)	58.0 (26-86)	43.9 (18-91)				
18-60	37	51	0.41 (0.20-0.84)	**0.014**		
> 61	33	19 (11.4)	2.39 (1.18-4.84)	**0.014**		
Gender						
Male	47 (67.1)	18 (25.7)	5.90 (2.83-12.27)	**0.000**		
Comorbidities						
Systemic arterial hypertension	33 (47.1)	16 (22.9)	3.01 (1.45-6.24)	**0.003**	**3.03 (1.09-8.38)**	**0.032**
Diabetes	23 (32.9)	12 (17.1)	2.36 (1.06-5.24)	**0.032**		
Renal insufficiency	23 (32.9)	11 (15.7)	2.62 (1.16-5.92)	**0.018**	**5.54 (1.29-23.82)**	**0.021**
Acute respiratory failure	10 (14.3)	1 (1.4)	11.50 (1.43-92.47)	**0.009***		
Smoking	0	6 (8.6)	1.09 (1.01-1.17)	**0.028***		
Hospitalization	28.0	17.1				
Mechanical ventilation	56 (80)	18 (25.7)	11.55 (5.22-25.56)	**0.000**	**17.85 (5.07-62.85)**	**0.000**
Urinary cateter	27 (38.6)	9 (12.9)	4.25 (1.82-9.95)	**0.001**		
Central venous cateter	37 (52.9)	12 (17.1)	5.41 (2.48-11.80)	**0.000**		
Nasoenteral tube	6 (8.6)	0	0.91 (0.85-0.98)	**0.028***		
Use of antimicrobials						
Carbapenems	39	20	3.14 (1.56-6.34)	**0.001**		
Cephalosporin	27	41	0.44 (0.22-0.87)	**0.018**		
Polymyxins	29	6	7.54 (2.88-19.75)	**0.000**	**3.24 (0.97-10.83)**	**0.055**
β-lactams	33	46	0.46 (0.23-0.91)	**0.027**	**0.08 (0.02-0.32)**	**0.000**
Deaths	48 (68.6)	15 (21.4)	8.00 (3.73-17.14)	**0.000**	**3.44 (1.22-9.67)**	**0.019**

*Fisher's Exact Test.

Table 4. Summary of factors associated with mortality in cases patients with COVID-19-MDR-GNB and COVID-19, MDR controls groups.

Groups	Factors	Deaths n (%)	Survivors n (%)	Univariable analysis OR (95% CI)	P-Value	Multi-variable analysis OR (95% CI)	P-Value
COVID-19-MDR-GNB	**Total**	48 (68.5)	22 (31.4)				
	Comorbidities						
	Systemic arterial hypertension	18 (37.5)	15 (68.1)	0.28 (0.09- 0.81)	**0.017**	**0.15 (0.04-0.60)**	**0.007**
	Hospitalization						
	Urinary cateter	23 (47.9)	4 (18.1)	4.14 (1.21- 14.05)	**0.020**	**6.00 (1.30-27.65)**	**0.021**
	Central venous cateter	30 (62.5)	7 (31.8)	3.57 (1.22- 10.41)	**0.017**		
	Length of stay (days)	20.5	37.8				
	Up to 30 days	40 (83.3)	13 (59.0)	3.46 (1.10-10.81)	**0.028**		
	Use of antibiotics						
	Polymyxins	25 (52.0)	4 (18.1)	4.89 (1.44-16.60)	**0.009**	**5.76 (1.36-24.42)**	**0.017**
	Origin of culture						
	Swabs	10 (20.8)	9 (81.8)	0.33 (0.10-1.01)	**0.049**	**0.23 (0.06-0.93)**	**0.040**
MDR-GNB	**Total**	35 (50)	35 (50)				
	Use of antibiotics						
	Carbapenems	23 (32.8)	15 (21.42)	2.55 (0.97-6.72)	**0.055**	**3.92 (1.27-12.11)**	**0.017**
	Origin of culture						
	Tracheal secretion	10 (14.2)	2 (2.85)	6.60 (1.32-32.84)	**0.023**	**7.70 (1.40-42.45)**	**0.019**
COVID-19	**Total**	25 (35.7)	45 (64.2)				
	Age (years)						
	>60	15 (60)	15 (60)	3.00 (1.09-8.25)	**0.031**	**4.59 (1.06-19.77)**	**0.041**
	Comorbidities						
	Renal insufficiency	6 (24)	2 (4.4)	6.78 (1.25-36.75)	**0.021**	**24.50 (2.96-202.54)**	**0.003**

Acute respiratory failure	10 (40)	2 (4.4)	14.33 (2.81-73.00)	**0.000**		
Hospitalization						
Urinary cateter	13 (52)	4 (8.8)	11.10 (3.05-40.42)	**0.000**		
Central venous catheter	14 (56)	3 (6.6)	17.81 (4.33-73.17)	**0.000**	**21.32 (4.29-105.82)**	**0.000**
Use of antibiotics						
Cephalosporin	9 (36)	31 (68.8)	0.25 (0.09-0.71)	**0.012**		
Polymyxins	6 (24)	4 (8.8)	3.23 (0.81-12.82)	0.151	**6.71 (1.06-42.46)**	**0.043**



Supplementary Figure 1. Flowchart of definition and selection of cases and controls included in the study of factors associated. Abbreviations: Severe Acute Respiratory Syndrome (SARS); COVID-19, coronavirus 19; GNB, Gram-negative bacillus; MDR, multidrug resistant; susceptible to carbapenems Gram-negative bacterium (GNB)

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APÊNDICE 2

Polymyxin B combined with carvacrol: a promising alternative strategy for combating polymyxin-resistant *Klebsiella pneumoniae* planktonic cells and biofilm

Gleyce Hellen de Almeida de Souza[a], Marcia Soares Mattos Vaz[a], Joyce Alencar dos Santos Radai[a], Thiago Leite Fraga[b], Luana Rossato [a], Simone Simionatto[a*]

[a]Laboratório de Pesquisa em Ciências da Saúde, Universidade Federal da Grande Dourados - UFGD, Dourados, Mato Grosso do Sul, Brazil.

[b]Centro Universitário da Grande Dourados – UNIGRAN, Dourados, Mato Grosso do Sul, Brazil.

***Corresponding author:** simonesimionatto@ufgd.edu.br

Abstract

Polymyxin-resistant *Klebsiella pneumoniae* infections represent a health challenge because of the limited treatment options for the patient. Thus, the development of antimicrobial alternatives is necessary. This study aimed to investigate the synergistic activity of carvacrol with polymyxin B against polymyxin-resistant *K. pneumoniae*. The checkerboard method evaluated the synergism of 48 combinations of carvacrol plus polymyxin B, of which 23 combinations indicated synergistic action (fractional inhibitory concentration index: 0.125–0.500). Evaluation of the time–kill of the synergistic combinations showed that carvacrol 70 µg/mL plus polymyxin B 1–0.001 µg/mL, carvacrol 35 µg/mL plus polymyxin B 2–0.06 µg/mL, and carvacrol 17 µg/mL plus polymyxin B 2–0.25 µg/mL exhibited *in vitro* bactericidal effect against polymyxin-resistant *K. pneumoniae*, killing all cells within 2 h after treatment. Of 23 combinations, 18 and 11 showed bactericidal and inhibitory effects on biofilm formation, respectively. The antimicrobial effect *in vivo* was determined using polymyxin-resistant *K. pneumoniae* in mice model of infection. Carvacrol 10 mg/kg plus polymyxin B 2 mg/kg was associated with increased survival and a significant reduction in bacterial load in the blood. To the best of our knowledge, this is the first study examining the combinations of carvacrol and polymyxin and their synergistic effects, which showed bactericidal activity against planktonic cells of polymyxin-resistant *K. pneumoniae* and biofilm, demonstrating its potential to be explored by the pharmaceutical industry.

Keywords: Multidrug-resistant; Gram-negative; synergy effect; monoterpene; natural product.

INTRODUCTION

Klebsiella pneumoniae is distinguished in the hospital environment as an opportunistic pathogen and could acquire resistance to multiple antimicrobials [1–3]. The spread of polymyxin resistance among clinical isolates threatens public health because it restricts available therapeutic options [4,5]. An estimated 4.95 million deaths annually are associated with bacterial resistance [6]. In 2050, approximately 10 million people could die because of ineffectiveness in controlling and combating antimicrobial resistance [7]. Thus, to combat antimicrobial resistance, the World Health Organization (WHO) has published a list of critical priority pathogens (including multidrug-resistant *K. pneumoniae*) to encourage research and development of new antibiotics.

In these circumstances, polymyxins are no longer used in medical clinics due to their side effects and nephrotoxic potential; however, they have re-emerged as the last option in treating infections caused by carbapenem-resistant *K. pneumoniae* [8,9]. Afterward, resistance to polymyxins gradually increased, with an estimated prevalence varying from 2.7% to >40% among clinical isolates of multidrug-resistant *K. pneumoniae*, resulting in therapeutic ineffectiveness [10,11]. Consequently, polymyxin-resistant *K. pneumoniae* infections are associated with high mortality [12,13]. The mechanisms of resistance to polymyxin have been associated with intrinsic, transferable, and mutational mechanisms [14]. One of the main mechanisms of resistance in *K. pneumoniae* is *mrgB* gene alteration [12,15].

Currently, where pan-resistant strains represent an emerging threat, research should prioritize developing new antimicrobials and synergistic use of combination therapies [16,17]. In this context, new therapeutic approaches have been considered, including investigating the antimicrobial potential of natural products, especially essential oils (EOs) [18].

Additionally, the investigation of synergistic interactions between EOs, EO constituents, and antibiotics has been recommended to contribute to determining new combinations with antimicrobial potential that can minimize the development of resistance [19,20].

Carvacrol (a phenolic monoterpenoid), the major constituent in the EO of *Origanum vulgare* and *Thymus vulgaris*, possesses antimicrobial properties [21,22]. However, to the best of our knowledge, this is the first study evaluating the *in vitro* and *in vivo* antibacterial effects of synergistic combinations of carvacrol and polymyxin B against polymyxin-resistant strains of *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial isolates

Bacterial strains of *K. pneumoniae* resistant-polymyxin B were obtained from patients admitted to a tertiary hospital in Dourados, Mato Grosso do Sul, Midwest, Brazil. Bacterial species were identified using the Phoenix 100® automated system (BD Diagnostic Systems, Sparks, MD, USA) and confirmed by matrix-assisted desorption time-of-flight mass spectrometry by using Microflex Spectrometer LT (Bruker Daltonics, Massachusetts, USA) as previously described [12]. Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to the Clinical and Laboratory Standards Institute [23]. Genomic DNA was extracted from fresh cultures using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) to investigate molecular events related to polymyxin resistance. Sequencing libraries were prepared using the Nextera library kit (Illumina, San Diego, CA, USA). The prepared libraries were sequenced with 150-bp paired-end reads by using the Illumina MiSeq Platform (Illumina, San Diego, CA, USA), as previously described [12]. The whole-genome sequences described were deposited in the European Nucleotide Archive (Project: PRJEB25746; accession numbers in supplementary Table 1).

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests for carvacrol were performed by a microdilution method using 96-well polystyrene microtiter plates in Mueller Hinton broth [24]. Briefly, cultures were grown overnight at 37 °C with constant agitation at 200 rpm. The next day, optical density was measured at 600 nm, and cultures were adjusted to match the 0.5 McFarland standard (1×10^8 CFU/mL, absorbance is 0.08–0.10 at 600 nm and a 1-cm path). Each microplate well was inoculated with bacterial concentration of 5×10^5 colony-forming units (CFU)/mL. Serial dilutions contained final concentrations from 8 to 0.06 µg/mL for polymyxin B and from 18 to 0.017 mg/mL for carvacrol. The plates were incubated at 37 °C (stationary) for ~18 h. Amikacin (16 mg/L) (Sigma-Aldrich Co., St Louis, MO, USA) was used as control for assays with polymyxin-resistant *K. pneumoniae* strains. *Escherichia coli* strain ATCC 25922 was used for quality control. The experiment was performed in triplicate, and the results were averaged.

Combinatorial assays between carvacrol and polymyxin B

Combinations of carvacrol and polymyxin B against polymyxin-resistant strains of *K. pneumoniae* were evaluated using the checkerboard assay. Double serial dilutions of the oils and antibiotics were prepared in the horizontal and vertical lines using a microtiter plate. Both antimicrobial agents were cross-diluted. The inoculum used was 5×10^5 CFU. The plates were incubated at 37 °C (stationary) for ~18 h. Subsequently, resazurin was used as a cell viability indicator [24]. The experiment was performed in triplicate, and the results were averaged and expressed as the fractional inhibitory concentration index (FICI). Each combination was calculated as the ratio of the MIC of the antimicrobial agent in combination versus the MIC of the antimicrobial agent alone. FICI is calculated using the following formula:

FICI = MIC of polymyxin B in combination/MIC of polymyxin B alone + MIC of carvacrol in combination/MIC of carvacrol alone.

The result interpretations were grouped into synergistic ($\Sigma FICI \leq 0.5$), additive ($0.5 < \Sigma FICI < 1.0$), indifferent ($1.0 < \Sigma FICI \leq 2.0$), and antagonistic effects for $FICI \geq 2$ ($FICI > 4.0$) [9]. The checkboard results were analyzed using the zero-interaction potency model for synergy [25] by using the free and open source SynergyFinder Software available at https://synergyfinder.fimm.fi.

Time–kill kinetics

Bacterial cultures and time–kill kinetics were performed as described for combination and checkerboard experiments, respectively. Aliquots (1 µL) were obtained from each well at 0, 2, 4, 6, and 12 h of incubation, then seeded onto Mueller Hinton agar plates and cultured at 37 °C [26]. Subsequently, the plates were examined for growth. Independent assays were performed in duplicate. Bacterial count values were transformed into CFU/mL and expressed as logs to ensure normal data distribution [27]. Negative controls (water, culture medium, and 0.5% Tween 80) and bacterial cultures (water, culture medium, 0.5% Tween 80, and bacterial suspension) were included. Amikacin was used as a positive standard.

Cell membrane integrity

Bacterial cell membrane integrity was monitored by the release of proteins from the cell to the supernatant after exposure to synergistic combinations based on the checkerboard method. The microplate was incubated at 37 °C for 4 h. The contents of each microplate were centrifuged at 2500 rpm for 5 min at 4 °C. The protein concentration released from the

cytoplasm was assessed in the supernatant using the Pierce™ BCA Protein Assay kit (Thermo Scientific, MA USA). Optical absorbance was read at 595 nm using the iMarkTM Microplate Absorbance Reader (Bio-Rad, São Paulo, SP, Brazil).

Assessment of the antibiofilm activity of carvacrol and polymyxin B compounds

The concentrations of the checkboard test were used to evaluate biofilm formation inhibition. The plates were incubated for 24 h at 37 °C under static conditions to allow bacterial growth and biofilm maturation, as described accordingly (RIBEIRO et al., 2015). A microplate reader was used to measure absorbance up to 595 nm. Biofilm inhibition ratio was calculated in relation to the amount of biofilm grown in the presence of synergism (defined as 100% biofilm) and the sterility control of the medium (defined as 0% biofilm) [28].

Intraperitoneal infection in mice and antimicrobial assay

All *in vivo* experiments used female Swiss mice (*Mus musculus*), aged 8–12 weeks, weighing approximately 20–30 g. The animals were kept in polypropylene cages with controlled temperature (22 ±3 °C), humidity (40%–60%), and light (12 h light–dark cycle), receiving standard commercial feed and water *ad libitum*. We assessed the survival, lethal dose, and longevity of infected animals, so humane endpoints were not used, and ensuring suffering was minimized. To evaluate the antibacterial activity of carvacrol and polymyxin B combinations, a murine infection model induced by polymyxin-resistant *K. pneumoniae* was developed, as previously described [21,26], with the following modifications. In brief, mice were randomly divided into treatment groups (n = 6 animals in each group) with the following regimens: polymyxin B (2 mg/kg, intraperitoneal [i.p.], 12/12 h), carvacrol 10 mg/kg plus polymyxin B at 2 mg/kg (12/12 h), and infected control, untreated, and naïve groups (without infection to assess the baseline indices). Amikacin (7.5 mg/kg every 12 h) was used as positive controls.

All animals infected with polymyxin-resistant *K. pneumoniae* strain (except the naive group) were injected with a 0.2 mL i.p. aliquot of 8.0×10^8 CFU/mL (median lethal dose: LD50). Treatments were performed 1 h after bacterial inoculation. The animals were observed for 24 h, and each group's mortality percentage was calculated. After 24 h of treatment, xylazine and ketamine (10 and 60 mg/kg, i.p., respectively) were injected muscularly to anesthetize the animals. Blood samples were collected (by cardiac puncture) to assess bacterial colonization, and they were plated on MacConkey agar to count CFU.

Statistical analysis

Data were expressed as a percentage and mean ±standard error (SE). One-way analysis of variance was used to assess the differences among the groups. Bacterial counts were transformed to $\log_{10}$ values. The data were analyzed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). P-value of ≤ 0.05 was considered significant.

Ethical standards

The Ethics Committee of the Federal University of Grande Dourados (UFGD) (numbers 877.292/2014 and 4.014.325/2020), the Ethics Committee on the Use of Animals of UFGD (n° 25/18), and Centro Universitário da Grande Dourados (Unigran) (n° 080/18) approved this study. *In vivo* tests were performed according to the norms of the National Council for the Control of Animal Experiments (CONCEA, 2016).

RESULTS

The bacterial strains evaluated were resistant to polymyxin, carbapenem, ceftazidime, and aztreonam, with sensitivity to tigecycline and amikacin (MICs described in Supplementary Table 1). Whole-genome sequencing showed that all isolates had several antimicrobial-resistant genes encoding resistance against beta-lactams, aminoglycosides, fluoroquinolones, tetracyclines, and mutational events related to polymyxin-resistance in the *mgr*B gene. The *in vitro* activity of carvacrol and different antibiotics were estimated in seven polymyxin-resistant *K. pneumoniae* strains. The MICs of carvacrol alone ranged from 140 to 280 µg/mL (Supplementary Table 1). The multilocus sequence typing technique, sequence type (ST) 11, was described as the most common among the samples. The results are summarized in the supplementary material (Supplementary Table 1).

Drug combination assay

In vitro evaluation of 48 polymyxin B/carvacrol combinations (Figure 1) showed that 23 combinations indicated a synergistic action (FICI range: 0.125–0.500) (Table 1). These data indicated that carvacrol MIC 70, 35, or 17.5 µg/mL when combined with decreasing concentrations of polymyxin B MIC (range, 2–0.003 µg/mL), exhibited a synergistic effect *in vitro* to combat strains of *K. pneumoniae* multidrug-resistant (MDR). Additionally, 8, 1, and 16 combinations indicated an additive (FICI range, 0.515–1.000), indifferent (FICI: 1.001), and 16 antagonistic actions (FICI: 2.000–4.02), respectively.

Table 1. Determination of FIC, FIC index and outcome of the interactions of the combination of carvacrol (A) and polymyxin B (B) (μg/ml) against polymyxin-resistant *K. pneumoniae*.

MIC (A) Combination	MIC (B) Combination	FIC (A)	FIC (B)	FICI	Effect	Outcome
562	2	4.0143	0.0156	4.0299	Antagonism	Inhibition
562	1	4.0143	0.0078	4.0221	Antagonism	Inhibition
562	0.5	4.0143	0.0039	4.0182	Antagonism	Inhibition
562	0.25	4.0143	0.0020	4.0162	Antagonism	Inhibition
562	0.125	4.0143	0.0010	4.0153	Antagonism	Inhibition
562	0.06	4.0143	0.0005	4.0148	Antagonism	Inhibition
562	0.003	4.0143	0.0000	4.0143	Antagonism	Inhibition
562	0.001	4.0143	0.0000	4.0143	Antagonism	Inhibition
281	2	2.0071	0.0156	2.0228	Antagonism	Inhibition
281	1	2.0071	0.0078	2.0150	Antagonism	Inhibition
281	0.5	2.0071	0.0039	2.0110	Antagonism	Inhibition
281	0.25	2.0071	0.0020	2.0091	Antagonism	Inhibition
281	0.125	2.0071	0.0010	2.0081	Antagonism	Inhibition
281	0.06	2.0071	0.0005	2.0076	Antagonism	Inhibition
281	0.003	2.0071	0.0000	2.0072	Antagonism	Inhibition
281	0.001	2.0071	0.0000	2.0072	Antagonism	Inhibition
140	2	1	0.0156	1.0156	Indifferent	Inhibition
140	1	1	0.0078	1.0078	Additive	Inhibition
140	0.5	1	0.0039	1.0039	Additive	Inhibition
140	0.25	1	0.0020	1.0020	Additive	Inhibition
140	0.125	1	0.0010	1.0010	Additive	Inhibition
140	0.06	1	0.0005	1.0005	Additive	Inhibition
140	0.003	1	0.0000	1.0000	Additive	Inhibition
140	0.001	1	0.0000	1.0000	Additive	Inhibition
70	2	0.5	0.0156	0.5156	Additive	Inhibition
70	1	0.5	0.0078	0.5078	Synergistic	Inhibition
70	0.5	0.5	0.0039	0.5039	Synergistic	Inhibition
70	0.25	0.5	0.0020	0.5020	Synergistic	Inhibition
70	0.125	0.5	0.0010	0.5010	Synergistic	Inhibition
70	0.06	0.5	0.0005	0.5005	Synergistic	Inhibition
70	0.003	0.5	0.0000	0.5000	Synergistic	Inhibition
70	0.001	0.5	0.0000	0.5000	Synergistic	Inhibition
35	2	0.25	0.0156	0.2656	Synergistic	Inhibition
35	1	0.25	0.0078	0.2578	Synergistic	Inhibition
35	0.5	0.25	0.0039	0.2539	Synergistic	Inhibition
35	0.25	0.25	0.0020	0.2520	Synergistic	Inhibition
35	0.125	0.25	0.0010	0.2510	Synergistic	Inhibition
35	0.06	0.25	0.0005	0.2505	Synergistic	Inhibition
35	0.003	0.25	0.0000	0.2500	Synergistic	Inhibition
35	0.001	0.25	0.0000	0.2500	Synergistic	Growth

17.5	2	0.125	0.0156	0.1406	Synergistic	Inhibition
17.5	1	0.125	0.0078	0.1328	Synergistic	Inhibition
17.5	0.5	0.125	0.0039	0.1289	Synergistic	Inhibition
17.5	0.25	0.125	0.0020	0.1270	Synergistic	Inhibition
17.5	0.125	0.125	0.0010	0.1260	Synergistic	Growth
17.5	0.06	0.125	0.0005	0.1255	Synergistic	Growth
17.5	0.003	0.125	0.0000	0.1250	Synergistic	Growth
17.5	0.001	0.125	0.0000	0.1250	Synergistic	Growth

FIC (A) = MIC of the combination/MIC (A) alone (140 µg/ml); FIC (B) = MIC of the combination/ MIC (B) alone (128 µg/ml) and FICI = FIC (A) + FIC (B). A = Carvacrol; B = Polymyxin B. The FICI was interpreted as follows: (1) a synergistic effect when FICI ≤0.5; (2) an additive effect when FICI >0.5 and 1; (3) a indifferent effect FICI >1.0 and ≤ 2.0 and antagonism as FICI >2. The outcome of the bactericidal effect of the combination were evaluated, such as inhibition (no microbial growth) and growth.

The analysis to verify the dose–response association of inhibition showed that polymyxin B >1 µg/mL increased inhibition (Figure 1.A), whereas carvacrol >200 µg/mL decreased the percentage of inhibition (Figure 1.B).

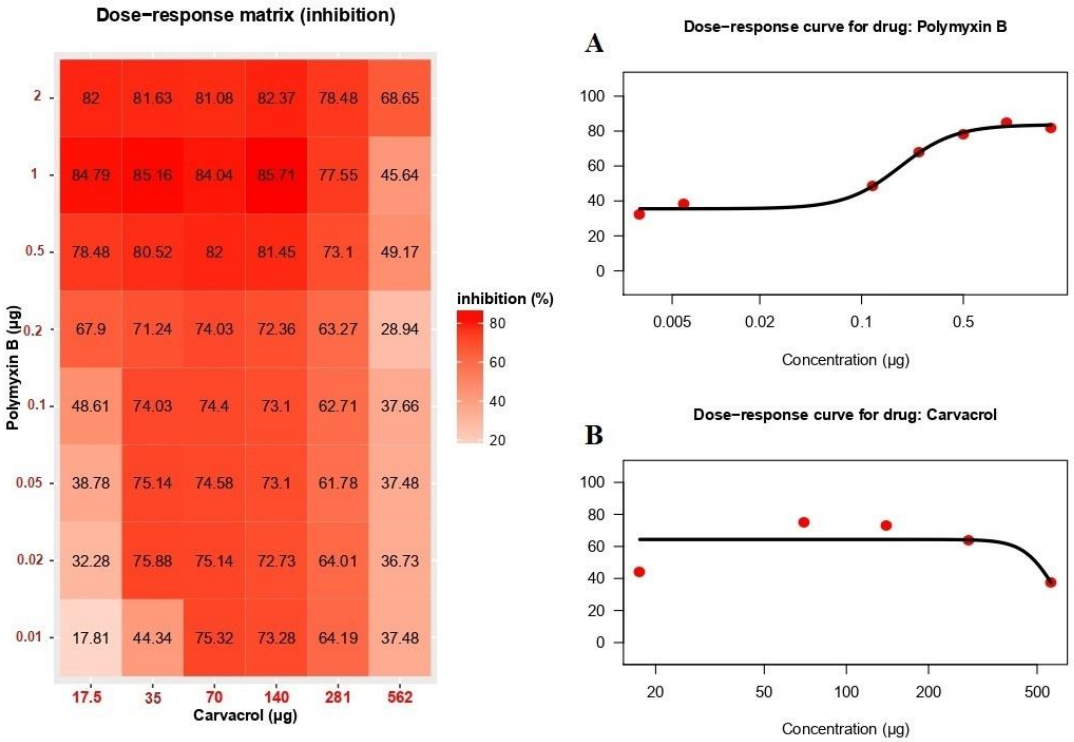

Figure 1. Dose-response matrix representing the percent inhibition percentage of the studied combinations. Inhibition curve for drugs: A) Polymyxin B; B) Carvacrol.

In the landscapes generated by this model (Figures 2A–2B), red and green indicated synergistic and additive interactions, respectively. In this model, synergistic, additive, and antagonistic interactions between drugs had a score of >10, between −10 to 10, and <−10, respectively. The landscapes for the interaction of carvacrol with polymyxin B were red, denoting synergism.

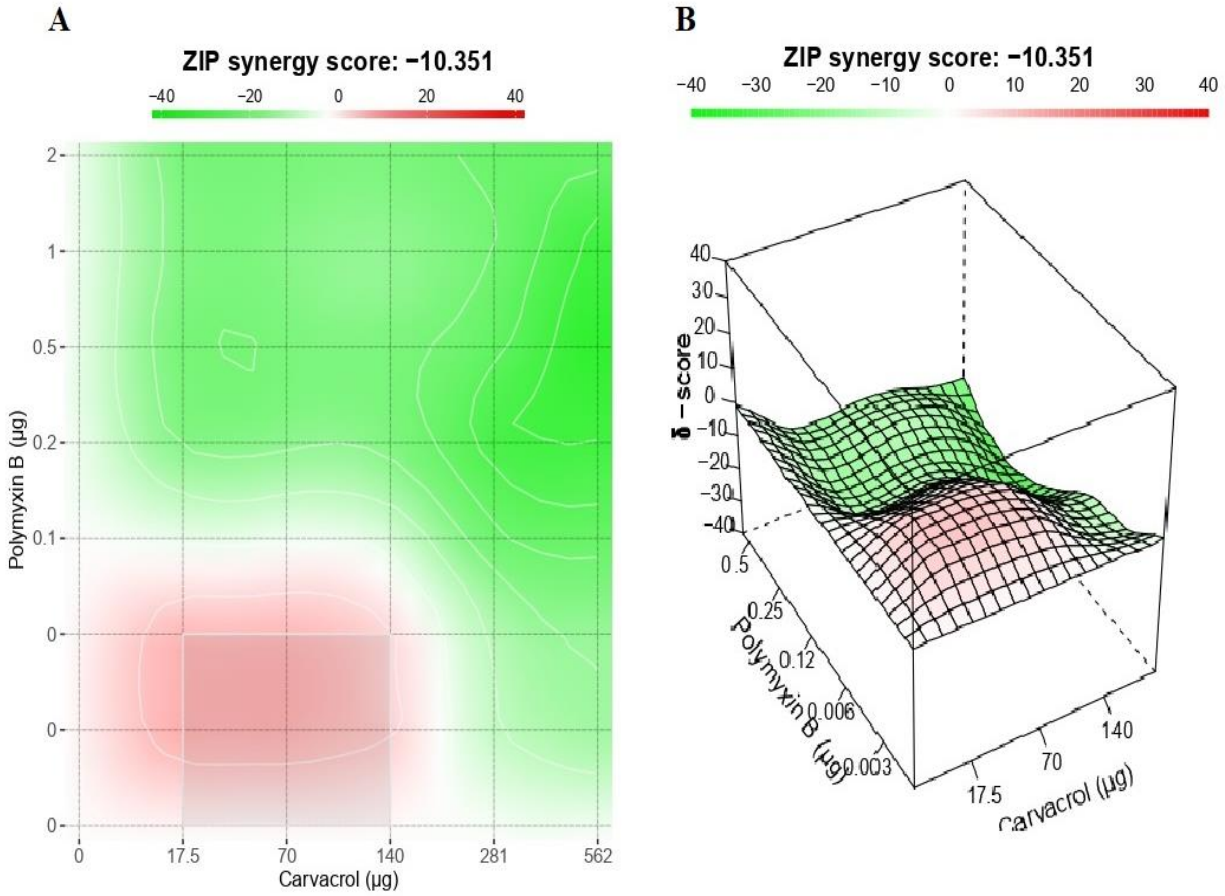

Figure 2. Calculation of synergy scores (A) and synergy maps for dose combinations (B).

Time–kill curves

Kinetic time-to-kill assays assessing synergies showed that carvacrol MIC 70 µg/mL combined with polymyxin B MIC 1–0.001 µg/mL eradicated all bacterial cells within 2 h. Carvacrol MIC 35 µg/mL combined with polymyxin B MIC 2–0.06 µg/mL exhibited an *in vitro* bactericidal effect against polymyxin-resistant *K. pneumoniae*, killing all cells within 2 h after treatment (Figure 3.A). A similar effect was observed in the combinations of carvacrol MIC 17 µg/mL and polymyxin B MIC 2–0.25 µg/mL (Figure 3.B). Therefore, 18 of the 23 synergistic combinations tested had a bactericidal outcome.

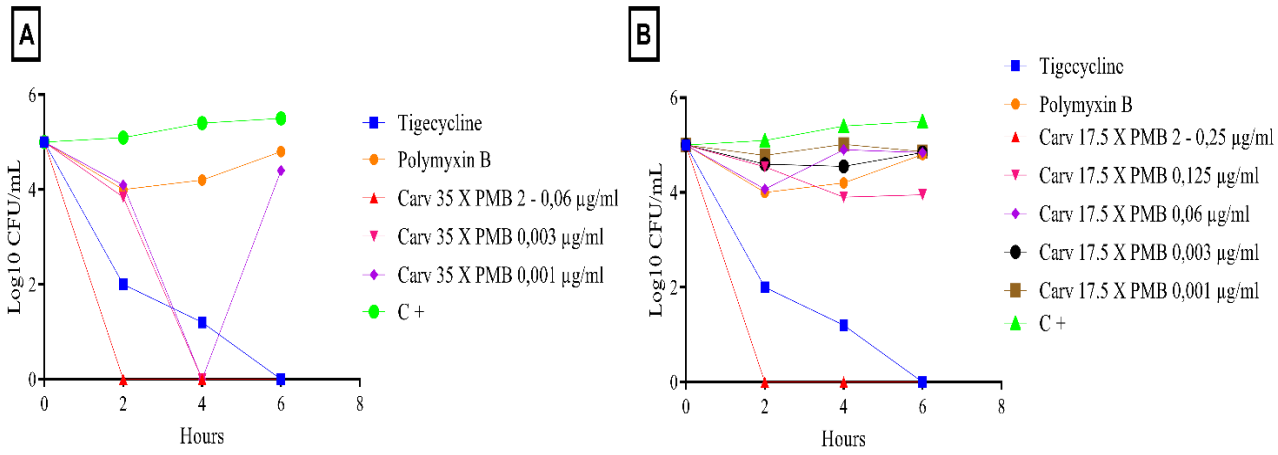

Figure 3. T**ime-kill curves of polymyxin-resistant *K. pneumoniae.*** (A) Carvacrol (Carv) at a concentration of 35 µg/mL combined with decreasing concentrations of polymyxin B (PMB) 2 - 0.001 µg/mL; (B) Carvacrol at a concentration of 17.5 µg/mL combined with decreasing concentrations of polymyxin B 2 - 0.001 µg/mL; C+: positive control (*K. pneumoniae* in Mueller Hinton broth).

The cells externalized the proteins when *K. pneumoniae* was treated with carvacrol plus polymyxin B. Higher doses of carvacrol showed higher levels of protein extravasation (Figure 4).

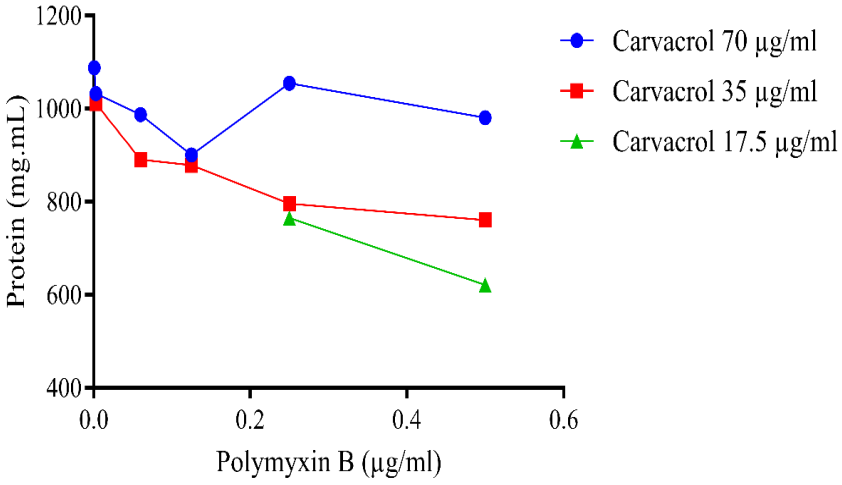

Figure 4. Quantification of *K. pneumoniae* proteins after 2 hours of exposure to synergistic combinations of carvacrol and polymyxin B.

Synergistic combinations of carvacrol 17.5 and 35 µg/mL combined with decreasing concentrations of polymyxin B 2–0.001 µg/mL were evaluated for their potential to inhibit

biofilm formation of polymyxin-resistant *K. pneumoniae*. Inhibitory action was observed in carvacrol 70, 35, and 17.5 µg/mL plus polymyxin B 2–0.25 µg/mL (Figure 5). Therefore, 11 of the 23 synergistic combinations showed antibiofilm action.

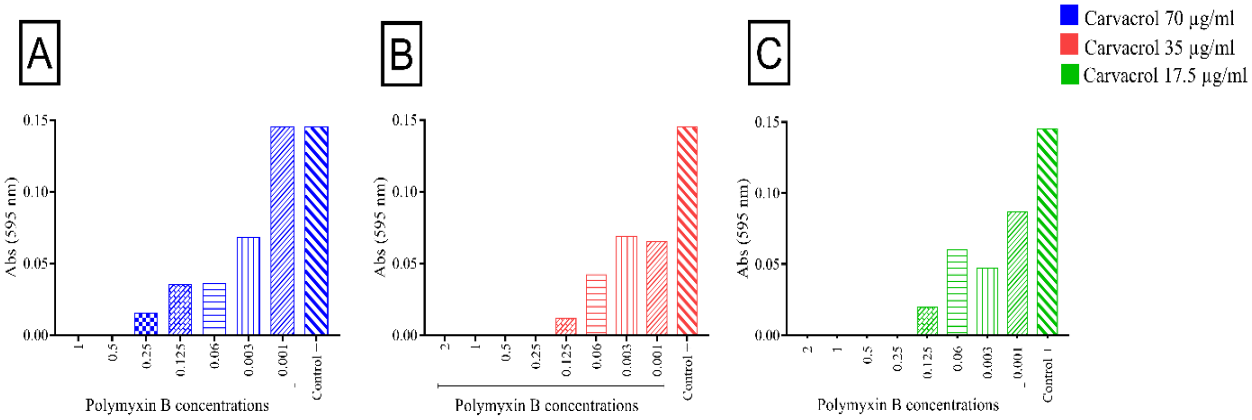

Figure 5. Effects of synergistic combinations on biofilm formation: (A) Carvacrol concentration of 70 µg/mL combined with decreasing concentrations of polymyxin B 1-0.001 µg/mL; (B) Carvacrol concentration of 35 µg/mL combined with decreasing concentrations of polymyxin B 2-0.001 µg/mL; (C) Carvacrol concentration of 17.5 µg/mL combined with decreasing concentrations of polymyxin B 2 - 0.001 µg/mL.

In vivo antibacterial activity

A polymyxin-resistant *K. pneumoniae* (KP10/ST11) infection model was used to evaluate *in vivo* antimicrobial activity for 24 h. All mice were infected with 8.0×10^8 CFU/mL (LD50). The mice were observed for 24 h after infection, and 50% of the bacterial control group (untreated) died. However, all mice treated with carvacrol and polymyxin B, alone or in combinations, remained alive (Figure 6.A).

The number of CFUs in the blood was determined to better characterize the observed difference in mortality rates between the bacterial control and treatment groups. The number of CFUs was significantly different for the following treatments: combination of carvacrol 10 mg/kg plus polymyxin B 2 mg/kg ($p < 0.01$) and amikacin 7.5 mg/kg was used as control ($p < 0.01$) (Figure 6.B).

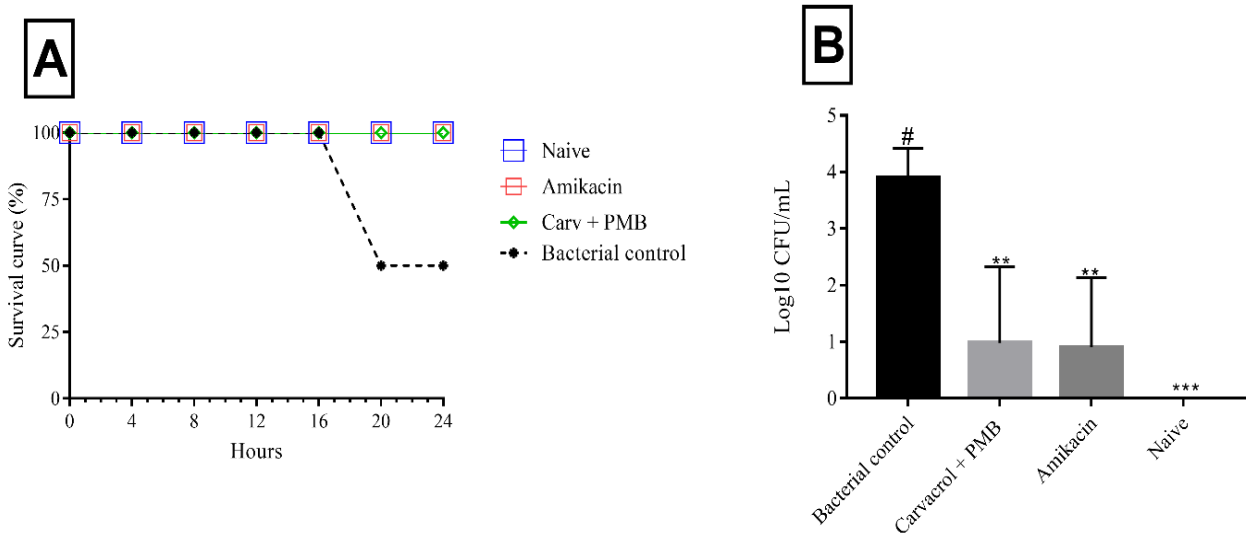

Figure 6. Antimicrobial activity in vivo: A) Survival curves of mice infected with polymyxin-resistant *K. pneumoniae* (8 × 10 8 CFU/mL) treated with amikacin (7.5 mg/kg) and combinations of carvacrol (10 mg/kg) plus polymyxin B (2 mg/kg); B) Effects of treatments on the number of CFUs in the blood. Differences between groups were analyzed by one-way ANOVA followed by Tukey's test. ***p<0.001, **p<0.01 and *p<0.05 in relation to the bacterial control (positive control).

DISCUSSION

Considering the increasing dissemination of polymyxin resistance in *K. pneumoniae*, especially the ST11 clone, this strain poses a potential threat to anti-infective treatment. Thus, developing alternatives to treat these infections is a global necessity [29–31]. Additionally, new antimicrobial approaches have been investigated to reduce the risks of resistance, including identifying bioactive compounds that can act alone or synergistically with antibiotics, producing effective therapeutic options in treating bacterial infections, wherein polymyxin monotherapy is ineffective [24,32,33].

Carvacrol has been emphasized as a bioactive compound due to its antimicrobial potential against a wide range of Gram-negative, Gram-positive, and mycobacteria [21,34,35]. Effective carvacrol–antibiotic combinations should be investigated because they are a potential therapeutic strategy against multidrug-resistant bacteria [35]. We evaluated the synergism of carvacrol plus polymyxin B combinations against polymyxin-resistant *K. pneumoniae* (by the *mgr*B alteration, belonging to the ST11 clone). The MICs of polymyxin B and carvacrol alone were 128 µg/mL and 140 µg/mL, respectively. However, combinations evaluated using the checkerboard method showed that MIC was remarkably reduced for carvacrol 70, 35, or 17.5

297 µg/mL and polymyxin B 2–0.003 µg/mL, of which 23 combinations showed synergistic results
298 (0.125–0.500).

299 Considering the importance of polymyxin, combination therapy has been used as an
300 antimicrobial strategy to circumvent resistance in the treatment of infections, wherein
301 polymyxin monotherapy is ineffective [24,33,36]. Previous studies have also demonstrated the
302 activity of carvacrol alone or in combination with meropenem against carbapenem-resistant *K.*
303 *pneumoniae* [21,22]. However, to the best of our knowledge, this is the first study that
304 described antimicrobial activity of polymyxin-associated carvacrol against polymyxin-resistant
305 bacteria.

306 Other studies have reported the combination of polymyxin B with adjuvant compounds
307 as an alternative in restoring antibiotic efficacy, decreasing MIC against resistant strains, and
308 eliminating microorganisms [24,37–41]. Corroborating our findings, the time–kill curves
309 showed synergistic combinations of carvacrol 70, 35, and 17.5 µg/mL with decreasing
310 polymyxin B 2–0.003 µg/mL concentrations, which exhibited a bactericidal effect against
311 polymyxin-resistant *K. pneumoniae* within 2 h after treatment.

312 Many studies suggested that carvacrol's mechanisms of action occur through
313 compromised cell membrane integrity, leading to extravasation of intracellular content resulting
314 in bacterial death [22,42,43]. In this context, we hypothesized that carvacrol could be involved
315 in bacterial membrane rupture. Thus, quantitative assays of proteins were performed to
316 elucidate the mode of action. We demonstrate that carvacrol's potential mechanism of action
317 occurs through compromised membrane integrity and leakage of cytoplasmic content, including
318 quantified proteins. We also confirmed dose-dependent disruption. Thus, additional studies
319 using transmission electron microscopy are required. Similar findings demonstrated that the
320 cell membrane in carbapenem-resistant *K. pneumoniae* was damaged after exposure to
321 carvacrol plus meropenem combination [22].

322 Additionally, few studies examined the effect of new antimicrobials on biofilm
323 formation, especially against polymyxin-resistant *K. pneumoniae* strains [44,45]. In our study,
324 we investigated whether synergistic combinations of carvacrol and polymyxin B exhibit
325 antibiofilm properties, of 11 combinations completely prevented biofilm formation,
326 constituting a promising approach against biofilms formed by polymyxin-resistant *K.*
327 *pneumoniae*. Carvacrol monotherapy showed antibiofilm action against strains of
328 *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella Typhimurium*, including
329 Gram-negative carbapenem producers [46–48]. However, additional studies should be

conducted to evaluate the mechanisms by which the compound acts on the biofilm and the use of these combinations in topical treatment [49,50].

We used a mouse model of peritonitis to assess the effect of carvacrol 10 mg/kg plus polymyxin B 2 mg/kg on polymyxin-resistant *K. pneumoniae*. The carvacrol and polymyxin B combination effectively reduced the number of CFUs in the blood and increased the survival of infected mice compared with the control (untreated) group, indicating a potential strategy for this combination. Further, additional studies should be conducted confirming that this combination is an alternative to the treatment of intractable polymyxin-resistant Gram-negative infections.

Our study has some limitations. We evaluated only one strain. Despite similar MICs, differences between strains could affect the results. In addition, a pharmacokinetic evaluation of the combination of carvacrol and polymyxin B in mice was not performed, which could help understand their mechanism of action.

CONCLUSIONS

The findings were promising and of great clinical importance. We demonstrate a synergistic combination of a safe bioactive compound associated with a commercial antibiotic that is effective in the *in vitro* eradication of planktonic cells and biofilm formation. Furthermore, we demonstrate the antimicrobial potential of carvacrol and polymyxin B combination in *in vivo* infection caused by polymyxin-resistant *K. pneumoniae.* In summary, our data suggest that carvacrol plus polymyxin B acts synergistically, showing potential to be explored by the pharmaceutical industry.

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Conflict of interest

The authors declare that they have no competing interests.

Supplementary material

Supplementary Table 1. Molecular characterization and susceptibility profile of polymyxin-resistant *K. pneumoniae* strains.

	Molecular characterization			MIC (mg/L)							
***K. pneumoniae* strains**	Relevant genotype	*mgrB* status	Run Accession in ENA	CARV	PXB	CTZ	AZT	IMI/ MEM	ERT	AMK	TGC
KP-RP03	ST11	*mgrB* repeated sequence at nt 89	*ERR2743730*	0,140	*32*	>254	>32	>16	>32	<64	<0.5
KP-RP05	ST11	Insertional inactivation, IS*Ecp1*at nt 124	ERR2743731	0,140	16	>254	>32	>16	>32	<64	<0.5
KP-RP10*	**ST11**	*mgrB* repeated sequence at nt 89	*ERR2743734*	0,140	*32*	>254	>32	>16	>32	<64	<0.5
KP-RP12	ST345	Insertional inactivation, IS*Kpn13* at nt 75	ERR2743736	0,140	16	>254	>32	>16	>32	<64	<0.5
KP-RP20	ST11	Insertional inactivation, IS*Kpn18* at nt 122	ERR2743738	0,140	32	>254	>32	>16	>32	<64	<0.5
KP-RP25	ST11	Insertional inactivation, IS903at nt 89	ERR2743739	0,280	16	>254	>32	>16	>32	<64	<0.5
KP-RP29	ST11	Insertional inactivation, IS5-like element at nt 89	ERR2743743	0,280	16	>254	>32	>16	>32	<64	<0.5

Abbreviations: KP: *K. pneumoniae*; ST: sequence typing; ENA: European Nucleotide Archive; **CARV**–Carvacrol; **PR**–polymyxin-resistant, **CTZ**–ceftazidime; **AZT**– aztreonam; **IM/ME**– imipenem and meropenem; **ERT**– ertapenem; **AMK**–amikacin; **PXB** – Polymyxin B; **TGC** – Tigecycline. *Strain used in synergistic *in vitro* and *in vivo* experiments

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REVIEW

Antimicrobial peptides against polymyxin-resistant *Klebsiella pneumoniae*: a patent review

Gleyce Hellen de Almeida de Souza[1] · Luana Rossato[1] · Alexandre Ribeiro de Oliveira[1] · Simone Simionatto[1]

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Abstract
The spread of polymyxin-resistant *Klebsiella pneumoniae* strains represents an emerging health challenge, limiting treatment options for the patients. Thus, the development of new antimicrobials is an urgent requirement. Antimicrobial peptides (AMPs) are a large class of compounds that are part of innate immune response; these peptides are promising compounds in the field of antimicrobial resistance and are present in all organisms. The present review evaluated patents on antimicrobial peptides tested against polymyxin-resistant *K. pneumoniae*, available on Espacenet as of September 2022. A total of 1313 patents were examined and 1197 excluded as they were out of focus for this review; 104 patents of peptides tested against *K. pneumoniae* were included; of which only 14 were tested against polymyxin-resistant *K. pneumoniae* strains. The results indicated that all AMPs evaluated were in the experimental or pre-clinical phase; the clinical phase is pending. Furthermore, a few peptides were tested effectively against polymyxin-resistant *K. pneumoniae*. Although, the research and patent filing alone are not enough to develop a suitable antimicrobial therapy, they can represent good starting point upon which to develop new antimicrobials. More investment is required to push these pharmaceuticals through the stages of development to introduce them into the market.

Keywords Gram-negative bacteria · Multidrug-resistant · Antimicrobial therapies

Introduction

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen that causes multidrug-resistant (MDR) infections (including pneumonia, urinary tract infections, and bloodstream infections) (Martin and Bachman 2018; Wang et al. 2020). In the last decade, it has emerged as a global health threat (Wyres et al. 2020; De Oliveira et al. 2020). This microorganism has an exceptional ability to develop and acquire genetic elements that encode resistance to multiple antibiotics, including carbapenems (Navon-Venezia et al. 2017; Yang et al. 2021).

Carbapenems are often used to treat MDR infections (Hansen 2021). However, carbapenem-resistant infections are difficult to treat because of the lack of effective and safe options (Soman et al. 2021). In these circumstances, polymyxins are antimicrobials of last resort for the treatment of infections caused by carbapenem-resistant *K. pneumoniae* (Rojas et al. 2016). Polymyxins were widely used in hospitals to treat infections, resulting in the current scenario of polymyxin-resistant strains (Zhang et al. 2021b). Resistance to polymyxin B, an antibiotic of last resort in the treatment of serious bacterial infections, leads to the failure of antibiotic treatment (Li et al. 2022).

Infections caused by polymyxin-resistant *K. pneumoniae* are associated with high mortality rates of around 60% (Da Silva et al. 2020). The main resistance mechanisms in polymyxin-resistant *K. pneumoniae* are mutations in *mgrB*, *phoPQ*, *pmrAB,* and *crrAB* and the presence of the *mcr* gene plasmid (De La Cadena et al. 2021). Therefore, limited therapeutic options are available to treat infections caused by polymyxin-resistant *K. pneumoniae* (Levin and Oliveira 2008). In this context, the introduction of conventional antimicrobial therapies often results in increased resistance (Nainu et al. 2021). In recent years, few new antibiotics have been approved, thereby compounding the scenario of antibiotic resistance (Shi et al. 2021). Therefore, initiatives for

✉ Simone Simionatto
simonesimionatto@ufgd.edu.br

[1] Laboratório de Pesquisa em Ciências da Saúde, Universidade Federal da Grande Dourados, Rodovia Dourados - Itahum, km 12, Cidade Universitária, Dourados, Mato Grosso do Sul 79804970, Brazil

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the development of new therapeutic alternatives are urgently required to control MDR *K. pneumoniae* (Felício et al. 2021; You et al. 2021).

Antimicrobial peptides (AMPs) are promising candidates for controlling infections caused by MDR microorganisms (Bin Hafeez et al. 2021). They are composed of 10–50 amino acids, are present in different organisms, and act against several pathogens, such as bacteria, viruses, fungi, and parasites (Rodríguez et al. 2021; Deshayes et al. 2022). AMPs have attracted interest as novel therapeutic agents because they exhibit potent and broad-spectrum antibiotic activities with a different mechanism of action from traditional antibiotics. Cationic AMPs interact and penetrate bacterial cell membranes, leading to bacterial death (Shi et al. 2021). In addition, AMPs have several mechanisms of action, which target intracellular action, acting on nucleic acids, cell wall synthesis, protein synthesis, and enzymatic activity, among others (Zhang et al. 2021a). In addition, they exhibit a broad spectrum of antibacterial, antifungal, and antiviral activities. Chemical structure optimization to create more effective synthetic peptides represents a promising strategy for the development of AMPs as a new drug class to prevent and treat systemic and topic infections (Cardoso et al. 2020). Additionally, they exhibit a reduced propensity to induce drug resistance when compared to conventional bacterial antibiotics (Wang et al. 2016). However, despite representing a promising therapy, it presents challenges for applications, which include potential toxicity in humans, poor specificity and costly manufacturing (Bahar and Ren 2013).

Several studies have described different antimicrobial peptides with distinct spectra of activity and mechanisms of action. But how many of these peptides have been patented for commercial use? How many peptides were tested against polymyxin-resistant *K. pneumoniae*? Patent content analysis is a vital tool to understand trends in the development of new drugs and explore the most promising target that can be produced commercially (Han 2007; Serafini et al. 2021). Therefore, the present study performed a patent review analysis to identify and explore innovation trends, therapeutic strategies, and the latest antimicrobial peptides for the treatment of polymyxin-resistant *K. pneumoniae* infections.

Search strategy

The flowchart of the present review was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al. 2021). The search for patents in the specialized online database: Espacenet from the European Patent Office (EPO), as described (Serafini et al. 2021) was performed with modifications. The present review evaluated relevant patents deposited until September 2022. This review did not include a meta-analysis, risk of bias assessment, assessment of causes of heterogeneity, or assessments of certainty (or confidence) or robustness. First, keywords were selected using the MeSH (Medical Subject Headings) for indexing articles for PubMed. The title, abstract, and full text of the articles were searched using a combination of "peptide", "*Klebsiella pneumoniae,*" and "resistance polymyxin." The PubMed literature database was searched using the same keywords to compare the number of articles with the number of patents identified from the patent databases.

Study selection

Two independent reviewers (GHAS and LR) screened the search results obtained from patent databases and PubMed for inclusion. Full text of the qualified patents and articles were screened by the same two independent reviewers. A third reviewer (SS) was called in to resolve any differences of opinion in order to form a consensus. Inclusion criteria were as follows: (1) studies with antimicrobial peptides tested against *K. pneumoniae*. The exclusion criteria were as follows: (1) Patents containing peptides that were not tested against *Klebsiella pneumoniae*; (2) duplicate and unavailable patents.

The search results (via Espacenet), containing the country, year, applicant type, mechanism of action, and International Patent Classification (IPC) code, were exported to a Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Results

A total of 1313 patents were identified for the preliminary evaluation of the database (Fig. 1). Of these, 1197 patents were excluded because they did not fit the scope or did not contain descriptive data. Moreover, we identified 116 patents for peptides tested against *K. pneumoniae*, of which 12 patents were excluded due to duplication. Out of those remaining 104, 14 eligible patents were included in this review. We emphasize that using the same keywords in the "Advanced search" option returned only 3 patents, of which two were in Chinese (no translation) and the other patent contained peptides that were not tested against polymyxin-resistant *K. pneumoniae*. Therefore, this search option is limiting as it could not search for patents within the scope. Because of this reason, the general search method was used.

The article search identified 1055 publications from 1990 to 2022. Of these, books, documents, meta-analyses, reviews, and systematic reviews (n = 85) were excluded, leaving 970 publications. Finally, to identify trials in the clinical phase that were being conducted against MDR *K.*

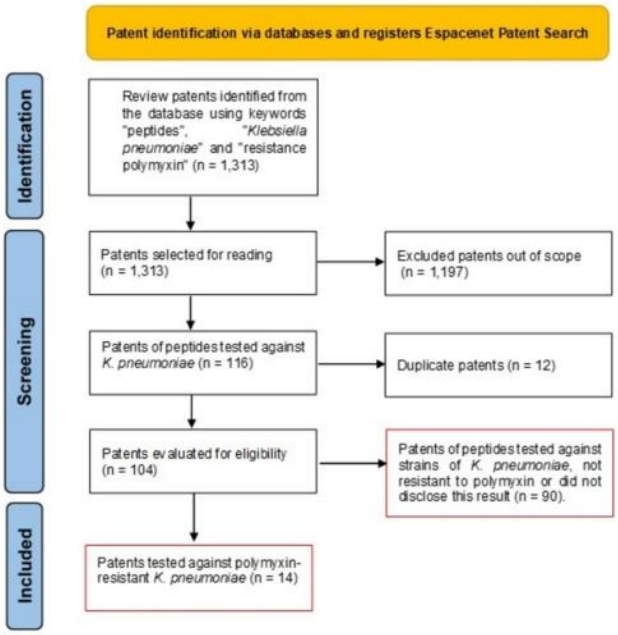

Fig. 1 PRISMA flow diagram, describing the selection and screening process

pneumoniae, a search was performed through the Clinical Trials (https://clinicaltrials.gov/) registry database (search terms: peptide | Infection, Bacterial); the search yielded no such trials.

In the last hundred years, more than 80 peptide drugs (for osteoporosis, multiple sclerosis, diabetes, cancer, human immunodeficiency virus infection, and chronic pain) have been introduced into the pharmacological market (Muttenthaler et al. 2021). Thus, patent analysis becomes essential to determine the generated innovations, predict new technological developments, and evaluate the progress of these innovations (Serafini et al. 2021). We identified an information gap regarding the development of peptides against MDR *K. pneumoniae*. Therefore, by analyzing patent filings, we can expose the main trends in the development of AMPs, including countries, filing years, and the action spectrum of peptides against several microorganisms (sensitive, MDR, and polymyxin-resistant).

Trends in the discovery of AMPs against polymyxin-resistant *K. pneumoniae*

Results indicated that patents were filed for AMPs against *K. pneumoniae* from 1993. In the last 30 years (1993–2022), 90 patents have been filed. The distribution of the number of patent deposits revealed both upward and downward trends over the years (Fig. 2A). However, since 2019, patent deposits have remained high, reaching a peak in 2021 with 14 patent deposits, the highest number ever registered since 1993. However, of these deposits, only 14 were peptide patents (2010–2022) tested against polymyxin-resistant *K. pneumoniae* (Fig. 2B). Between 2016 (n = 1) and 2017 (n = 7), the number of patent deposits increased. This may be due to the increase in the reporting of polymyxin-resistant isolates and the description of the first plasmid-mediated resistance mechanism against polymyxins (*mcr-1*) in China (Liu et al. 2016). Thus, as bacteria continue to develop resistance, initiatives are being established to develop new antibacterial agents (Stephens et al. 2020).

External funding for performing high-tech research leading to publications and patents is an increasingly prominent desire in the scientific community (Krauss and Kuttenkeuler 2021). However, the total number of patent publications was lower compared with the number of scientific articles published in the same period (Fig. 2C). Furthermore, since 2019, this number has decreased, possibly due to the global closing as a result of the COVID-19 pandemic.

Universities and companies leading the discovery of AMPs against polymyxin-resistant *K. pneumoniae*

The requirement to develop new antimicrobial drugs that are effective against MDR pathogens has spurred the research community to invest in various drug discovery strategies. (Farha and Brown 2019). Universities and industrial companies around the world claim the majority of patents on AMPs (Fig. 3) against polymyxin-resistant *K. pneumoniae.* Notably, the number of scientific publications does not follow the number of patent applications (Fig. 2A–C). This may be because the results of the articles do not qualify for the patentability criteria (such as industrial application, novelty, and inventive step) or the patent applications take a long time to be processed.

China and the United States lead the patent ranking in the discovery of AMPs against polymyxin-resistant *K. pneumoniae*

AMPs are promising compounds to address antimicrobial resistance, thereby representing a starting point for the development of new antimicrobials (Annunziato and Costantino 2020). For this reason, some countries have focused their research on this area. The United States (US) represents the country with the highest number of inventions, followed by China (Fig. 4A). This data corroborates the Worldwide Intellectual Property Office (WIPO) https://www.wipo.int/ipstats/en/statistics/country_profile indicators, where China and the US lead the ranking of patents in 2020, with 1,441,085 and 495,883 patents, respectively. This result is likely due to the US's stable economy and investment in technology and intellectual property (Serafini et al. 2021).

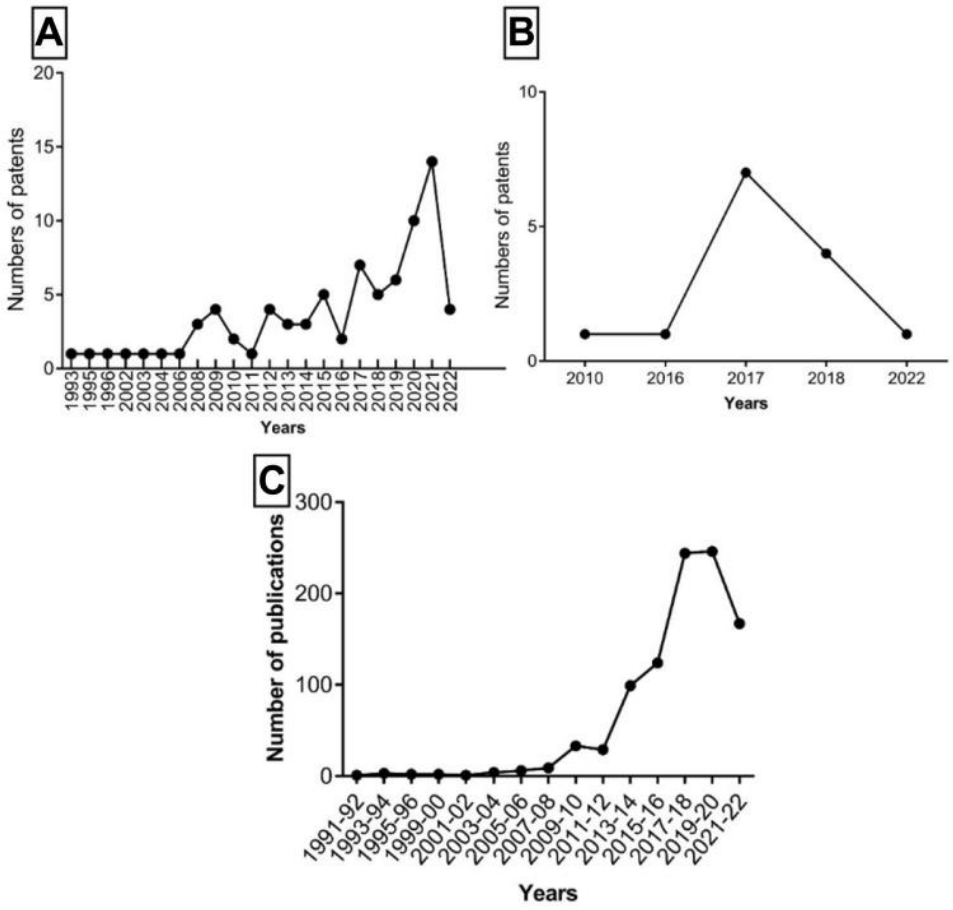

Fig. 2 Frequency per year. **A** Patents of antimicrobial peptides against *K. pneumoniae*; **B** Patents of polymyxin-resistant *K. pneumoniae* antimicrobial peptides among the years 2010–2022; **C** Scientific articles published in the PubMed, using the terms "peptide", "*Klebsiella pneumoniae*", "resistance polymyxin", database by year from 1990 to o October 10, 2022 (books, meta-analysis, review and systematic review, were not considered)

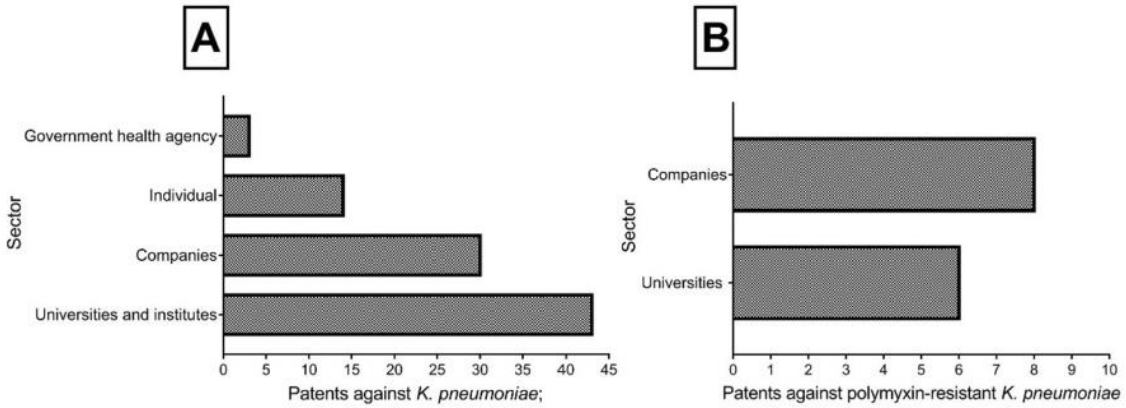

Fig. 3 Number of patents publications per sector. **A** Antimicrobial peptides against *K. pneumoniae*; **B** Polymyxin-resistant *K. pneumoniae* antimicrobial peptides

Classification of AMP patents against polymyxin-resistant *K. pneumoniae*

The IPC code is a universal application classification system that is administered by WIPO; it classifies patents according to technical fields to establish an orderly tool for searching patent documents (World Intellectual Property 2022). Most AMP patents against *K. pneumoniae* were classified in category A (human needs [n = 80]; highlighting: A61K [53], A01N [16], A61P [8]) or in category C (chemistry [n = 10]; especially C07K [8]; Fig. 5A). Among

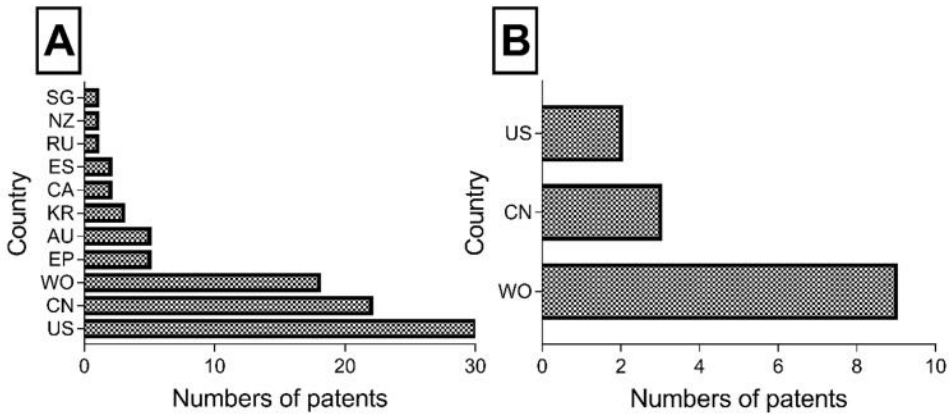

Fig. 4 Number of patent publications by country. **A** Antimicrobial peptides against *K. pneumoniae*; **B** Polymyxin-resistant *K. pneumoniae* antimicrobial peptides. *SG* Singapore, *NZ* New Zealand, *RU* Russian Federation, *ES* Spain, *CA* Canada, *KR* Korea, *AU* Australia, *EP* European Patent Office (EPO), *WO* World Intellectual Property Organization (WIPO), *CN* China, *US* United States of America

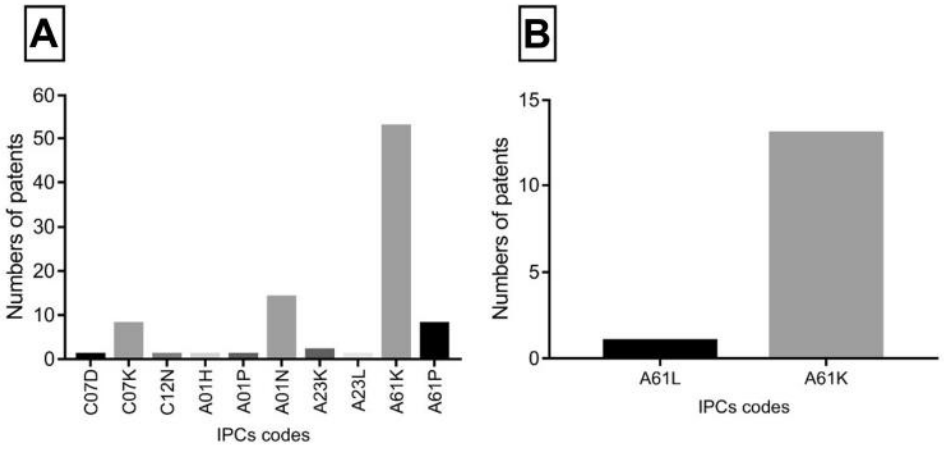

Fig. 5 Patents per International Patent Classification (IPC). **A** Antimicrobial peptides against *K. pneumoniae*; **B** Polymyxin-resistant *K. pneumoniae* antimicrobial peptides. C12N: microorganisms or enzymes; compositions there of; propagating, preserving, or maintaining microorganisms; mutation or genetic engineering; culture media; C07K: peptides; A01H: new plants or processes for obtaining them; plant reproduction by tissue culture techniques; A01P: biocidal, pest repellent, pest attractant, or plant growth regulatory activity of chemical compounds or preparations; A01N: preservation of bodies of humans or animals or plants or parts thereof; A23: foods or foodstuffs; treatment thereof, not covered by other classes; A61L: methods or apparatus for sterilizing materials or objects in general; disinfection, sterilization, or deodorization of air; chemical aspects of bandages, dressings, absorbent pads, or surgical articles; materials for bandages, dressings, absorbent pads, or surgical articles; A61K: preparations for medical, dental, or toilet purposes; A61P: specific therapeutic activity of chemical compounds or medicinal preparations

AMP patents against polymyxin-resistant *K. pneumoniae*, 92.8% were categorized as A61K (Fig. 5B).

Effective peptides against polymyxin-resistant *K. pneumoniae*

After reviewing data on AMPs against *K. pneumoniae* (n = 90), patents tested against polymyxin-resistant strains (n = 14) were selected for a detailed review. We hereby summarize key trends in peptide drug discovery and development by including data on sources, structures, modes of action, and the resistance profile of the tested microorganisms (Table 1).

Discussion

In this manuscript, we performed a critical patent review on advances in the development of antimicrobial peptides against polymyxin-resistant *K. pneumoniae*. The patent application with the registration of "WO2010130007A1" (Li et al. 2010) refers to synthetic peptide antibiotics and polymyxin analog compounds that are effective against Gram-negative bacteria, including polymyxin-sensitive and MDR-resistant Gram-negative bacteria (colistin MICs > 128 mg/L). Specifically, for polymyxin-resistant *K. pneumoniae* the in vitro results demonstrated that the compound caused bacterial cell death. No toxicity and

Table 1 Final selection of antimicrobial peptide patents that test against polymyxin-resistant *K. pneumoniae*

Publication number/status	Title	Country/year	Applicants	Microrganisms	IPC
WO2010130007A1/ Application filed	Antimicrobial compounds (Li et al. 2010)	WO/2010	UNIV MONASH [AU];	*K. pneumoniae* (polymyxin-resistant)	A61K38/12; A61P31/04; C07K7/62
CN108467424A CN108467424B / Active	Linear antibacterial oligopeptide SLAP-S25 and application thereof (Kui et al. 2018)	CN/2019	UNIV CHINA AGRICULTURAL [CN]	*K. pneumoniae* (gene *mcr*-1, *mcr*-6)	A61K38/08; A61P31/04; C07K7/06
WO2020014642A2 WO2020014642A3/ Application filed	Antibacterial peptide monomers and combinations for co-therapy (Kraus and Otvos 2020a)	WO/2020	ARREVUS INC [US]	*K. pneumoniae* (polymyxin-resistant)	A61K39/02; A61K31/40; A61K31/56; A61K31/58; A61K31/675; A61K38/04; A61K38/10
US2020323950A1/ Abandoned	Antibacterial peptides and combinations for co-therapy (Kraus and Otvos 2020b)	US/2020	ARREVUS INC [US]	*K. pneumoniae* (polymyxin-resistant)	A61K38/12; A61K38/16; A61K45/06; A61P31/04
WO2019200378A1/ Application filed	Bactericidal peptides and uses thereof (Kao et al. 2019)	WO/2019	UNIV INDIANA TRUSTEES [US]	*K. pneumoniae* (polymyxin-resistant)	A61K38/00; C07K1/107; C07K14/00
WO2017172929A1/ Application filed	Bactericidal peptides and uses thereof (Kao 2017)	WO/2017	UNIV INDIANA RES & TECH CORP [US]	*K. pneumoniae* (polymyxin-resistant)	A61K38/17; A61P37/04; C07K14/435
WO2020014501A1 WO2020014501A8/ Application filed	Compositions and methods for the treatment of bacterial infections (Balkovec et al. 2020)	WO/2020	CIDARA THERAPEUTICS INC [US]	*K. pneumoniae* (mutation in *pho*Q)	A61K38/04; A61K38/12; C07K7/62
WO2019126353A2 WO2019126353A3/ Application filed	Compositions and methods for the treatment of bacterial infections (Akers-Rodriguez et al. 2019)	WO/2019	CIDARA THERAPEUTICS INC [US]	*K. pneumoniae* (mutation in *pho*Q)	A61K47/64; A61K38/04; A61K38/12; A61K38/14
WO2019028463A1/ Application filed	Linear lipopeptide paenipeptins and methods of using the same (Huang et al. 2019)	WO/2019	BIOVENTURES LLC [US]	*K. pneumoniae* (polymyxin-resistant)	A61K35/66; A61K38/14; A61P31/04; C07K14/47; C07K7/06
WO2019084628A1/ Application filed	Peptide antibiotics (Cooper et al. 2019)	WO/2019	UNIV QUEENSLAND [AU]	*K. pneumoniae* (polymyxin-resistant)	A61K38/00; A61P31/04; C07K7/62
CN110582507A/ Pending	Engineered antimicrobial amphiphilic peptides and methods of use (Steckback 2019)	CN/2019	PEPTIDE LOGIC [US]	*K. pneumoniae* (polymyxin-resistant)	A61K38/00; A61L27/22; A61P31/04; A61P31/10; A61P31/12; A61P35/00; C07K14/00; C07K7/08
US2020207821A1/ Active	Stabilized anti-microbial peptides for the treatment of antibiotic-resistant bacterial infections (Walensky and Mourtada 2020)	US/2020	DANA FARBER CANCER INST INC [US]	*K. pneumoniae* (polymyxin-resistant)	A61L27/22; A61P31/04; C07K1/113; C07K14/46; C12Q1/18; C07K5/11
WO2022173981A1/ Application filed	Intravenous administration of engineered antimicrobial amphiphilic peptides (Steckbeck et al. 2022)	WO/2022	PEPTIDE LOGIC [US]	*K. pneumoniae* (polymyxin-resistant)	A61K38/17; A61P31/04; A61P31/12; A61P35/00; C07K14/47
CN111386283A/ Pending	Peptide antibiotic complexes and methods of use thereof (Smith et al. 2020)	CN/2020	GENENTECH INC [CN]	*K. pneumoniae* (CDC 0106)	A61K38/07; A61K38/55; C07K14/81; C07K5/02; C07K5/10; C07K5/11

no adverse effects were observed in vivo using rat and mice models. The in vivo results using the mouse lung infection model corroborate the in vitro results, confirming the antibacterial activity. The compounds were subjected to antibacterial activity measurements against MDR strains, including polymyxin-resistant *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *K. pneumoniae*. The minimum bactericidal concentration for compound 1 was 10.3 mg/L. Moreover, compounds 2–5 caused bacterial cell death. In contrast, colistin was inactive (even at 128 mg/L) against the resistant strain. A neutropenic mouse lung infection model demonstrated the superior in vivo efficacy of compound 3 compared with that of colistin ($p < 0.045$).

"CN108467424A" (Kui et al. 2018) refers to the field of biotechnology, in particular, to the linear antibacterial oligopeptide, designated SLAP-S25. Short linear antibacterial peptides (SLAP) have the advantages of simple structure, low production cost, high safety, and superior application prospects. Assays evaluating the antimicrobial effect against Gram-negative bacteria demonstrated that the SLAP-S25 oligopeptide has a minimum inhibitory concentration (MIC) of 0.5–32 μg/mL (including against resistant bacteria). Researchers evaluated the synergistic effect of SLAP-S25 and antibacterial drugs (tetracycline, vancomycin, ofloxacin, ampicillin, imipenem, rifampicin, or polymyxin) by the checkerboard method against polymyxin-resistant *K. pneumoniae* (*mcr*-1, *mcr*-6) and demonstrated a strong synergistic effect ($\Sigma FIC < 0.5$) for rifampicin (0.065), ofloxacin (0.127), and tetracycline (0.129). The synergistic treatment effectively reduced the number of colonies in the thighs of mice compared with that of the polymyxin-treated group. In the bacteremia experiment in mice, treatment with SLAP-S25 increased the survival rate, and no hemolytic activity was detected. Therefore, SLAP-S25 demonstrated antimicrobial activity in vivo and in vitro against resistant *K. pneumoniae*. When evaluating the interaction between SLAP-S25 and lipopolysaccharides (LPSs), the researchers identified that SLAP-S25 acted in a similar way to LPS-targeted antibiotics (Song et al. 2020).

Similarly, "US2020323950A1" and "WO2020014642A2" (Kraus and Otvos 2020a, b) patent applications are about combinations of antibacterial peptide monomers and analogs derived from antibiotics; the applications also include methods of using the combination to increase the sensitivity of antibiotic-resistant bacteria to an antibiotic, thereby broadening the therapeutic index of the AMP.

Proline-rich peptide dimers, such as A3-APO, exhibit antibacterial properties, and their potential increases when used in combination with antibiotics. Thus, "US2020323950A1" (Kraus and Otvos 2020b) patent application comprises 8 peptides with a composition of A3-APO analogs, derivatives or oligomers there of, or a pharmaceutically acceptable salt and an antibiotic. The activity of the peptide against MDR *K. pneumoniae* K97/09 (resistant to ceftazidime, ceftriaxone, imipenem, meropenem, ciprofloxacin, gentamicin, and colistin) was determined in vitro. The MIC value of A3-APO alone against polymyxin-resistant *K. pneumoniae* was 32 mg/L; A3-APO exhibited synergism when combined with colistin ($FIC = 0.08$), and an additive activity for the A3-APO/imipenem combination ($FIC = 0.53$) was also observed. In vivo, the combination of A3-APO with colistin was tested in a mouse model with systemic *K. pneumoniae* infection. In the same model, the combination of A3-APO significantly reduced colistin doses and prolonged their survival. A3-APO monotherapy at 0.5 mg/kg or 1.0 mg/kg resulted in a 20%–40% prolonged survival and reduction in blood bacterial counts.

The monomer peptides described herein, when administered with other antibiotics, are highly effective against bacterial infections, either alone or in combination with other antibiotics. The patent application "WO2020014642A2"(Kraus and Otvos 2020a) comprises monomer peptides, such as Chexl-Arg20 (commercially being developed as ARV-1502) or its dimeric form, A3-APO. The activity of A3-APO against polymyxin-resistant *K. pneumoniae* (K97/09) was determined in vitro, and an MIC value of 32 mg/L was obtained. The mechanism of action of A3-APO or ARV-1502 is non-membrane disruptive and is mostly related to the activation of host defense mechanisms rather than direct bacterial killing (Ostorhazi et al. 2011; Otvos Jr. et al. 2018).

Likewise, "WO2019200378A1" and "WO2017172929A1"(Kao 2017; Kao et al. 2019) patent applications are about peptide compositions with bactericidal activities; they describe a method of treating bacterial infections using compositions of antimicrobial peptides or variants. Thus, "WO2019200378A1"(Kao et al. 2019) provides antimicrobial peptide compositions that are selected from the peptide group consisting of SEQ ID NOs: 1–11. The peptide named B22a was tested against 20 strains of Gram-negative bacteria by using the broth dilution assay to determine the MIC; results demonstrated that it has antimicrobial activity against members of the *Enterobacteriaceae*. B22a resulted in only a two-fold increase in MIC value (8 μM) when tested against polymyxin-resistant *K. pneumoniae*. Thus, B22a inhibits the growth of MDR clinical isolates, including polymyxin-resistant ones. Additionally, the peptide named PB22N (MIC: 8 μM) was also effective in inhibiting polymyxin-resistant clinical isolates of *K. pneumoniae*.

This invention "WO2017172929A1" (Kao 2017) identifies compositions for antimicrobial activities that are selected from the group of peptides (cathelicidin family) consisting of SEQ ID NOs: 11–13 (BMAP-27A, BMAP-27B, and BMAP-27C), SEQ ID NOs: 15–17 (SMAP-29B,

SMAP-29C, and SMAP-29D), and SEQ ID NOs. 18–19 (BMAP-24 and B22); a total of 8 peptides. Of these, only 2 peptides, named BMAP-27B and SMAP-29D, effectively kill colistin-resistant bacteria. Furthermore, peptides B22 and BMAP-24 exhibited MICs of 2 µM and 4 µM against carbapenem and polymyxin-resistant *K. pneumoniae*, respectively. The results indicated that B22 and BMAP-24 inhibited colistin and carbapenem-resistant *K. pneumoniae*. Probably, the differences in the mechanism of action between cathelicidins and colistin are responsible for BMAP-27B and SMAP-29D-mediated killing of colistin-resistant bacteria. BMAP-27B and SMAP-29D bind to bacterial membrane lipids through basic amino acids, whereas colistin binds to bacterial membranes through its N-terminal hydrophobic region and positive regions (Velkov et al. 2013; Kao et al. 2016).

In addition, "WO2020014501A1" and "WO2019126353A2" (Balkovec et al. 2020) are about peptide compositions, including compounds containing a cyclic heptapeptide or conjugates, that can be used in the treatment of infections caused by Gram-negative bacteria. In "WO2020014501A1"(Akers-Rodriguez et al. 2019; Balkovec et al. 2020) 46 compounds containing a cyclic heptapeptide are described. In the MIC test against *K. pneumoniae* (polymyxin-resistant clinical isolate having a phoQ mutation [T244N]), compounds 10, 37, 47, and 39 exhibited MICs of 16, 32, 32, and 64 ug/mL, respectively. The results revealed the antimicrobial activity of these compounds against polymyxin-resistant *K. pneumoniae*.

In the invention "WO2019126353A2", (Akers-Rodriguez et al. 2019) 79 conjugates, containing monomers or dimers of cyclic heptapeptides, are described. In the evaluation of in vitro activity through the MIC test against *K. pneumoniae* (polymyxin-resistant clinical isolate carrying a mutation in phoQ [T244N]), conjugates 23, 38, and 48 exhibited MICs of 16 µg/mL; the conjugates 25, 30, 31, 34, 35, 40, 43, 44, 48, 49, 51, 52, 53, 57, 59, 60, and 77 exhibited MICs of 32 µg/mL. The results revealed the antimicrobial activity of 20 of these conjugates against polymyxin-resistant *K. pneumoniae*. However, in vivo test was not performed.

The "WO2019028463A1" (Huang et al. 2019) refers to Paenipeptin analogs that are novel synthetic linear lipopeptides consisting of a lipophilic terminus, an amino terminus, and a peptide interposed between the lipophilic terminus and the amino terminus or a salt. The 17 lipopeptide analogs mentioned in the application are compositionally similar to a natural mixture of linear and cyclic lipopeptides produced by *Paenibacillus* sp. The antibacterial activities of these 17-synthetic linear lipopeptides analogs were determined against reference strains, carbapenem-resistant isolates, and 6 polymyxin-resistant strains. The linear lipopeptide 17 exhibited potent activity with an MIC of 0.5–2 µg/mL against carbapenem-resistant clinical isolates (including *A. baumannii*, *Enterobacter cloacae, Escherichia coli, K. pneumoniae,* and *P. aeruginosa*). In addition, the linear lipopeptide 17 was active against polymyxin-resistant strains of *E. coli* (n = 3) and *K. pneumoniae* (n = 3), thereby exhibiting potent in vitro activity. However, it was not evaluated in vivo. Paenipeptin analogs exhibit a high affinity for LPS on the outer membrane of Gram-negative bacteria. The results demonstrated that the bactericidal activity of paenipeptins is linked to the disruption and damage of cytoplasmic membranes, as the paenipeptin analog 17 depolarizes the membrane potential (Moon et al. 2017).

"WO2019084628A1" (Cooper et al. 2019) mentions novel cyclic peptide compounds. A total of 171 peptides were tested in vitro and demonstrated antimicrobial efficacy against different strains of MDR bacteria, including polymyxin-resistant *P. aeruginosa* (n = 2), *A. baumannii* (n = 1), and *K. pneumoniae* (n = 2). These compounds demonstrated advantageous properties when used in combination with other antibiotics (rifampicin and minocycline), thereby exhibiting synergy (FIC ≤ 0.5).

The patent application with the registration of "CN110582507A" (Steckback 2019) focuses on synthesized peptides consisting of a polypeptide sequence of 13 formulations, named SEQ ID NO: 1 to SEQ ID NO: 13. In vitro evaluation of peptide SEQ ID NO: 1 revealed its antimicrobial activity against MDR *K. pneumoniae* isolates (n = 101; 28 polymyxin-resistant ones). Peptide SEQ ID NO: 1 demonstrated an MIC of 8 µg/mL against most isolates; against polymyxin-resistant *K. pneumoniae,* the MIC ranged from 4 to 16 µg/mL. The MIC 50/90 was observed to be 8/16 µg/mL. The "US2020207821A1" describes numerous sequences of stabilized antimicrobial peptides (StAMP) that show antimicrobial activity against Gram-negative colistin-resistant bacteria (eg *A. baumannii, E. coli, K. pneumoniae*) with MIC less than 10.0 µg/mL(Walensky and Mourtada 2020).

The patent "WO2022173981A1" refers to the amphiphilic peptide named SEQ ID NO:1, which showed antimicrobial activity against multidrug resistant strains of K. pneumoniae (n = 101), including polymyxin resistant (27.7%), demonstrating MIC between 2 to > 16 pg/ml and MIC50/90 of 8/ > 16 pg/ml (Steckbeck et al. 2022). The patent application with the registration number "CN111386283A" (Smith et al. 2020) are peptide inhibitors, the final molecule, named G0775. focused on peptide inhibitor G0775. The MIC of G0775 was determined against a group of 49 clinical isolates of MDR *E. coli* and *K. pneumoniae*.

In the antimicrobial evaluation, G0775 maintained effective activity against all 49 multidrug-resistant isolates, including polymyxin-resistant *K. pneumoniae* (n = 1). In the antimicrobial evaluation, G0775 maintained effective activity against all 49 MDR isolates, including polymyxin-resistant *K. pneumoniae* (n = 1). In vivo efficacy was evaluated

using a bacterial lung infection model (polymyxin-resistant *K. pneumoniae*); results demonstrated its bacteriostatic (2 mg/kg) and bactericidal (20 mg/kg) effects. No toxicity was observed in mammalian cells. The mechanism of action of G0775 is through the inhibition of essential bacterial signal peptidase type I (Smith et al. 2018).

Our results indicated that academic sectors possess the highest number of patents published against *K. pneumoniae* (Fig. 3A). This may be due to improvements in research and increased knowledge of intellectual property in academia. However, regarding patent filings containing AMPs against polymyxin-resistant *K. pneumoniae*, companies filed more than universities, with 8 and 6 patents, respectively. The possible explanation (especially for universities) may be related to the lack of financial resources to cover patent registration and maintenance fees. Regarding the status of these peptide patent registrations, only 2 are legally active (for example, US2020207821A1; CN108467424A); while 9 were archived; 2 are pending and 1 abandoned (Table 1). Evidencing that few patents were granted, and most were archived, probably due to non-payment of an annuity. In conclusion, the number of studies aiming at the development of new molecules and products to combat MDR infections is increasing steadily. All evaluated AMPs were in the experimental or preclinical phase; therefore, the clinical phase remains pending. In conclusion, research and patent filings alone are not enough to develop adequate antimicrobial therapy, and more investment is required to push these pharmaceuticals through the development stages to introduce them into the market.

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Author contributions GHAS and LR conducted the formal analysis. ARO contributed the methodology and data review. GHAS, LR and SS wrote the manuscript. SS and LR conceived, designed and supervised the research. All authors read and approved the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest, financial or otherwise.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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CONCLUSÕES

Esse estudo permitiu que tivéssemos a seguintes conclusões:

I. No que tange ao estudo de caso-controle, descrevemos dados epidemiológicos sobre o impacto do isolamento de BGN-MR em pacientes com COVID-19 no Brasil, os quais aumentaram a porcentagem de letalidade. Além disso, descrevemos fatores associados à ocorrência de BGN-MR e a mortalidade em pacientes com e sem COVID-19. Os resultados, nessa amostra populacional, reforçam a necessidade de minimizar o uso de dispositivos invasivos e a exposição previa a antimicrobianos, a fim de contribuir na contenção de BGN-MR, com o intuito de melhorar o prognóstico dos pacientes.

II. As combinações sinérgicas de carvacrol associado a polimixina B, demonstraram potencial antimicrobiano *in vitro* e *in vivo* frente a *K. pneumoniae* resistente à polimixina, representando uma alternativa a ser explorada no desenvolvimento de novos antibacterianos e/ou adjuvantes.

III. Ao revisar os depósitos de patentes, sobre peptídeos antimicrobianos testados frente a *K. pneumoniae* resistente à polimixina, identificou-se que os peptídeos testados efetivamente frente a *K. pneumoniae* resistente à polimixina, estavam em fase experimental ou pré-clínica, sendo necessários investimentos adicionais, a fim de progredir nos estágios de desenvolvimento, para que sejam introduzidos no mercado farmacêutico.

IV. Portanto, este estudo corrobora com as preconizações da Organização Mundial da Saúde e do plano de ação global para contenção da resistência antimicrobiana, contribuindo para monitoramento da resistência a nível local, a fim de mitigar riscos assistenciais, com o intuito de melhorar a compreensão sobre o impacto da resistência antimicrobiana.

ANEXOS A: CONTRIBUIÇÕES EM ARTIGOS CIENTÍFICOS

Artigo 1

- ✓ Revista: Revista do Instituto de Medicina Tropical de São Paulo (Impact Factor (IF) 2.160)
- ✓ Carbapenem-resistant *Pseudomonas aeruginosa* strains: a worrying health problem in intensive care units
- ✓ DOI: http://doi.org/10.1590/S1678-9946202163071

Artigo 2

- ✓ Revista: Industrial Crops and Products (IF 6.4)
- ✓ *Zingiber officinale* Roscoe essential oil: An alternative strategy in the development of novel antimicrobial agents against MDR bacteria
- ✓ DOI: https://doi.org/10.1016/j.indcrop.2022.115065

Artigo 3

- ✓ Revista: Scientific Data (IF 8.0)
- ✓ Large Scale Genome-Centric Metagenomic Data from the Gut Microbiome of Food-Producing Animals and Humans
- ✓ DOI: https://doi.org/10.1038/s41597-022-01465-5

Artigo 4

- ✓ Revista: Microbiology spectrum (IF 9.0)
- ✓ Panorama Exploring the Bacteriome and Resistome of Humans and Food-Producing Animals in Brazil
- ✓ DOI: https://doi.org/10.1128/spectrum.00565-22

Artigo 5

- ✓ Revista: Antibiotics (IF 5.22)
- ✓ Genetic Diversity of Virulent Polymyxin-Resistant *Klebsiella aerogenes* Isolated from Intensive Care Units
- ✓ DOI: https://doi.org/10.3390/antibiotics11081127

Capítulo de livro

- ✓ Livro: 365 DIAS DE PANDEMIA DE COVID-19 E A REALIDADE BRASILEIRA: PESQUISAS E REFLEXÕES
- ✓ Capitulo 41: DIFICULDADES DA PESQUISA CIENTÍFICA NO CONTEXTO DO ENFRENTAMENTO A PANDEMIA DE COVID-19: UM RELATO
- ✓ DOI: 10.36926/editorainovar-978-65-86212-89-1

Artigo 6: Status submetido

- ✓ Revista: Diagnostic Microbiology & Infectious Disease
- ✓ Antimicrobial activity of cinnamaldehyde against multidrug-resistant *Klebsiella pneumoniae*: *in vitro* and *in vivo* study

ANEXO B: APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA (CEP)

UFGD - UNIVERSIDADE FEDERAL DA GRANDE DOURADOS / UFGD-MS

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Caracterização dos mecanismos de resistência antimicrobiana e desenvolvimento de alternativas terapêuticas para o controle de bactérias multirresistentes

Pesquisador: Simone Simionatto
Área Temática:
Versão: 2
CAAE: 30781220.6.0000.5160
Instituição Proponente: FUNDACAO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.255.410

Apresentação do Projeto:
As informações elencadas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram retiradas do arquivo Informações Básicas da Pesquisa (PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1611388_E1.pdf, de 20/08/2020).

Introdução
As infecções hospitalares contribuem para maiores índices de morbidade e mortalidade, tempos de internação prolongados, altos custos e principalmente trazem a ameaça constante de disseminação de bactérias multirresistentes (Nordmann et al., 2011). As taxas de infecção continuam crescendo em todo o mundo, principalmente no Brasil. É uma preocupação mundial controlar as infecções de origem hospitalar e a disseminação da resistência bacteriana aos antimicrobianos (Viale et al., 2015). Muitos programas de controle foram lançados desde o fim da década de 1990, todos visando à conscientização sobre o uso racional de antimicrobianos, o controle da disseminação de cepas resistentes, pesquisa sobre os mecanismos de transmissão da resistência e novos fármacos que possam ser usados para controlar cepas multirresistentes (Tavares, 2001). O aumento da resistência antimicrobiana em bactérias responsáveis por infecções hospitalares é um grande desafio para a Saúde Pública e um problema de grandes proporções para o tratamento de pacientes imunodeprimidos e/ou internados em unidades de terapia intensiva

Endereço: Rua Melvin Jones, 940
Bairro: Jardim América **CEP:** 79.803-010
UF: MS **Município:** DOURADOS
Telefone: (67)3410-2853 **E-mail:** cep@ufgd.edu.br

(Poirel, et al., 2007). As bactérias Gram-negativas são importantes causas de infecções urinárias, pneumonias, sepse, bacteremias, meningites, entre outras infecções (Tavares, 2001) e são responsáveis por um número significativo de mortes no mundo todo, principalmente por acometerem indivíduos em ambiente hospitalar adquirindo assim a capacidade de se disseminar facilmente. O tratamento de infecções causadas por estes micro-organismos tem sido crítico, em função da emergência da resistência frente a várias classes de antibióticos, o que limita as opções terapêuticas (Poirel et al., 2007; Nordmann et al., 2011). Em 2017, a Organização Mundial da Saúde (OMS) lançou uma lista de agentes patogênicos que representam a maior ameaça para a saúde humana. As bactérias Gram-negativas A. baumannii, P. aeruginosa e as enterobactérias estavam entre as três primeiras da
lista, consideradas prioritárias para pesquisa e desenvolvimento (P&D) de novos antibióticos, como parte dos esforços da OMS para enfrentar a crescente resistência global aos antimicrobianos (WHO, 2017).A emergência de bactérias Gram-negativas resistentes a -lactâmicos é um problema global, uma vez que estes medicamentos são muito utilizados na clínica médica (Markovska et al., 2014; Temkin et al., 2014). A resistência aos -lactâmicos constitui um dos principais desafios aos laboratórios clínicos e as equipes de saúde, uma vez que se trata de uma categoria formada por
várias classes de medicamentos bastante utilizados pela clínica médica. Vários mecanismos podem contribuir para a resistência a essa classe de antibióticos, como a impermeabilidade da membrana externa, hiper-expressão de bombas de efluxo, porém, a produção de carbapenemases é considerada o mecanismo de resistência mais importante (Tenke et al., 2014).Com a disseminação mundial de bactérias resistentes a carbapenêmicos resultou no aumento do uso de polimixinas e com isso o inevitável risco da emergência desta resistência (Liu et al., 2015). No final de 2015 foi descrita a primeira bactéria com resitência a polimixina mediada pelo gene mcr1 na China (Liu et al., 2015), em seguida foi descrita na Bélgica (Xavier et al., 2016), Estados Unidos da América (McGann et al., 2016) e Brasil (Fernandes et al., 2016). Embora o gene mcr1 seja responsável pela resistência a polimixina, outros mecanismos podem estar envolvidos na resistência. O aumento da resistência a essa classe de
antimicrobianos diminui as opções terapêuticas, demonstrando a urgente necessidade de desenvolvimento de estratégias alternativas e inovadoras que previnam o agravamento da situação e contribuam para a diminuição das taxas de morbidade e mortalidade dos pacientes acometidos por essas infecções (Braasch et al., 2002). Uma das estratégias para tentar conter essa disseminação é compreender a magnitude do problema,

conhecer os principais mecanismos de resistência e a dinâmica epidemiológica destes microorganismos. Apesar de muitos mecanismos de resistência terem sido elucidados, pouco se conhece como o uso indiscriminado de antibióticos na área animal tem contribuído para a alta prevalência de resistência á polimixinas nas bactérias. É importante ressaltar que a Agência Européia de Medicamentos (EMA) preocupou-se com o crescente risco para os seres humanos devido ao uso de polimixina em animais (Sun et al., 2018), pois a sua rápida disseminação e a prevalência global do mcr-1 representam um sério desafio para a produção agrícola e a saúde pública no mundo todo
(Liu et al., 2015). A região Centro-Oeste é essencialmente pecuária, provavelmente com elevada prevalência no uso de polimixinas na criação animal. Desta forma, o levantamento epidemiológico de isolados bacterianos resistentes a polimixina terá um impacto significativo nesta região.Recentemente nosso grupo realizou a caracterização fenotípica e molecular de 50 cepas de enterobactérias resistente a polimixina
envolvidas em um surto de infecção hospitalar e busca identificar os fatores de risco envolvidos na aquisição desta resistência. A presença do gene blaMCR-1 foi pesquisada, porém o mesmo não foi identificado nestas cepas, indicando que, possivelmente, existe um novo mecanismo de resistência nestas cepas, até o momento desconhecido. A aprovação desta proposta irá oportunizar a continuidade das pesquisas que já vem sendo realizadas na UFGD, como a realização do sequenciamento do genoma destas cepas a fim de elucidar os mecanismos moleculares envolvidos na resistência bacterian. Além disso, permitirá ampliar as pesquisas na busca de entender a disseminação destas bactérias no ambiente hospitalar, em animais de produção e meio ambiente. Acredita-se que este estudo permitirá entender melhor a cadeia de disseminação da resistência bacteriana, contribuindo assim para compreender o surgimento, a propagação da resistência aos antimicrobianos, bem como sua dispersão em seres humanos, animais e ambiente.

Hipótese

Com o aumento no número de casos de infecção hospitalar causada por bactérias multirresistentes, a identificação dos mecanismos genéticos envolvidos na aquisição da resistência e a busca de novas terapias para o tratamento dos pacientes têm grandes implicações no aperfeiçoamento de medidas de redução e contenção da disseminação desses micro-organismos.

Metodologia Proposta

Isolados bacterianosAs amostras bacterianas serão coletadas no período de Junho/2020 a

Junho/2025 provenientes de diferentes fontes: (i) pacientes internados em unidades de terapia intensiva de hospitais da região centro-oeste; (ii) superfícies inanimadas de ambiente hospitalar; (iii) efluentes de hospitais da região centro-oeste e abatedouros de animais e (iv) animais destinados ao consumo (aves e suínos). As espécies bacterianas serão identificadas pelo sistema automatizado Phoenix 100® (BD Diagnostic Systems), e cepas que apresentarem perfil de resistência a-lactâmicos e/ou carbapenêmicos serão incluídas no estudo. A identificação das espécies bacterianas será confirmada através de espectrometria de massa (MALDI-TOF MS) usando o espectrômetro Microflex LT (BrukerDaltonics, Massachusetts, EUA) como descrito previamente (Carvalhaes et al., 2013).Perfil de susceptibilidadeO perfil de susceptibilidade antimicrobiana das cepas incluídas no estudo será confirmado através do teste de Concentração Inibitória Mínima pelo método de microdiluição em caldo, seguindo as recomendações do Clinical and Laboratory Standards Institute
(CLSI 2018). A triagem de cepas produtoras de carbapenemases será realizada através de espectrometria de massa (MALDI-TOF MS) pelo teste de hidrólise ao ertapenem (2 e 4 horas de incubação) usando o espectrômetro Microflex LT (BrukerDaltonics, Massachusetts, EUA) (Carvalhaes et al., 2013).Identificação dos genes codificadores de carbapenemases Será realizada por PCR utilizando primers específicos para os genes (blaTEM, blaSHV, blaCTX-M-1, blaCTX–M-2, blaCTX-M-8, blaCTX-M-14, blaGES-I, blaKPC-2, blaSME, blaNDM-I, blaIMP, blaSPM, blaVIM, blaSIM, blaGIM, blaOXA-48 e blaMCR-1). Os produtos de PCR serão confirmados por sequenciamento.Análise de proteínas de membrana externa(OMPs): As OMPs serão analisadas através de eletroforese em gel de poliacrilamida na presença de dodecilsulfato de sódio (SDS-PAGE) e os géis serão corados com Coomassie blue para visualização das proteínas. Alterações nos genes codificadores de porinas específicas para cada espécie bacteriana incluídas
no estudo serão investigadas através de PCR e sequenciamento(Correa et al., 2013).Expressão de bombas de efluxoSerão realizados testes de inibição por microdiluição na presença e ausência de um desacoplador da força próton motiva, carbonil cianida mclorofenilhidrazona (CCCP). E os genes codificadores de bombas de efluxo serão investigados através de PCR e seqüenciamento (Correa et al., 2013). Tipagem MolecularA relação genética entre os isolados bacterianos será avaliada pelo método de eletroforese em gel de campo pulsado (PFGE). Os padrões de restrição serão
analisados pelo software BioNumerics 2.0 (AppliedMaths, Bélgica) (Fehlberg et al., 2014).Sequenciamento do genoma As bactérias que não tiverem seus mecanismos de resistência elucidados pelos mecanismos acima descritos, serão submetidas ao sequenciamento do genoma completo, a fim de identificar os mecanismos genéticos envolvidos na resistência. O DNA

genômico e as bibliotecas serão preparados utilizando o kit Nextera (Illumina). As amostras de DNA serão submetidas ao sequenciamento através da plataforma Illumina MiSeq2000 (Illumina, San Diego, EUA). Estudo dos fatores de risco associadosOs dados clínicos dos pacientes serão analisados, buscando determinar o perfil clínico e epidemiológico das cepas
multirresistentes. Para identificar os fatores de risco associados à infecção será realizado um estudo caso-controle. Pacientes internados entre Junho de 2020 a junho de 2025 em hospitais localizados na região centro-oeste serão incluídos neste estudo. Casos serão definidos como pacientes em que houve isolamento de cepas resistentes. Os controles serão pacientes em que o isolamento de cepas resistentes não for observado durante as primeiras 48 horas de admissão. Para cada caso, um controle será selecionado.

Metodologia de Análise de Dados
Os dados serão analisados através da combinação de diferentes metodologias propostas neste protocolo. Os resultados obtidos com as técnicas moleculares serão associados buscando entender a dinâmica de transmissão de bactérias multirresistentes. Através da revisão de prontuários de pacientes internados nos hospitais será possível identificar os fatores de riscos associados à infecção ou colonização por micro-organismos multirresistentes de interesse clínico. Também serão realizadas investigações sobre a relação entre a gravidade das infecções dos pacientes e a
aquisição dos isolados resistentes, a influência do tempo de exposição ao ambiente hospitalar sobre a aquisição destes agentes infecciosos.

Critério de Inclusão
Ter aptidão mental e intelectual para compreender o estudo;Aceitar participar da pesquisa e assinar o Termo de Consentimento Livre e Esclarecido (TCLE) ou o Termo de Assentimento Livre e Esclarecido (TALE);

Critério de Exclusão
Não assinar o Termo de Consentimento Livre e Esclarecido (TCLE) ou o Termo de Assentimento Livre e Esclarecido (TALE);

Objetivo da Pesquisa:
Objetivo Primário:
Caracterizar os mecanismos de resistência em bactérias Gram-negativas multirresistentes isoladas

de hospitais, animais e meio ambiente, buscando
elucidar a cadeia de transmissão da resistência bacteriana, bem como propor futuras estratégias de controle da sua disseminação. Além disso, objetiva dar continuidade à interação existente entre os grupos de pesquisa das instituições envolvidas e formar recursos humanos qualificados, de modo a promover avanços científicos e tecnológicos em áreas estratégicas para o desenvolvimento do estado do Mato Grosso do Sul e do país.

Objetivo Secundário:
Isolar bactérias Gram-negativas multirresistentes de amostras clínicas de pacientes internados em hospitais do Centro-Oeste, bem como de superfícies hospitalares, animais de produção e meio ambiente. - Estudar os mecanismos genéticos de resistência nas bactérias Gram-negativas isoladas.- Caracterizar os mecanismos de resistência através do sequenciamento do genoma das cepas multirressitentes.- Associar os dados genômicos e epidemiológicos, buscando identificar os fatores de risco envolvidos na aquisição da resistência.- Estabelecer a relação genética e a cadeia de disseminação da resistência de modo a sugerir como a transmissão dos clones ocorre.- Identificar fatores de risco associados à mortalidade causada por bactérias Gram-negativas multirresistentes isoladas de pacientes internados em hospitais do Centro-Oeste.- Gerar informações úteis para melhoria dos serviços de controle de infecção hospitalar, buscando diminuir a disseminação dessas bactérias.- Organizar e
fortalecer o sistema de vigilância epidemiológica de bactérias multirresistentes no ambiente hospitalar.- Avaliar novas estratégias para inibição dos mecanismos de resistência através do silenciamento da expressão de genes.- Avaliar a atividade antimicrobiana in vitro de peptídeos sintéticos, óleos essenciais e compostos naturais.- Desenvolver modelos experimentais de infecção e tratamento de peptídeos sintéticos, óleos essenciais e compostos naturais.- Contribuir na formação de alunos de graduação e pós-graduação, fixar recém-doutores dinamizando o intercâmbio com
instituições de pesquisa nacionais e estrangeiras envolvidas nesta proposta;- Fortalecer um núcleo emergente de pesquisadores no desenvolvimento de novos produtos biotecnológicos para saúde humana, estratégicos para o estado do Mato Grosso do Sul e pais.Identificar fatores de risco associados à infecções causadas por bactérias multirresistentes isoladas em pacientes internados em hospitais do Centro-Oeste, com ou sem co-infecção pelo coronavírus-2019 (Covid-19). -Avaliar as prescrições de antimicrobianos, associando com os dados microbiológicos e de resistência dos pacientes internados.

Avaliação dos Riscos e Benefícios:

Riscos

Os riscos que a pesquisa poderá lhe causar estão relacionados ao questionário que poderão suscitar lembranças negativas, e com isso dano psicológico. A nossa equipe está treinada para minimizar tais riscos, e caso aconteça, garantimos assistência integral e gratuita a danos causados em decorrência do estudo. Você tem garantido pela resolução do Conselho Nacional de Saúde no 466 de 2012, direito a indenização e ressarcimento decorrentes da pesquisa. O pesquisador principal se responsabiliza por futuras indenizações e ressarcimentos que possam ocorrer decorrente dessa pesquisa.

Benefícios

- Contribuir para a elucidação dos mecanismos de resistência e relação genética de bactérias Gram-negativas isoladas de hospitais do CentroOeste, animais de produção e meio ambiente;
- Colaborar no controle da disseminação de bactérias multirresistentes com ações de biossegurança, conscientização e formação de profissionais para atuar na área da saúde;
- Contribuir a médio e longo prazo para melhoria dos indicadores de saúde pública, bem como na redução dos custos de tratamento de pacientes internados através do fortalecimento do sistema de vigilância epidemiológica de bactérias multirresistentes no ambiente hospitalar;
- Interagir com o sistema produtivo na conscientização do uso racional de antimicrobianos, com atuação em áreas estratégicas para o desenvolvimento do estado do Mato Grosso do Sul e do país;

Comentários e Considerações sobre a Pesquisa:

O presente trabalho propõe realizar um estudo de epidemiológico de bactérias Gram negativas multirresistentes isoladas de pacientes internados em hospitais, de animais e meio ambiente, buscando elucidar a cadeia de transmissão da resistência, bem como propor futuras estratégias para controle da sua disseminação através do desenvolvimento de novas terapias antimicrobianas. As bactérias multirresistentes isoladas serão

submetidas a caracterização fenotípica e molecular e os dados genômicos e epidemiológicos serão associados, buscando identificar os fatores de risco envolvidos na aquisição da resistência e a taxa de mortalidade causada por estas bactérias. Acredita-se que este estudo permitirá entender melhor a cadeia de disseminação da resistência bacteriana, contribuindo assim para a compreensão do surgimento, propagação da resistência aos antimicrobianos, bem como sua dispersão em seres humanos, animais e ambiente. A realização deste projeto irá contribuir para o conhecimento dos

fatores de riscos envolvidos na resistência, causas de óbitos e o nível endêmico de bactérias nos hospitais, auxiliando os centros de controle de infecção hospitalar no sentido de traçar medidas de prevenção de surtos e disseminação de bactérias multirresistentes dentro dos hospitais.

Considerações sobre os Termos de apresentação obrigatória:

Vide "Conclusões ou Pendências ou Lista de Inadequações"

Recomendações:

Vide "Conclusões ou Pendências ou Lista de Inadequações"

Conclusões ou Pendências e Lista de Inadequações:

A pesquisadora submeteu um emenda com a seguinte justificativa:

"EMENDA ADITIVA Considerando que com a Pandemia de COVID-19 muitos pacientes internados nos hospitais com infecções bacterianas podem apresentam co-infecção com SARS-CoV-2, estamos propondo não excluir estes pacientes do nosso estudo. Reforçamos que não será realizada nenhuma metodologia nova no estudo, estamos apenas solicitando avaliar as bactérias e características clinicas dos pacientes internados, sem a exclusão dos que apresentarem co-infecção com SARS-CoV-2. Sendo assim, acrescenta-se aos objetivos específicos do projeto, que passa a ter a seguinte redação: EMENTA

Identificar fatores de risco associados à infecções causadas por bactérias multirresistentes isoladas em pacientes internados em hospitais do Centro-Oeste, com ou sem co-infecção pelo coronavírus-2019 (Covid-19). Avaliar as prescrições de antimicrobianos, associando com os dados microbiológicos e de resistência dos pacientes internados. JUSTIFICATIVA DE EMENDA A inclusão deste objetivo específico ao projeto, justifica-se pelo fato de que ao submetermos anteriormente este projeto ao CEP, não havíamos previsto a pandemia por Covid-19. Desse modo, com a disseminação do Covid-19 no Brasil, e tendo em vista que: 1) pacientes internados em unidades de terapia intensiva por COVID-19 compartilham doenças subjacentes associadas e fatores de risco a infecções; 2) Infecções secundárias foram encontradas em 50% dos pacientes com desfechos de mortalidades por COVID-19; 3) Infecções ou co-infecções secundárias bacterianas são fatores prováveis que afetam a mortalidade de pacientes com COVID-19; 4) Na assistência hospitalar, tem aumentado o número de procedimentos invasivos, uso de antibióticos e a superlotação nos serviços de saúde, os quais podem contribuir para o surgimento e disseminação de fatores de resistência e de microorganismos mais virulentos nas infecções associadas aos cuidados de saúde. Desse modo, faz-se necessário a inclusão desse objetivo ao

projeto, a fim de que possamos contribuir com a produção de evidências sobre a eficácia da intervenção antimicrobiana em pacientes internados, visto que muitos destes pacientes podem apresentar co-infecção por SARS-CoV-2, e os fatores de risco associados as infecções."

Esse CEP considera que essa solicitação de EMENDA não descaracteriza o projeto original.

Considerações Finais a critério do CEP:

Diante do exposto, o CEP/UFGD, de acordo com as atribuições definidas na Resolução CNS nº 510 de 2016, na Resolução CNS nº 466 de 2012 e na Norma Operacional nº 001 de 2013 do CNS, manifesta-se pela APROVAÇÃO da referida EMENDA ao protocolo de pesquisa.

Conforme orientações das resoluções vigentes que regem a ética em pesquisa com seres humanos:

* o pesquisador deve comunicar qualquer evento adverso imediatamente ao Sistema CEP/CONEP;
* O pesquisador deve apresentar relatório parcial e final ao Sistema CEP/CONEP.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1611388_E1.pdf	20/08/2020 17:44:06		Aceito
Outros	EMENDA.docx	20/08/2020 17:41:53	Simone Simionatto	Aceito
Outros	portariaFCBA.pdf	15/04/2020 18:23:16	Simone Simionatto	Aceito
Folha de Rosto	folhaDeRosto.pdf	15/04/2020 18:18:11	Simone Simionatto	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	15/04/2020 16:58:43	Simone Simionatto	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TALE.pdf	15/04/2020 16:57:45	Simone Simionatto	Aceito
Orçamento	Orcamento.docx	15/04/2020 11:48:36	Simone Simionatto	Aceito
Declaração de Instituição e	declaracaoLAB_FCBA.pdf	14/04/2020 20:19:36	Simone Simionatto	Aceito

Infraestrutura	declaracaoLAB_FCBA.pdf	14/04/2020 20:19:36	Simone Simionatto	Aceito
Outros	parecerCAPEHU2020.pdf	14/04/2020 01:57:25	Simone Simionatto	Aceito
Parecer Anterior	PB_PARECER_CONSUBSTANCIADO_CEP_877292_E1.pdf	14/04/2020 01:46:23	Simone Simionatto	Aceito
Outros	ResolucaoFCBA2019.pdf	14/04/2020 01:44:09	Simone Simionatto	Aceito
Outros	QuestionarioProntuarios.docx	14/04/2020 01:38:24	Simone Simionatto	Aceito
Declaração de Pesquisadores	declaracaocoordeandor.pdf	14/04/2020 01:32:24	Simone Simionatto	Aceito
Projeto Detalhado / Brochura Investigador	projetoUFGD2020.pdf	14/04/2020 01:13:44	Simone Simionatto	Aceito
Cronograma	CRONOGRAMA.docx	14/04/2020 01:12:08	Simone Simionatto	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

DOURADOS, 02 de Setembro de 2020

Assinado por:
Leonardo Ribeiro Martins
(Coordenador(a))

ANEXO C: APROVAÇÃO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS (CEUA)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA

Dourados-MS, 17 de agosto de 2020.

CERTIFICADO

Certificamos que a proposta intitulada ***"Avaliação da atividade antibacteriana da Cinnamomum cassia L. em enterobactérias produtoras de carbapenemases in vitro e in vivo."***, registrada sob o protocolo de nº 25/2018, sob a responsabilidade de *Simone Simionatto e Márcia Soares Mattos Vaz* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 14/09/2018. No dia 03/06/2020, houve alteração prorrogando este certificado até a data descrita abaixo. Passando a ter o número de protocolo *25/2018-02. Em 17/08/2020 esta Comissão autorizou de modo ad referendum o acréscimo de animais, descrito abaixo, neste protocolo para novos testes com Zingiber officinale roscoe contra Klebsiella pneumoniae resistente a carbapenêmicos/polimixina e Cinnamaldehyde + Carvacrol + Meropenem contra Klebsiella pneumoniae resistente a carbapenêmicos/polimixina, seguindo o mesmo protocolo já estabelecido e aprovado anteriormente. Passando a ter o número de protocolo 25/2018-03.*

Comissão de Ética no Uso de Animais – CEUA/UFGD – Rua João Rosa Góes, 1761 – Vila Progresso.
Dourados/MS. E-mail: ceua@ufgd.edu.br

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*10/02/2019 a 31/03/2021*
Espécie/linhagem/raça	*Mus musculus*
Nº de animais	160 *Swiss*
Peso/idade	30 dias
Sexo	Fêmeas
Origem	Biotério Central UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA