ÁGHATA CARDOSO DA SILVA RIBEIRO

CARACTERIZAÇÃO do GENE *fosA* CROMOSSÔMICO e AVALIAÇÃO da ATIVIDADE SINÉRGICA de FOSFOMICINA contra *Klebsiella pneumoniae*

Tese de Doutorado apresentada ao Programa de Pós-graduação em Infectologia da Universidade Federal de São Paulo - Escola Paulista de Medicina como pré-requisito para obtenção do título de Doutor em Ciências

São Paulo

2023

ÁGHATA CARDOSO DA SILVA RIBEIRO

CARACTERIZAÇÃO do GENE *fosA* CROMOSSÔMICO e AVALIAÇÃO da ATIVIDADE SINÉRGICA de FOSFOMICINA contra *Klebsiella pneumoniae*

Tese de Doutorado apresentada ao Programa de Pós-graduação em Infectologia da Universidade Federal de São Paulo - Escola Paulista de Medicina para obtenção do título de Doutor em Ciências.

Orientadora: Profª. Drª. Ana Cristina Gales
Coorientadora: Profª. Drª. Luciene Andrade da Rocha Minarini

Este trabalho foi realizado com auxílio financeiro fornecido pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Demanda Social (CAPES).

São Paulo

2023

Ficha catalográfica elaborada pela Biblioteca Prof. Antonio Rubino de Azevedo,
Campus São Paulo da Universidade Federal de São Paulo, com os dados fornecidos pelo(a) autor(a)

Cardoso da Silva Ribeiro, Ághata
CARACTERIZAÇÃO do GENE fosA CROMOSSÔMICO e AVALIAÇÃO da ATIVIDADE SINÉRGICA de FOSFOMICINA contra Klebsiella pneumoniae / Ághata Cardoso da Silva Ribeiro. - São Paulo, 2023.
xvi, 96f.

Tese (Doutorado) - Universidade Federal de São Paulo, Escola Paulista de Medicina. Programa de Pós-Graduação em Programa de Pós Graduação em Infectologia.

Título em inglês: Chromosomic fosA gene characterization and evaluation of synergy activity of Fosfomycin against Klebsiella pneumoniae.

1. Resistência aos antimicrobianos. 2. Klebsiella pneumoniae. 3. Fosfomicina. 4. fosA.

UNIVERSIDADE FEDERAL DE SÃO PAULO

ESCOLA PAULISTA DE MEDICINA

DISCIPLINA DE INFECTOLOGIA

Chefe do Departamento de Medicina:

Prof. Dr. Álvaro Pacheco e Silva Filho

Coordenadora do curso de Pós-graduação em Infectologia:

Profª. Drª. Ana Cristina Gales

Chefe da Disciplina de Infectologia:

Prof. Dr. Paulo Roberto Abrão Ferreira

São Paulo

2023

ÁGHATA CARDOSO DA SILVA RIBEIRO

CARACTERIZAÇÃO do GENE *fosA* CROMOSSÔMICO e AVALIAÇÃO da ATIVIDADE SINÉRGICA de FOSFOMICINA contra *Klebsiella pneumoniae*

Presidente da Banca:

Professora Doutora Ana Cristina Gales

Banca Examinadora:

1. Doutor Diego Olivier Andrey

2. Professor Doutor Jorge Luiz Mello Sampaio

3. Professora Doutora Maria Cristina Bronharo Tognim

4. Professora Doutora Rosa Maria Silva

DEDICATÓRIA

Dedico este trabalho à minha família. Minha força motriz, meu aconchego, meu porto seguro, meu presente de Deus.

AGRADECIMENTOS

Meu agradecimento maior é ao meu Deus. Autor da vida, meu guia, meu Senhor. Se cheguei até aqui, com certeza foi porque a Sua poderosa mão me acompanhou.

Durante a caminhada do doutorado pude contar com o apoio e ajuda de muitas pessoas, dentro e fora do ambiente de trabalho, às quais sou e serei eternamente grata.

Quero agradecer à minha família por ser meu amparo. Por me incentivarem tanto e me darem suporte para que eu conseguisse concluir mais essa etapa.
Em especial, quero agradecer ao meu esposo Jônathas por ser companheiro nessa jornada, sempre compreensivo e amoroso.
Ao meu filhinho Pietro por compreender a mamãe e o trabalho dela, por me amar tanto.
Aos meus pais, Débora e Adilson, por todo apoio, segurança e amor que me dão. Um dia quero ser só um pouquinho como vocês.

Não posso deixar de agradecer à minha querida orientadora Professora Ana Gales que tanto me ensinou, apoiou e inspirou nesses últimos anos. Como é enriquecedor aprender com uma das melhores pesquisadoras do Brasil. Obrigada por receber-me. Sou muito feliz por fazer parte da sua equipe.

Agradeço também à minha co-orientadora, Professora Luciene Minarini e ao Professor Rodrigo Cayô por todo apoio durante esses anos de doutorado. Com certeza sem vocês essa caminhada seria bem mais árdua.

Por fim, mas não menos importante, quero agradecer a cada um dos membros do Laboratório Alerta. Essa caminhada foi construída com muitas mãos, e sem vocês com certeza seria impossível. Obrigada por tanto apoio, ajuda, palavras de incentivo, palavras de ânimo. Vocês me ajudaram a crescer pessoalmente e profissionalmente. E por isso serei eternamente grata. Todos são importantes para mim. Entretanto, nominalmente quero deixar registrado meu carinho e eterna gratidão aos meus amigos: Dra. Fernanda Fernandes, MsC. Tiago Valiatti, MsC. André Valêncio, Raissa Fidélis, Yohanna Chikhani, MsC. Michael Lenzi, Dr. Willames Martins e Dr. Ikechuko Moses. Muito obrigada!

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – PDS (88882.430562/2019-01).

"Eu aprendi qual é o valor que eu 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"

É só o começo – Vocal Livre

RESUMO

A Resistência aos Antimicrobianos (RAM) é considerada pela Organização Mundial da Saúde como uma das maiores ameaças à saúde pública. Com o acelerado surgimento de linhagens bacterianas multi- e pan-resistentes, associada ao número limitado de antimicrobianos eficazes, a busca por alternativas terapêuticas tem sido cada vez mais comum. A fosfomicina dissódica é uma opção terapêutica potencial para tratar infecções de difícil tratamento, especialmente quando combinada com outros antimicrobianos. Neste estudo, avaliamos a atividade da fosfomicina em combinação com meropenem ou polimixina B contra 17 isolados clínicos contemporâneos de *K. pneumoniae* produtores de KPC-2. A atividade sinérgica destes antimicrobianos foi avaliada *in vitro* por meio dos ensaios de *checkerboard* e *time-kill* e *in vivo* em *Galleria mellonella*. Sabendo que *K. pneumoniae* é considerada uma espécie reservatório do gene *fosA,* um gene de resistência à fosfomicina, pesquisou-se a expressão do gene *fosA* cromossômico por PCR quantitativo em tempo real, seu impacto no fenótipo de resistência à fosfomicina nesse patógeno, bem como o contexto genético envolvido com este gene por meio de dados obtidos do sequenciamento de genoma completo. No presente estudo, por meio da análise da sequência de *fosA* cromossômico, conseguiu-se diferenciar clones de *K. pneumoniae* do CC258/11 de outros STs, bem como identificar clones dos STs 16, 15, 307 e 101. Os resultados de sinergismo obtidos *in vitro* apontaram para a recuperação da atividade de meropenem e de polimixina B em 82,4% e em 47% dos isolados, respectivamente, quando combinados com fosfomicina. Observou-se sinergismo entre fosfomicina e meropenem contra 58,8% dos isolados, com redução do crescimento bacteriano variando de 3,0 a 6,7 $\log_{10}$ CFU/mL; e entre fosfomicina e polimixina B contra 47,1% dos isolados, com redução de 6,0 a 6,2 $\log_{10}$ CFU/mL, quando usadas as concentrações de pico. O ensaio em *G. mellonella* demonstrou atividade de fosfomicina em combinação com meropenem, uma vez que todas as larvas tratadas com essa combinação sobreviveram. Observou-se também a atividade sinérgica de fosfomicina com meropenem ou polimixina B na desestruturação do biofilme produzido por *K. pneumoniae* produtora de KPC (redução de 1,481 a 3,470 vezes na detecção do biofilme). Por fim, observou-se um aumento de 4,69 a 7,73 vezes na expressão de *fosA* nos isolados resistentes à fosfomicina. Em média, o gene *fosA* nos isolados resistentes à fosfomicina apresentaram-se cerca de cinco vezes mais expressos do que nos isolados sensíveis à fosfomicina. Neste trabalho demonstraram-se bons resultados *in vitro* e *in vivo* da atividade de fosfomicina em combinação com meropenem ou polimixina B, apontando para o seu promissor uso como terapia contra infecções de difícil tratamento. Entretanto, o aumento da expressão de *fosA* cromossômico observado nos isolados resistentes à fosfomicina apontam para a permanente necessidade de avaliação da possibilidade de surgimento de resistência à fosfomicina. Este fato reforça o uso de fosfomicina em terapia combinada para tratamento de infecções causadas por *K. pneumoniae*.

Descritores: Fosfomicina; *fosA*; *Klebsiella pneumoniae*; sinergismo.

ABSTRACT

Antimicrobial Resistance (AMR) is considered by the World Health Organization as one of the greatest threats to the public health. With the accelerated increase on multi- and pan-resistant bacterial pathogens, associated with the limited number of effective antimicrobials, the search for therapeutic alternatives has become increasingly common. Fosfomycin disodium is a potential therapeutic option to treat difficult-to-treat infections, especially when combined with other antimicrobials. In this study, we evaluated the activity of fosfomycin in combination with meropenem or polymyxin B against 17 contemporaneous KPC-2-producing *K. pneumoniae* clinical isolates. The synergistic activity of these antimicrobials was evaluated *in vitro* using checkerboard and time-kill assays and *in vivo* in *Galleria mellonella*. *K. pneumoniae* is considered a reservoir species of the *fosA* gene, a fosfomycin resistance gene. Thus, the expression of the chromosomal *fosA* gene was assessed by real-time quantitative PCR as well as its impact on the fosfomycin resistance phenotype. Also, we analyzed the *fosA* genetic context through the data obtained from whole genome sequencing. In the present study, through the analysis of the chromosomal *fosA* sequence, it was possible to differentiate clones of *K. pneumoniae* from CC258/11 from other STs, as well as to identify clones of STs 16, 15, 307 and 101. The results of *in vitro* synergism assays showed recovery of meropenem and polymyxin B activity in 82.4% and 47% of the isolates, respectively, when combined with fosfomycin. Synergism between fosfomycin and meropenem was observed against 58.8% of the isolates, with a reduction in bacterial growth ranging from 3.0 to 6.7 $\log_{10}$ CFU/mL; and between fosfomycin and polymyxin B against 47.1% of the isolates, with a reduction of 6.0 to 6.2 $\log_{10}$ CFU/mL, when using the peak concentrations. The assay with *G. mellonella* demonstrated fosfomycin activity in combination with meropenem, as all larvae treated with this combination survived. The synergistic activity of fosfomycin with meropenem or polymyxin B in disrupting the biofilm produced by KPC-producing K. pneumoniae was also observed (1.481 to 3.470-fold reduction in biofilm detection). Finally, a 4.69 a 7.73-fold increase in *fosA* expression was observed in the fosfomycin-resistant isolates. On average, the *fosA* gene expression in the fosfomycin-resistant isolates was about 5-fold increase compared with the fosfomycin-susceptible isolates. In this work, good *in vitro* and *in vivo* results were demonstrated for the activity of fosfomycin in combination with meropenem or polymyxin B, pointing to its promising use as a therapy against difficult-to-treat infections. However, the increase in chromosomal *fosA* expression observed in the fosfomycin-resistant isolates points to the permanent need to evaluate the possibility of the emergence of fosfomycin resistance. This fact reinforces the use of fosfomycin in combination therapy for the treatment of infections caused by *K. pneumoniae*.

Keywords: Fosfomycin; *fosA*; *Klebsiella pneumoniae*; synergism.

RESUMO POPULAR

Sabe-se que a Resistência aos Antimicrobianos é um problema grave de saúde pública. Existem algumas bactérias que são bastante importantes, pois se apresentam resistentes a muitos antibióticos, de modo que restam poucas opções para tratar infecções causadas por esses patógenos. Este estudo teve como objetivo buscar uma alternativa para o tratamento de infecções hospitalares complicadas causadas por uma bactéria chamada *Klebsiella pneumoniae.* Essa espécie bacteriana pode produzir algumas proteínas, denominadas enzimas, que são capazes que inativar alguns dos mais importantes antibióticos usados na rotina clínica para tratamento de infecções. Por isso, tratar infecções causadas por essa bactéria tem se tornado cada vez mais difícil. Neste trabalho nós testamos a atividade de duas diferentes combinações de antibióticos contra essa espécie bacteriana: fosfomicina+meropenem e fosfomicina+polimixina B. Além disso, nós buscamos entender o quanto um gene presente no DNA dessa bactéria, o gene *fosA,* poderia interferir na atividade do antibiótico fosfomicina. Os resultados desse estudo são importantes para a sociedade uma vez que indicam uma nova possibilidade de tratamento para infecções graves causadas por *K. pneumoniae* resistentes no Brasil.

SUMÁRIO

DEDICATÓRIA ..... ***viii***

AGRADECIMENTOS ..... ***ix***

RESUMO ..... ***xi***

ABSTRACT ..... ***xii***

RESUMO POPULAR ..... ***xiii***

LISTA DE ABREVIATURAS ..... ***xv***

LISTA DE FIGURAS ..... ***xvi***

1. INTRODUÇÃO 17

2. REVISÃO BIBLIOGRÁFICA 22

2.1 Fosfomicina, um antigo antimicrobiano como uma nova opção terapêutica 22

2.2 Mecanismo de Ação da Fosfomicina 23

2.3 Mecanismos de Resistência à Fosfomicina 24

2.4 Uso clínico de fosfomicina em combinação com outros antimicrobianos 27

2.5 *Klebsiella pneumoniae*, um patógeno de grande importância clínica 28

3. OBJETIVOS 32

3.1 Objetivo Geral 32

3.2 Objetivos Específicos 32

4. RESULTADOS 33

ARTIGO 1 34

ARTIGO 2 47

ARTIGO 3 73

5. DISCUSSÃO 84

6. CONCLUSÃO 88

LISTA DE ABREVIATURAS

ANVISA – Agência Nacional de Vigilância Sanitária

CC – Complexo Clonal

CIM – Concentração Inibitória Mínima

COVID-19 – *Coronavirus Disease 2019*

ESBL – *Extended Spectrum β-lactamases*

GlpT- Glicerol-3-fosfato

IDSA – *Infectious Diseases Society of America*

IS – *Insertion Sequence*

ITU – Infecção do Trato Urinário

KPC – *Klebsiella pneumoniae Carbapenemase*

MDR – *Multidrug resistance*

Mn^{2+}- íon manganês

MRSA – *Meticillin-resistant Staphylococcus aureus*

MurA - UDP-N-acetilglicosamina-enolpiruvil transferase

NDM – *New-Delhi Metallo β-lactamase*

OXA - oxacilinase

PEP - enolpiruvil do fosfoenolpiruvato

RAM – Resistência aos Antimicrobianos

ST – *Sequence Type*

UhpT - Glicose-6-fosfato

UNAG - UDP-N-acetilglicosamina

UTI – Unidade de Terapia Intensiva

VRE – *Vancomycin-resistant Enterococcoccus*

FOS - Fosfomicinases

FOM – Fosfomicina quinases

LISTA DE FIGURAS

Figura 1- Estruturas químicas das moléculas de fosfomicina, glicose-6-fosfato e glicerol-3-fosfato.

Figura 2- Mecanismo de ação da fosfomicina na enzima MurA.

1. INTRODUÇÃO

A Resistência aos Antimicrobianos (RAM) é considerada pela Organização Mundial da Saúde como uma das maiores ameaças à saúde pública. Recentemente, foi estimado que mundialmente 1,27 milhões de mortes foram diretamente atribuídas à RAM e 4,95 milhões de óbitos foram associados à RAM em 2019, refletindo a necessidade urgente de um plano de ação global para o combate à essa importante ameaça (MURRAY et al., 2022; WORLD HEALTH ORGANIZATION, 2017).

Nesse contexto, com o acelerado surgimento de linhagens bacterianas multi- e pan-resistentes, associada ao número limitado de antimicrobianos eficazes, a busca por alternativas terapêuticas tem sido cada vez mais comum (KAYE; GALES; DUBOURG, 2017). Na última década, tem-se observado um aumento na pesquisa e desenvolvimento de novos antimicrobianos. Entretanto, a pesquisa e desenvolvimento de novos antimicrobianos nem sempre é atrativa para a indústria, visto que novos mecanismos de resistência podem surgir. Além disso, o processo de aprovação de um novo antimicrobiano é moroso, o que limita ainda mais as opções disponíveis. No Brasil especificamente, os novos antimicrobianos recentemente aprovados e/ou em fase de aprovação são, de maneira geral, inacessíveis à maior parte da população que atendida na rede pública de saúde, visto apresentarem alto custo. Sendo assim, na prática clínica, diante da necessidade de tratamento destas infecções, é comum a busca por antimicrobianos mais antigos que mantenham sua atividade contra isolados multirresistentes. A terapia com fosfomicina se limitou por longos anos apenas ao tratamento de infecções do trato urinário (ITU), administrando-se por via oral a fosfomicina trometamol. Entretanto, com a alta frequência de bactérias resistentes aos carbapenêmicos e às polimixinas, o uso de fosfomicina dissódica (apresentação endovenosa) combinado a outros antimicrobianos tem sido prescrito como uma alternativa terapêutica também contra infecções sistêmicas em diferentes países, onde essa formulação/apresentação é disponível (FALAGAS et al., 2010a; HASHEMIAN; FARHADI; FARHADI, 2019; YANG; LU; TSENG, 2019).

A fosfomicina foi descoberta, em 1969, por Hendlin e colaboradores na Espanha. É um antibiótico produzido por algumas espécies bacterianas de *Streptomyces* spp (HENDLIN et al., 1969) e apresenta como mecanismo de ação a interferência na etapa inicial de biossíntese da

parede celular bacteriana, ligando-se irreversivelmente à enzima MurA (UDP-GlcNAc-3-enolpiruvil transferase), responsável por catalisar a síntese dos monômeros de mureína, que se polimerizam formando cadeias de peptidoglicano que, por sua vez, compõe a estrutura da parede celular bacteriana. Para adentrar à célula bacteriana, este antimicrobiano se utiliza dos transportadores de glicose-6-fosfato (UhpT) e glicerol-3-fosfato (GlpT), mimetizando seus ligantes naturais (GOLLA et al., 2019; KAHAN et al., 1974).

A resistência bacteriana à fosfomicina pode ocorrer por conta de mutações na proteína alvo (MurA), e nos transportadores (UhpT e GlpT), bem como em seus genes reguladores (FALAGAS et al., 2019). O principal mecanismo de resistência à fosfomicina se dá pela inativação enzimática deste antimicrobiano, por meio da ação das fosfomicinases (enzimas Fos) e fosfomicina-quinases (YANG; LU; TSENG, 2019), codificadas pelos genes *fos* e *fom*, respectivamente. Ainda, por apresentar estrutura e mecanismo de ação únicos, a resistência cruzada de fosfomicina com outros antimicrobianos é incomum (FALAGAS et al., 2019).

Nos últimos anos, o uso de fosfomicina endovenosa tem sido apontada como uma alternativa para o tratamento de infecções causadas por bactérias multirresistentes (MDR, do inglês *multidrug-resistant*) e estudos recentes têm indicado uma melhor atividade dessa droga quando em combinação com outros antimicrobianos (WISTRAND-YUEN et al., 2020). Sabe-se que combinações terapêuticas têm como objetivo potencializar o efeito bactericida da terapia e minimizar a emergência de linhagens MDR. Nesse âmbito, explorar potenciais combinações de fosfomicina com outros antimicrobianos tem se mostrado bastante promissor, especialmente como terapia para infecções de difícil tratamento (BONCOMPAGNI et al., 2022). Os resultados observados em diversos países utilizando-se fosfomicina endovenosa são expressivos, inclusive contra patógenos MDR, como *Klebsiella pneumoniae* resistente aos carbapenêmicos (BURASTERO et al., 2022; OLIVA et al., 2021; RODRÍGUEZ et al., 2018). Entretanto, cabe ressaltar que até o presente momento, no Brasil, ainda não há aprovação para o uso da formulação endovenosa de fosfomicina, evidenciando a necessidade latente de que esse processo seja acelerado.

K. pneumoniae resistente aos carbapenêmicos é considerado um patógeno crítico para guiar a pesquisa e o desenvolvimento de novos fármacos (WORLD HEALTH

ORGANIZATION, 2017) e é um dos microrganismos de maior importância clínica em todo o mundo (MURRAY et al., 2022). Importante carreador de genes de resistência e virulência, esse patógeno apresenta alta frequência de disseminação, especialmente algumas linhagens como as do Complexo Clonal (CC) 258, tipo de sequência (ST, do inglês *Sequence type*) 15, ST101, ST307 e ST16 (WYRES; HOLT, 2021). De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA), no Brasil, *K. pneumoniae* é o segundo patógeno mais frequentemente isolado de infecções de corrente sanguínea associadas a cateter venoso central em pacientes adultos internados em Unidades de Terapia Intensiva (UTI), apresentando altas taxas de resistência aos carbapenêmicos (63,2%) e polimixinas (15%) (ANVISA, 2021). Mundialmente, foi estimado que *K. pneumoniae* causou mais de 150.000 mortes em 2019, e que destas, aproximadamente 15% ocorreram na região da América Latina e Caribe. Além disso, globalmente, 55.700 foram atribuídas à *K. pneumoniae* resistente aos carbapenêmicos (MURRAY et al., 2022).

No Brasil, até 2020 o mais frequentemente reportado mecanismo de resistência aos carbapenêmicos em *K. pneumoniae* era a produção da enzima KPC (*Klebsiella pneumoniae carbapenemase*). Entretanto, durante a pandemia por SARS-CoV-2, observou-se um aumento na frequência da detecção de carbapenemase do tipo NDM (*New-Delhi* metalo-β-lactamase), e ainda co-produção de KPC e NDM (BRASIL, 2022; VÁSQUEZ-PONCE et al., 2022; WINK et al., 2021) nessa mesma espécie bacteriana. Este fato é preocupante, visto que antimicrobianos recentemente lançados no mercado, como ceftazidima-avibactam não possuem ação contra patógenos produtores de metalo-β-lactamases (DAVIDO et al., 2017).

Na ordem dos Enterobacterales, à qual pertence *K. pneumoniae,* a resistência à fosfomicina se dá majoritariamente pela presença das enzimas codificadas pelo grupo de genes *fos,* mais especificamente a família *fosA* (YANG; LU; TSENG, 2019). *K. pneumoniae* apresenta de forma intrínseca o gene *fosA* cromossômico, sendo, portanto, uma espécie reservatório desse mecanismo genético de resistência à fosfomicina (ITO et al., 2017b). Por este motivo, e pelo fato de ter sido observado o surgimento de resistência em ensaios *in vitro*, o uso de fosfomicina em monoterapia para tratamento de infecções causadas por *K. pneumoniae* não tem sido recomendada (TAMMA et al., 2022; WISTRAND-YUEN et al., 2020). Entretanto, pouco se

sabe sobre a expressão desse gene intrínseco no cromossomo de *K. pneumoniae.* Diversos estudos têm demonstrado taxas relativamente altas de sensibilidade *in vitro* à fosfomicina em *K. pneumoniae*, inclusive entre linhagens MDR (FALAGAS et al., 2010b; LIU et al., 2020). Até o presente momento, somente um caso de resistência à fosfomicina em *K. pneumoniae* atribuído à expressão do gene *fosA* cromossômico foi relatado (KIEFFER et al., 2020a). Geralmente, a elevação das concentrações inibitórias mínimas (CIM) para fosfomicina em *K. pneumoniae* tem sido associada à aquisição do gene *fosA* plasmideal, como *fosA3*, como ocorre também para outras enterobactérias não portadoras de *fosA* cromossômico, como *Escherichia coli* (TSENG et al., 2017).

Tendo em vista os altos índices de resistência bacteriana e a dificuldade no manejo clínico de infecções de difícil tratamento, comumente causadas por patógenos MDR, observa-se a latente necessidade por alternativas terapêuticas. Sendo assim, explorar combinações eficazes de fosfomicina com outros antimicrobianos é importante para ampliar as alternativas terapêuticas vigentes, especialmente no Brasil, em que o acesso a novos antimicrobianos é restrito. Estudos conduzidos sobre a ação de fosfomicina em isolados clínicos brasileiros são de extrema importância para avaliar a utilidade clínica potencial da fosfomicina, especialmente em um cenário em que fosfomicina-trometamol tem sido utilizada para tratamento de ITU na comunidade. Além disso, diante das poucas informações sobre o impacto do gene *fosA* cromossômico de *K. pneumoniae* em seu fenótipo de resistência à fosfomicina, se faz necessária uma maior compreensão sobre a expressão deste gene, a fim de orientar o uso deste antimicrobiano em casos de infecções causadas por *K. pneumoniae*.

O presente estudo teve como objetivo avaliar o sinergismo *in vitro* de fosfomicina em combinação com outros antimicrobianos disponíveis em hospitais públicos brasileiros contra isolados clínicos brasileiros de *K. pneumoniae* resistentes aos carbapenêmicos e/ou à polimixina B, buscando reduzir a CIM desses antimicrobianos quando em combinação com fosfomicina. Além disso, pesquisar a expressão do gene *fosA* cromossômico e seu impacto no fenótipo de resistência à fosfomicina nesse patógeno faz parte do escopo deste estudo.

Espera-se que este estudo forneça importantes resultados obtidos *in vitro* e *in vivo* sobre a avaliação da utilidade clínica potencial da fosfomicina endovenosa para o tratamento de

infecções causadas por microrganismos multirresistentes no Brasil, com destaque para *K. pneumoniae,* trazendo perspectivas quanto ao uso dessa droga, especialmente em combinação com outros antimicrobianos.

2. REVISÃO BIBLIOGRÁFICA

2.1 Fosfomicina, um antigo antimicrobiano como uma nova opção terapêutica

Descoberta em 1969, na Espanha, por Hendlin e colaboradores a fosfomicina é um antibiótico produzido naturalmente por espécies bacterianas de *Streptomyces* spp, como *Streptomyces fradiae, Streptomyces viridochromogenes* e *Streptomyces wedomorensis* (HENDLIN et al., 1969). Entretanto, pode também ser produzido por meio de reações de síntese orgânica. Sua estrutura química é simples, e compreende um grupo epóxido, que é essencial para sua atividade em seu alvo, e um grupo ácido fosfônico, que faz sua molécula se assemelhar com os ligantes naturais dos transportadores dos quais se utiliza para adentrar à célula bacteriana (KAHAN et al., 1974) (Figura 1).

É uma molécula de baixo peso molecular (138 g/mol), solúvel em água e que apresenta baixa ligação com proteínas plasmáticas e se dissemina facilmente para a maioria dos tecidos. Comercialmente, existem as formulações oral (fosfomicina-trometamol e fosfomicina cálcica) e endovenosa (fosfomicina dissódica) (DÍEZ-AGUILAR, 2019; FALAGAS et al., 2016; KAHAN et al., 1974). Historicamente, o uso da fosfomicina se fez mais presente para o tratamento de ITUs. Entretanto, por longos anos, esteve em segundo plano até mesmo para o tratamento desse tipo de infecção, com o extensivo uso de fluoroquinolonas para este fim. Porém, com o aumento das taxas de resistência às fluoroquinolonas e com a contra-indicação do uso dessa classe de antimicrobianos para profilaxia e tratamento de ITUs não complicadas devido ao aumento dos relatos de eventos adversos com essa classe de antimicrobianos, o uso de fosfomicina aumentou. Inicialmente, na Europa (EMA, 2018) e, posteriormente, nos demais continentes, atingindo também o Brasil, o uso de fosfomicina-trometamol tornou-se mais frequente, sendo hoje, a principal recomendação para tratamento de ITUs não complicadas (DE ROSSI et al., 2020; EMA, 2018).

Nos últimos anos, com a intensa crescente nas taxas de multirresistência bacteriana, a fosfomicina foi revisitada como uma alternativa terapêutica para uso endovenoso em diversos países. Diferentes estudos têm apontado a sua ação contra cepas produtoras de β-lactamases de espectro estendido (ESBL, do inglês *Extended-Spectrum β-lactamases*) e de carbapenemases

(FALAGAS et al., 2010b). Shortgridge et al. (2018) apontou sensibilidade à fosfomicina em 98% das *E. coli* ESBL e em 80,6% de *K. pneumoniae* ESBL (Shortridge et al. 2018). Ainda, foi reportado em outro estudo que 79% das *K. pneumoniae* resistentes aos carbapenêmicos era sensível à fosfomicina (LIU et al., 2020).

Nesse panorama, esse antigo antibiótico tem ganhado destaque como uma pungente opção terapêutica para tratamento de infecções de difícil tratamento causadas por microrganismos reconhecidamente resistentes, como *K. pneumoniae* resistente aos carbapenêmicos (PERDIGÃO NETO et al., 2020).

2.2 Mecanismo de Ação da Fosfomicina

A fosfomicina apresenta como mecanismo de ação a interferência na etapa inicial de biossíntese da parede celular bacteriana. Para tanto, ela se utiliza de dois diferentes transportadores presentes na membrana plasmática da bactéria, mimetizando seus substratos naturais, visto possuir estrutura química semelhante a estes. Ao mimetizar os ligantes naturais dos transportadores UhpT e GlpT, a fosfomicina induz a expressão dos genes que codificam esses transportadores, favorecendo ainda mais a entrada de moléculas de fosfomicina na bactéria (DÍEZ-AGUILAR, 2019; GOLLA et al., 2019; KAHAN et al., 1974) (Figura 1).

Quando no citoplasma, a fosfomicina se liga irreversivelmente à enzima MurA (UDP-N-acetilglicosamina-enolpiruvil transferase), responsável por catalisar a síntese de UDP-N-acetilglicosamina (UNAG) por meio da transferência da porção enolpiruvil do fosfoenolpiruvato (PEP) para o grupo 3'-hidroxil do UNAG. Essa etapa é essencial para a formação dos monômeros de mureína, imprescindíveis na biossíntese de pepetideoglicano que compõe a estrutura da parede celular bacteriana (DÍEZ-AGUILAR, 2019; FALAGAS et al., 2016). A fosfomicina se liga covalentemente ao grupo tiol de uma cisteína (usualmente a Cys115) no sítio ativo da MurA, consequentemente impedindo a formação de UNAG e comprometendo a integridade da parede celular, com subsequentes lise e morte celular, caracterizando a fosfomicina como um antimicrobiano bactericida (Figura 2) (ESCHENBURG; PRIESTMAN; SCHÖNBRUNN, 2005; GOLLA et al., 2019).

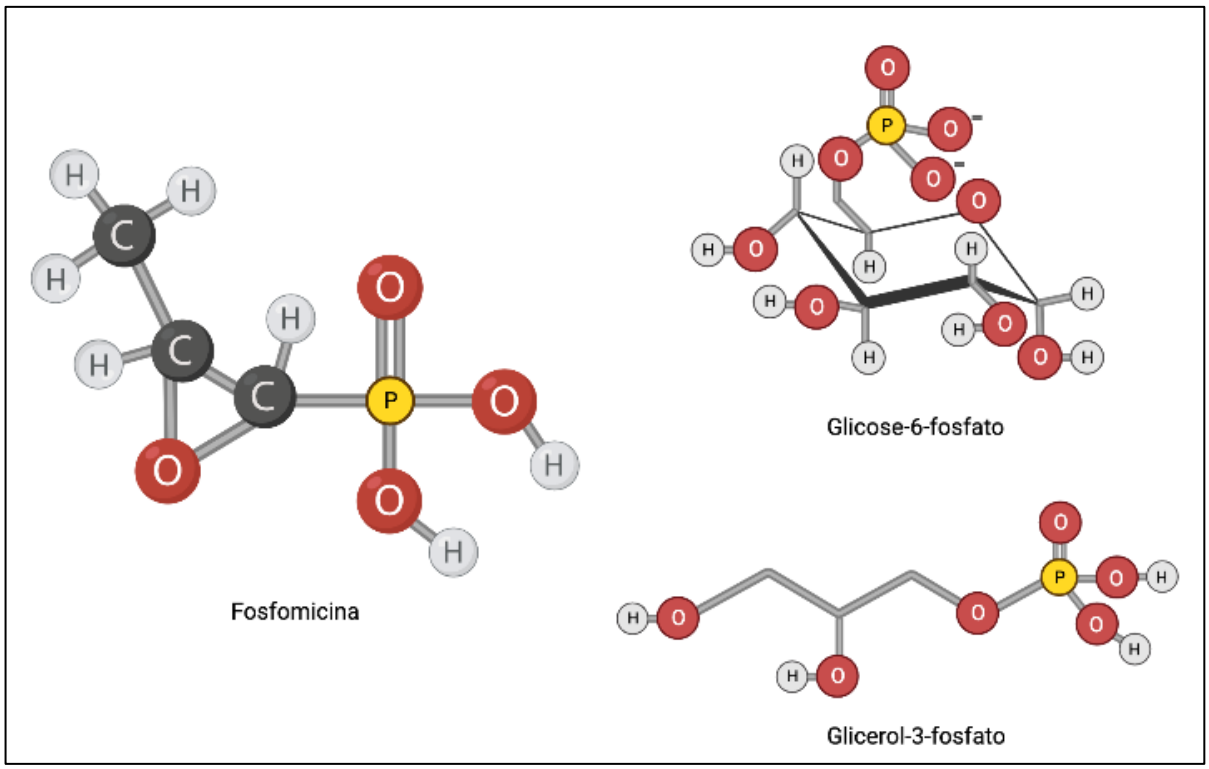

Figura 1: Estruturas químicas das moléculas de fosfomicina, glicose-6-fosfato e glicerol-3-fosfato. Imagem elaborada por meio da plataforma Biorender.

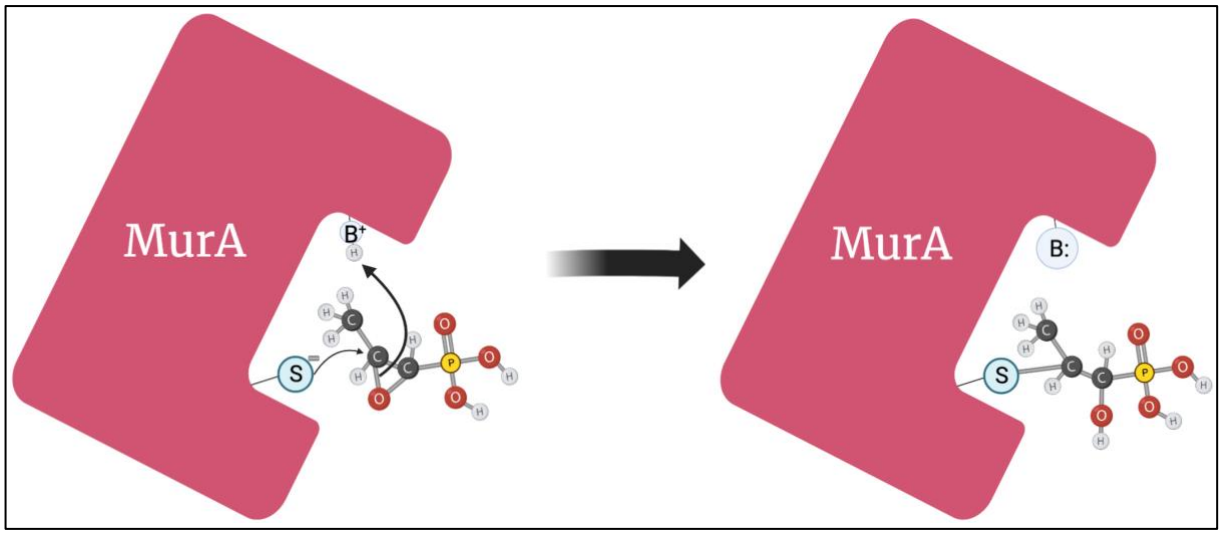

Figura 2. Mecanismo de ação da fosfomicina na enzima MurA. O grupo tiol da cisteína presente no sítio ativo da enzima MurA ataca nucleofilicamente a molécula de fosfomicina, promovendo o rompimento do anel epóxido. Com isso, a molécula de fosfomicina se liga covalentemente à enzima, impedindo que esta tenha ação em seu ligante natural, ou seja, a enzima é inativada. Imagem elaborada por meio da plataforma Biorender.

2.3 Mecanismos de Resistência à Fosfomicina

Embora a fosfomicina conserve atividade frente a bactérias gram-negativas e gram-positivas, abrangendo patógenos MDR, como *Staphylococcus aureus* resistentes à meticilina

(MRSA), *Enterococcus* resistentes à vancomicina (VRE) e bactérias gram-negativas produtoras de ESBL e carbapenemases, diferentes mecanismos de resistência à fosfomicina já foram descritos. Mesmo que os índices de resistência ainda sejam baixos, alguns estudos demonstraram que estes têm aumentado com o decorrer dos anos, como é o caso de Peretz et al. (2019) que apontaram taxas resistência de 20.7% em 2015 e 30.9% em 2016 em bactérias gram-negativas (PERETZ et al., 2019).

Os mecanismos de resistência à fosfomicina envolvem, principalmente:

i. Redução da permeabilidade da membrana: a) mutações nos genes codificadores dos sistemas de transporte (GlpT e UhpT) responsáveis pela entrada do antimicrobiano na célula bacteriana, bem como mutações em seus genes regulatórios, como *uhpA*; b) mutações em *ptsI* e *cyaA* provocam uma redução dos níveis intracelulares de AMP cíclico, o que acarreta em uma menor expressão de GlpT e UhpT (CASTAÑEDA-GARCÍA; BLÁZQUEZ; RODRÍGUEZ-ROJAS, 2013a; FALAGAS et al., 2019);

ii. Modificações no alvo do antimicrobiano: a) mutações em MurA, principalmente substituição do resíduo Cys115 por aspartato; b) aumento da transcrição de MurA, embora esse mecanismo nunca tenha sido reportado em isolados clínicos (FALAGAS et al., 2019);

iii. Modificações na molécula de fosfomicina: enzimas conhecidas como fosfomicinases (Fos) e fosfomicina-quinases (Fom) respectivamente rompem e modificam o anel epóxido presente na estrutura do antimicrobiano, inativando-o (CASTAÑEDA-GARCÍA; BLÁZQUEZ; RODRÍGUEZ-ROJAS, 2013a; FALAGAS et al., 2019; YANG; LU; TSENG, 2019).

É importante ressaltar que mutações no gene que codifica a enzima MurA são raras, assim como mutações genéticas que proporcionam modificações estruturais em GlpT e UhpT. Além disso, esses mecanismos de resistência não são apontados como biologicamente importantes para as bactérias, por conta do alto custo biológico que eles geram para esses microrganismos (NILSSON et al., 2003; SILVER, 2017).

Sendo assim, o mecanismo de maior destaque e alvo da maioria dos estudos, é a produção das enzimas inativadoras deste antimicrobiano: as fosfomicinases e as fosfomicina-quinases. As fosfomicina-quinases (FomA, FomB, FosC, FosC2) fosforilam o grupo fosfonato na

molécula de fosfomicina, inativando-a (DÍEZ-AGUILAR, 2019). Já as fosfomicinases, rompem o anel epóxido presente na molécula, também inativando-a. Dentre as fosfomicinases, a primeira a ser identificada foi a FosA (MENDOZA et al., 1980), uma glutationa-S-transferase dependente de Mn^{2+}. Desta, originou-se a superfamília FosA, que é composta por 10 variantes (FosA1-10) (YANG; LU; TSENG, 2019). Enzimas FosA codificadas por genes *fosA* plasmideais têm demonstrado exercer importante papel na resistência à fosfomicina, apresentando CIM acima de 256 μg/mL em cepas de *E. coli* (ponto de corte de sensibilidade BRCAST, < 8 μg/mL para uso via oral e < 32μg/mL para uso endovenoso) e de *K. pneumoniae* (ponto de corte de sensibilidade BRCAST, < 32μg/mL para uso endovenoso) (BRCAST - BRAZILIAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING, 2022). O impacto da resistência causada por essas enzimas fica evidente em testes de sensibilidade realizados na presença de fosfonoformato (Foscarnet®), um reconhecido inibidor de FosA, quando as CIMs para fosfomicina são reduzidas em mais do que três diluições (TOMICH et al., 2019).

O gene *fosA* plasmideal é frequentemente encontrado em um contexto genético característico, sendo que o gene *lysR* apresenta-se truncado por sequências de inserção (IS), e por isso o *locus lysR-fosA* apresenta-se flanqueado à montante e/ou à jusante, normalmente por IS10 (KIEFFER et al., 2020a).

Algumas espécies bacterianas, entretanto, são consideradas reservatórios naturais de genes *fos,* como é o caso da *K. pneumoniae* que apresenta o gene *fosA* intrinsecamente em seu cromossomo. O gene $fosA^{KP}$ está localizado à jusante do gene regulador *lysR,* sobrepondo-se à sua porção final. Baseados na presença desse gene cromossômico, alguns clínicos não recomendam o uso da fosfomicina para o tratamento de infecções causadas por *K. pneumoniae*, inferindo que esse determinante seja um fator de predisposição ao desenvolvimento de resistência a esse antimicrobiano (ITO et al., 2017b; KIEFFER et al., 2020a). A Sociedade Americana de Doenças Infecciosas (IDSA, do inglês "*Infectious Diseases Society of America")*, inclusive, sugere que a fosfomicina não seja utilizada para tratamento de infecções por *K. pneumoniae* por conta desse determinante cromossômico (TAMMA et al., 2022). Entretanto, apesar de apresentar esse gene intrinsecamente em seu cromossomo, cepas selvagens de *K.*

pneumoniae se mantém sensíveis à fosfomicina, apresentando CIM normalmente < 16 μg/mL, o que sugere que este gene cromossômico não seja expresso ou seja pouco expresso nessas cepas. De fato, pouco se sabe sobre a influência da expressão do gene *fosA* em *K. pneumoniae* sobre o perfil de sensibilidade à fosfomicina. Até o presente momento, apenas um relato de resistência à fosfomicina atribuído ao gene $fosA^{KP}$ cromossômico de *K. pneumoniae* foi reportado, sendo associado a um promotor localizado em uma sequência de inserção IS*Ecp1* à montante do *locus lysR-fosA* (KIEFFER et al., 2020a).

Interessantemente, durante o presente estudo, observou-se que o gene *fosA* cromossômico em *K. pneumoniae* é bastante conservado entre as diferentes linhagens dessa espécie, de modo que cada STs ou CC apresenta a mesma sequência de *fosA* (RIBEIRO et al., 2022). Essa observação deu origem ao **Artigo científico 1** contido no item Resultados Parciais.

2.4 Uso clínico de fosfomicina em combinação com outros antimicrobianos

Recentemente, diferentes combinações entre fosfomicina e outros antimicrobianos têm sido exploradas contra diferentes patógenos. Scudeller et al. (2021) elencaram em sua revisão sistemática o uso de fosfomicina em combinação com as polimixinas como sendo as mais bem exploradas. Nesse estudo, eles apontaram taxas de sinergismo altas e moderadas entre fosfomicina e polimixinas contra *K. pneumoniae*, além de discutirem estudos que obtiveram bons resultados dessa combinação contra isolados de *Acinetobacter baumannii* (SCUDELLER et al., 2021). Ademais, Antonello et al. (2020) apontou em sua revisão notáveis interações sinérgicas entre fosfomicina e diferentes antimicrobianos contra diferentes patógenos, destacando-se as combinações com β-lactâmicos contra Enterobacterales e *Pseudomonas aeruginosa,* com polimixinas contra *K. pneumoniae* e com daptomicina e linezolida contra *Staphylococcus* spp (ANTONELLO et al., 2020).

Diversos relatos de caso, estudos clínicos e observacionais reportaram sucesso no uso de fosfomicina em combinação com outros antimicrobianos como terapia alternativa contra infecções de difícil tratamento. Como exemplo, podemos citar o uso de fosfomicina combinada com meropenem-vaborbactam ou com ceftazidima-avibactam para o tratamento de infecções graves causadas por *K. pneumoniae* produtora de KPC (OLIVA et al., 2022, 2021); também

fosfomicina e ceftazidima-avibactam para tratamento de pneumonia associada à ventilação mecânica causada por *Stenotrophomonas maltophilia* (BURASTERO et al., 2022); e fosfomicina combinada com ceftazidima tratando infecção causada por *Serratia marcescens* produtora de KPC (RODRÍGUEZ et al., 2018), dentre outros.

Recentemente, estudos brasileiros também exploraram a atividade *in vitro* de fosfomicina em combinação com β-lactâmicos contra *Pseudomonas aeruginosa* e *K. pneumoniae* resistentes aos carbapenêmicos. Albiero et al. (2019) e Cuba et al. (2020) obtiveram resultados sinérgicos entre fosfomicina e meropenem contra *K. pneumoniae*, e entre fosfomicina e ceftolozane-tazobactam contra *P. aeruginosa,* respectivamente (ALBIERO et al., 2019; CUBA et al., 2020).

Em 2016, Albiero et al. Avaliaram a atividade de fosfomicina combinada com meropenem contra *K. pneumoniae* produtoras de KPC isoladas entre 2008 e 2010 e pertencentes aos STs 11, 17, 70, 133, 340, 437 e 617, obtendo resultados sinérgicos com importante redução da CIM_{50} (ALBIERO et al., 2016).

Entretanto, estudos brasileiros explorando combinações de fosfomicina com outros antimicrobianos ainda são escassos, evidenciando a latente necessidade de dados que possam avaliar a utilidade clínica potencial da fosfomicina para quando for aprovada para uso endovenoso no Brasil.

2.5 *Klebsiella pneumoniae*, um patógeno de grande importância clínica

Klebsiella pneumoniae é uma espécie bacteriana pertencente à ordem Enterobacterales e faz parte da microbiota intestinal humana. Entretanto, esse microrganismo pode configurar um patógeno oportunista, causando diferentes tipos de infecções, como ITUs, pneumonias e infecções de corrente sanguínea, sendo atrelada a altos índices de morbi-mortalidade, especialmente em ambientes nosocomiais (ANDREY et al., 2020; MURRAY et al., 2022).

Recentemente, foi estimado que 150 mil mortes no mundo foram atribuídas diretamente a *K. pneumoniae* resistente, dentre elas 55.700 mortes causadas por *K. pneumoniae* resistente aos cabapenêmicos somente no ano de 2019 (MURRAY et al., 2022). No Brasil, de acordo com

dados epidemiológicos da ANVISA, *K. pneumoniae* é o segundo patógeno mais frequentemente isolado de infecções de corrente sanguínea associadas a cateter venoso central em pacientes adultos internados em UTIs. Destes isolados, 63,2% apresentaram-se resistentes aos carbapenêmicos e 15% à polimixina B (ANVISA, 2021). Andrey e colaboradores (2020) observaram uma taxa de mortalidade em 30 dias de 60% em pacientes com infecção de corrente sanguínea causados por *K. pneumoniae* resistente aos carbapenêmicos internados em um hospital terciário brasileiro evidenciando a importância desse patógeno no Brasil (ANDREY et al., 2020). Ainda, um recente estudo multicêntrico apontou taxas de mortalidade em 30 dias por *K. pneumoniae* resistente aos carbapenêmicos de 23% nos Estados Unidos da América e 28% na América do Sul, demonstrando a importância desse patógeno como uma grande ameaça à saúde pública em âmbito global (WANG et al., 2021).

Reconhecida como uma das mais altas prioridades para pesquisa e desenvolvimento de novos antimicrobianos, *K. pneumoniae* resistente aos carbapenêmicos possui um importante papel na disseminação de genes de resistência intercontinentalmente, especialmente aqueles que codificam ESBL e carbapenemases, com destaque para os genes *bla*$_{\text{CTX-M}}$ e *bla*$_{\text{KPC}}$, respectivamente. A carbapenemase mais frequente entre linhagens de *K. pneumoniae* resistentes aos carbapenêmicos é a *Klebsiella pneumoniae* carbapenemase (KPC), e sua disseminação mundial tem sido atrelada a clones específicos dessa espécie, como as pertencentes ao complexo clonal (CC) 258, que compreende os STs 11, 258, 340, 437, por exemplo, e também aos STs 15, 101 e 16 (LEE et al., 2016; WORLD HEALTH ORGANIZATION, 2017). Além disso, nos últimos anos têm sido observada uma crescente nas taxas de detecção de *K. pneumoniae* produtora de NDM, com destaque para clones emergentes como o ST 307 (CEJAS et al., 2022). Preocupantemente, no cenário durante e pós-pandemia COVID-19, houve um aumento no relato de casos de detecção de NDM, bem como de co-produção de KPC e NDM, tornando cada vez mais restritas as opções terapêuticas disponíveis (BRASIL, 2022; VÁSQUEZ-PONCE et al., 2022; WINK et al., 2021).

Agravando ainda mais o panorama, *K. pneumoniae* resistente aos carbapenêmicos pode apresentar-se hipervirulenta, driblando o sistema imune do hospedeiro e a ação dos antimicrobianos. Recentemente foi reportado na China um primeiro surto causado por *K.*

pneumoniae ST11 hipervirulenta. A aquisição do plasmídeo de virulência do tipo pLVPK-like por cepas ST11 resistentes aos carbapenêmicos, rapidamente tornou uma cepa de *K. pneumoniae* ST11 "normal" em uma cepa resistente aos carbapenêmicos e hipervirulenta (GU et al., 2018). Apesar desse episódio ter ocorrido na China, ele pode ter grande impacto no Brasil, uma vez que o ST 11 é predominante na América do Sul e essas cepas apresentam alta capacidade de disseminação (WANG et al., 2021).

Nesse contexto, o combate às infecções por linhagens causadas por *K. pneumoniae* resistentes aos carbapenêmicos, majoritariamente apresentando um fenótipo de multirresistência, tem se tornado cada vez mais difícil. Nos últimos anos, foram aprovados para uso clínico novos antimicrobianos, especialmente novos inibidores de β-lactamases associados com β-lactâmicos já comercializados, como ceftazidima-avibactam, meropenem-vaborbactam e imipenem-relebactam, que possuem ação contra carbapenemases de classe A, como a KPC, sendo que a ceftazidima-avibactam também mantém ação contra OXA-48. Ainda, pode-se citar o cefiderocol, um novo antimicrobiano que possui atividade contra isolados produtores de metalo-β-lactamases (CARCIONE et al., 2021; KARAISKOS et al., 2019; WANG et al., 2022b). Entretanto, no Brasil, até o presente momento, somente a combinação ceftazidima-avibactam foi aprovada para uso clínico e está disponível para comercialização, apresentando ainda um alto valor agregado, o que inviabiliza o seu uso na maioria dos casos.

Ademais, associados aos altos índices de resistência bacteriana, ao restrito número de novos antimicrobianos desenvolvidos, aos altos preços atribuídos a estes fármacos e ao processo demorado de aprovação para uso clínico no Brasil, muitas vezes depara-se com a falta de antimicrobianos específicos, como o episódio ocorrido em 2021, quando enfrentou-se um período de desabastecimento de polimixina B no mercado brasileiro, afetando principalmente os serviços públicos de saúde, que ainda possuem um acesso restrito aos novos antimicrobianos, como ceftazidima-avibactam. Circunstâncias desse tipo requerem dos clínicos decisão rápida para indicar alternativas de tratamento. Portanto, muito se tem discutido e estudado a respeito do uso de combinações de diferentes antimicrobianos que já fazem parte do cotidiano clínico, como uma alternativa ao combate às infecções causadas por patógenos multirresistentes (SUGATHAN; MANDAL, 2019).

Nesse contexto, mundialmente passou-se a explorar combinações de diferentes antimicrobianos já presentes no uso clínico. Um desses antimicrobianos é a fosfomicina, um antibiótico antigo, por muito tempo destinado apenas ao tratamento de ITUs, mas que recentemente recebeu atenção como uma alternativa para o tratamento de infecções sistêmicas causadas por patógenos de difícil tratamento, como *K. pneumoniae* resistente aos carbapenêmicos (FALAGAS et al., 2010a).

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar a atividade de fosfomicina isolada e em combinação com outros antimicrobianos contra *K. pneumoniae* produtoras de KPC e resistentes à polimixina B isoladas no Brasil, bem como pesquisar a expressão do gene *fosA* cromossômico e seu impacto no fenótipo de resistência à fosfomicina nesse patógeno.

3.2 Objetivos Específicos

3.2.1 Analisar as sequências de *fosA* cromossômico em diferentes linhagens de *K. pneumoniae*, bem como o contexto genético envolvido – **Artigo científico 1.**

3.2.2 Avaliar a atividade *in vitro* e *in vivo* de fosfomicina isolada e combinada com meropenem ou polimixina B contra diferentes clones brasileiros de *K. pneumoniae* produtores de KPC e resistentes às polimixinas, bem como observar o efeito dessas combinações sobre o biofilme bacteriano – **Artigo científico 2.**

3.2.3 Comparar a expressão de *fosA* cromossômico em isolados clínicos de *K. pneumoniae*, bem como analisar e caracterizar o contexto genético de *fosA* em isolados resistentes à fosfomicina – **Artigo científico 3.**

4. RESULTADOS

Os resultados desse estudo serão apresentados na forma de três artigos científicos, sendo que o **ARTIGO 1** foi publicado em 1 de novembro de 2022, na revista *Microbial Drug* Resistance (Fator de Impacto: 2.706), o **ARTIGO 2** foi publicado em 23 de janeiro de 2023 na revista *Antibiotics* (Fator de Impacto: 5.222), e o **ARTIGO 3** será submetido na revista *Journal of Global Antimicrobial Resistance* (Fator de Impacto: 4.349).

4.1 O **ARTIGO 1** trata da análise das sequências de *fosA* cromossômico em *K. pneumoniae* pertencentes a diferentes STs. O trabalho obteve como principal resultado uma alternativa de baixo custo para auxiliar na tipagem de linhagens de *K. pneumoniae* disseminadas em todo o mundo e, portanto, de grande importância epidemiológica. Essa nova ferramenta, além de mais rápida, seria uma opção economicamente mais acessível para países de baixa e média renda, que não possuem proventos suficientes para utilizar a tipagem por metodologias convencionais. De maneira geral, é uma ferramenta eficiente para discriminar o CC258 de outros clones de *K. pneumoniae.*

4.2 O **ARTIGO 2** traz como resultados a análise da atividade de fosfomicina em monoterapia e em combinação com meropenem ou polimixina B contra diferentes linhagens brasileiras de *K. pneumoniae* que albergam diferentes genes de resistência. Os experimentos foram realizados *in vitro* pelas metodologias de *Checkerboard* e *Time-kill*, e *in vivo* por meio do modelo de sobrevivência em *Galleria mellonella.* Ainda, foi avaliada a atividade desses antimicrobianos isolados e em combinação contra o biofilme bacteriano.

4.3 O **ARTIGO 3** explora os diferentes determinantes de resistência à fosfomicina em isolados clínicos de *K. pneumoniae.* Por meio da análise dos dados do sequenciamento de genoma completo, dos resultados de testes fenotípicos para detecção da produção de FosA e dos resultados obtidos por PCR quantitativo em tempo real para análise da expressão de *fosA*, com o objetivo de comparar a expressão de *fosA* cromossômico em isolados clínicos de *K. pneumoniae*, bem como analisar e caracterizar o contexto genético de fosA em isolados resistentes à fosfomicina .

ARTIGO 1 - Sequencing of *fosA*: A Rapid and Inexpensive Method for discriminating *Klebsiella pneumoniae* CC258 from other Clones

(https://doi.org/10.1089/mdr.2022.0081)

Ághata Cardoso da Silva Ribeiro[1*], Fernanda Fernandes Santos[1], Ikechukwu Benjamin Moses[1,3], Luciene Andrade da Rocha Minarini[2], Ana Cristina Gales[1*]

[1]Laboratório Alerta. Division of Infectious Diseases, Department of Internal Medicine. Escola Paulista de Medicina/Universidade Federal de São Paulo - UNIFESP, Rua Pedro de Toledo, 781, São Paulo-SP, Brazil.

[2]Universidade Federal de São Paulo - UNIFESP, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Laboratório Multidisciplinar em Saúde e Meio Ambiente, Departamento de Ciências Farmacêuticas, Rua São Nicolau, 210, Diadema - SP, Brazil.

[3]Department of Applied Microbiology, Faculty of Science, Ebonyi State University, P.M.B. 053, Abakaliki, Nigeria.

**Corresponding Author*
e-mail address: aghata.cardoso@unifesp.br
Universidade Federal de São Paulo
Rua Pedro de Toledo, 781 - 6º andar
Vila Clementino - 04025-010 - São Paulo – SP

Abstract

Typing carbapenem-resistant *Klebsiella pneumoniae* (CR-KPN) is crucial in controlling their dissemination and solving outbreaks. In this context, we searched for an effective, faster, and cheaper alternative technique to type KPN by analyzing the *fosA*^{KP} sequence. We analyzed the nucleotide (nt) sequences of chromosomal *fosA*^{KP} gene in 350 KPN genomes (70 per ST or CC). Assembly genomes were randomly downloaded from NCBI and annotated using RAST in PATRIC platform. The isolates STs were verified using MLST 2.0 by CGE. Chromosomally encoded *fosA*^{KP} was confirmed in MLplasmid, and the sequences alignment were performed in Clustal Omega. The amino acid sequences (aa) were analyzed using SNAP2 and SMART platforms. Out of the 70 genomes analyzed for each ST/CC, we observed 100% *fosA* sequence identity for CC258/11, ST15, ST307 and ST101. For ST16, only two *fosA* sequences were

different from each other. We observed differences in aa sequences at positions 25 and 79 (ST16) and 86 (ST16, ST101). The C-terminal (aa 138, 139, 140) was different for each cluster. None of these polymorphisms is related to the protein active site. Moreover, L25Q (ST16) polymorphism was predicted to probably affect the protein function. We observed that chromosomal *fosA*^{KP} sequences from KPN are highly conserved in ST15, ST307, ST16, ST101, and CC258/11, suggesting *fosA*^{KP} sequencing as an alternative, easier, faster, and less expensive technique in identifying epidemiological STs for KPN, and discriminating them from CC258/11.

Keywords: *Klebsiella pneumoniae*; *fosA* sequence; sequence typing; typing technique.

Introduction

Klebsiella pneumoniae (KPN) is ubiquitous enterobacteria found in animals, humans, and the environment. Besides being a member of the human microbiota, this species can be pathogenic and cause difficult-to-treat infections [1]. Carbapenem-resistant *K. pneumoniae* (CR-KPN) has been recognized as a major public health problem because it has been associated with high *morbidity and mortality rates,* especially due to carbapenemase production [2]. Currently, multilocus sequence typing (MLST) technique is used to get a comprehensive understanding of the global CR-KPN epidemiology (www.pasteur.fr/mlst/Kpneumoniae.html). The global dissemination of *Klebsiella pneumoniae* carbapenemase (KPC) has been attributed to specific clones of KPN such as Clonal Complex (CC) 258/11, Sequence Type (ST) 16, ST15, and ST101, with ST258 being the most prevalent worldwide [2,3]. In the last years, ST307 has gained importance because its widely spread around the world and associated with the emergence of ceftazidime/avibactam resistance [4]. In addition, KPN ST11 clones carrying *bla*$_{OXA-48}$ gene have been frequently reported in Spain, Argentina, and Turkey. In contrast, *bla*$_{OXA-48}$ was observed in KPN ST15 and ST101 in the USA, Spain, Czech Republic, Libya, India, Germany, Finland, France, and Japan [3]. Reports from various countries in Asia and Europe showed that KPN ST11 clones also harbored *bla*$_{NDM}$ gene [1]. *bla*$_{NDM}$ was also found in KPN ST340 in Spain, Serbia, and Korea, and ST437 in Singapore, Pakistan, and Spain [1,2].

Although KPC-2-producing KPN CC258/11 was endemic in many Brazilian hospitals before COVID-19 pandemic, the emergence of "new" STs was also reported [3,5]. Furthermore, KPC-2 producing KPN ST16 was associated with a higher virulence and 30-day mortality rate [3]. Although Whole Genome Sequence (WGS) has been widely applied for outbreak investigations worldwide, the turnaround time for results availability is sometimes long. In addition, this methodology is expensive for most institutions in low-and middle-income countries (LMIC). For this reason, it would be highly desirable to have an easier, faster, and less expensive alternative technique to easily discriminate new emerging STs. In fact, during the COVID-19 pandemic, an alarming increase in New Delhi Metallo-beta-lactamase (NDM)-producing KPN were detected in many Latin American countries, including Brazil [6,7].

$FosA^{KP}$ is a chromosomally encoded glutathione transferase which inactivates fosfomycin in KPN [8]. The *fosA*^{KP} gene is located downstream of the transcriptional regulator *lysR* gene, overlapping its end [9]. Furthermore, *fosA*^{KP} can be mobilized by mobile genetic elements to other species; especially *Escherichia coli*, leading to fosfomycin resistance [8]. In the present study, we evaluated the sequencing of *fosA*^{KP} as an alternative for discriminating KPN CC258/11 from other STs.

Material and Methods

Bacterial Isolates and Genomes analysis

A total of 23 KPC-2-producing KPN strains belonging to six different STs were previously characterized [3]. During that analysis, it was noticed that KPN not belonging to the CC258/11 had distinct *fosA* sequences. To evaluate the correlation between *fosA* sequence and specific STs, we included other 327 KPN genomes obtained from Pathosystems Resource Integration Center (PATRIC) and National Center for Biotechnology Information (NCBI) data banks in our analysis, summing-up to 350 KPN genomes (70 KPN strains each from CC258/11, ST15, ST16, ST101 and ST307).

The 280 KPN genomes were annotated using RAST in PATRIC platform (https://www.patricbrc.org/app/Annotation) while the sequence of *fosA*^{KP} gene was obtained in fasta format and the sequences were then compared by nucleotide sequence alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and MView (https://www.ebi.ac.uk/Tools/msa/mview/). Furthermore, the ST of each strain was confirmed using MLST 2.0 by Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/services/). Table S1 shows the accession numbers of the strains included in this study. The chromosomal location of *fosA*^{KP} was confirmed by MLplasmid® software (https://sarredondo.shinyapps.io/mlplasmids/). The SNAP2 software was used to estimate the functional effects caused by amino acid changes (https://rostlab.org/services/snap2web/). In addition, the protein domain was searched by SMART platform (http://smart.embl-heidelberg.de/).

Results

fosA gene nucleotide sequence analysis

After performing the nucleotide and amino acid sequence alignment of *fosA* and FosA respectively for the first 23 KPN strains, four clusters were identified, and all the strains belonging to a specific cluster shared identical *fosA* nucleotide and FosA amino acid sequences. Interestingly, each cluster corresponded to a ST or CC, being divided into CC258/11, ST16, ST15, and ST101.

By enlarging the number of KPN representatives, more genomes were downloaded, annotated, and analyzed, amounting to 70 strains in each cluster including ST307, an emergent ST around the world. The alignment among the *fosA*^{KP} sequences of each ST/CC was showed in Figure 1. The percentage of *fosA*^{KP} identity among the STs obtained by *fosA*^{KP} sequence analysis of each ST and the distance between each cluster was depicted in Figure 2. For CC258/11 represented by isolates of ST258, ST11, ST340, and ST437, the 70 *fosA*^{KP} gene and FosA protein sequences shared 100 % identity, and the same was observed for ST15, ST101, and ST307. Only two (2.8 %) out of the 70 KPN ST16 strains showed different *fosA*^{KP}

sequences, sharing 95.0 % nucleotide and 96.4 % amino acid identities. These two sequences shared 100 % identity with *fosA*^{KP} genes from KPN ST17, a closely related sequence type. Furthermore, we observed that *fosA*^{KP} identity between ST15 and ST307 was 100% (Figure 2).

FosA protein amino acids sequence analysis

After performing the FosA alignment from the different STs and the previously reported FosA variants recognized to be originated from KPN [FosA5, FosA6, and FosA10] [10–12], we observed the presence of 139 amino acids in FosA from ST15, ST101, ST307 and CC258/11, while FosA from ST16 presented 140 amino acids. Furthermore, we found amino acid polymorphisms as L25Q and S79R changes in ST16, and A86V change in ST16 and ST101. At position 91, the CC258/11, ST16, and ST101 showed the amino acid valine (V) while ST15 and ST307 had an isoleucine (I) like FosA6 and FosA10. It is important to highlight that none of these polymorphisms were within the protein active site [13], but in the glyoxalase protein domain, according to Pfam 34.0 [14]. Also, the final portion of the amino acid sequences (residues 138, 139, and 140) varied (Figure 3).

Moreover, a single polymorphism was predicted by SNAP2 to probably affect the protein function: the FosA from ST16 presented the amino acid glutamine instead of leucine (L25Q) as presented by ST15, ST101, CC258/11, ST307, FosA5, FosA6, and FosA10.

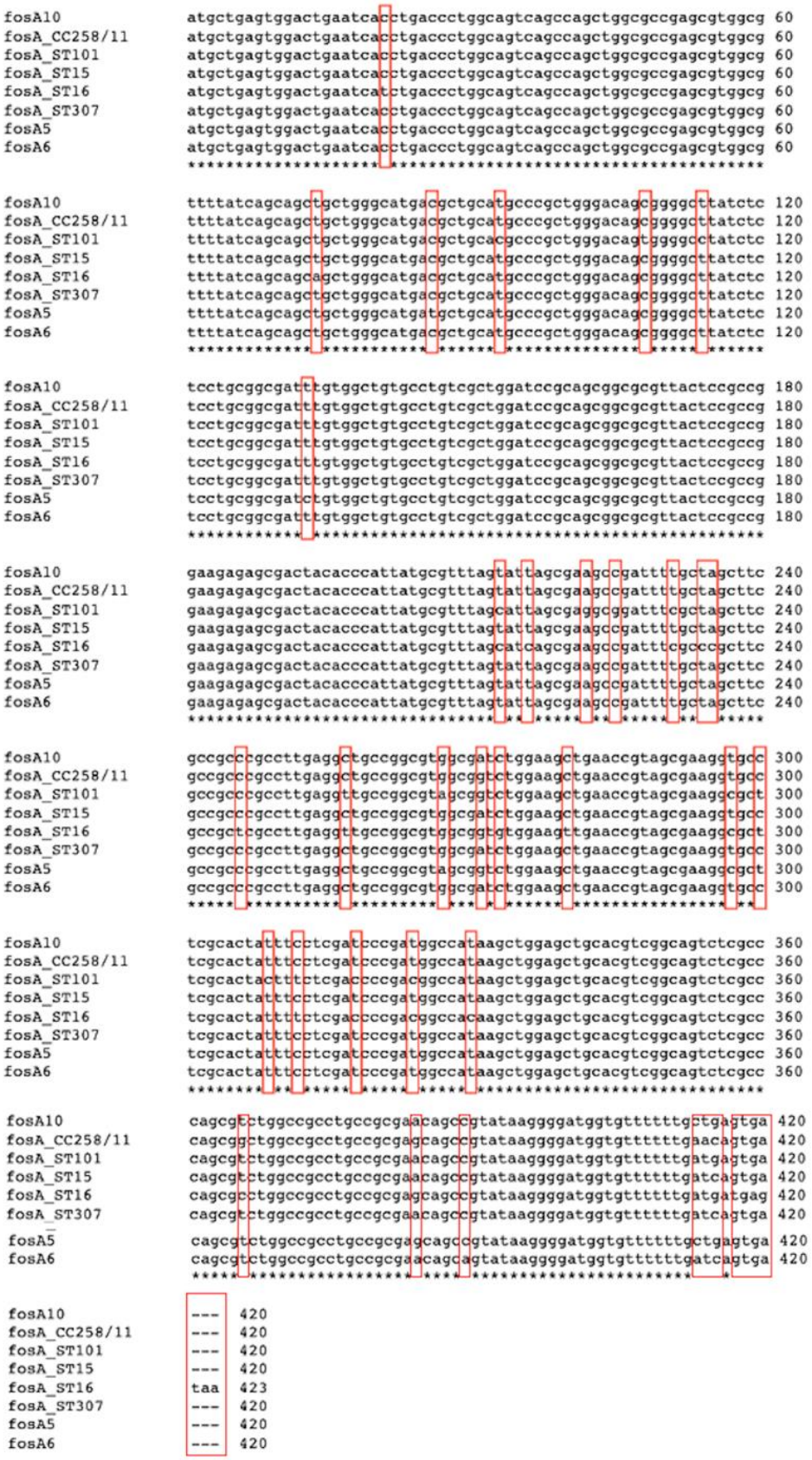

Figure 1 - Alignment between the STs. The nucleotides in the boxes are those presenting mutations in at least one of the sequences. ST, sequence type.

	*fosA*_ST16	*fosA*_ST101	*fosA*_ST307	*fosA*_CC258/11
*fosA*_ST15	93.4%	96.0%	100%	99.0%
*fosA*_ST16	-	94.1%	94.0%	93.6%
*fosA*_ST101	-	-	96.0%	95.5%
*fosA*_ST307	-	-	-	99.0%

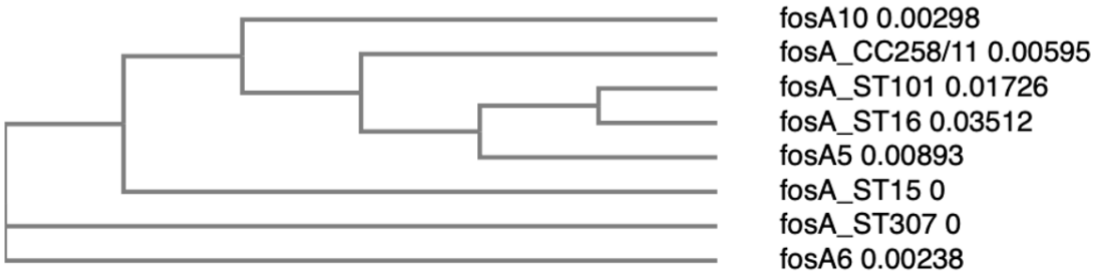

Figure 2 - Phylogram comparing the *fosA* gene from the different clusters and *fosA5, fosA6, fosA10* and the identity percentages among STs/CCs. CCs, clonal complexes.

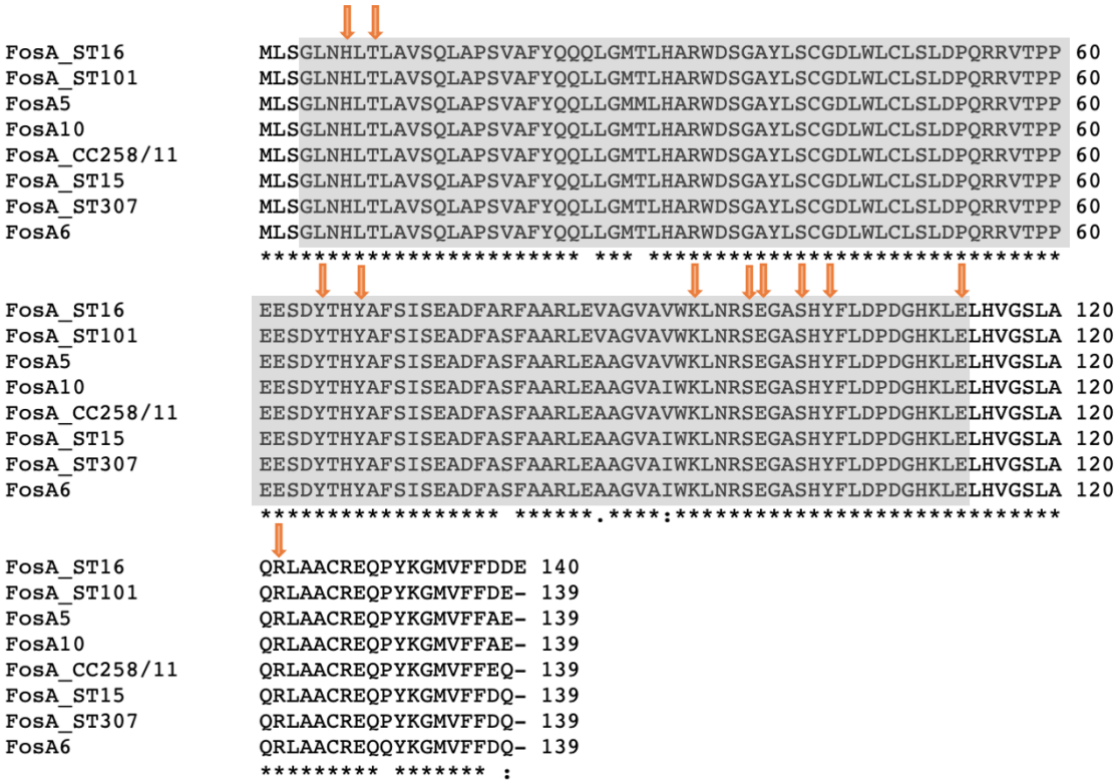

Figure 3 - Alignment among the FosA protein of each cluster and FosA5, FosA6, and FosA10. The arrows indicate the residues of the protein active site. In gray indicates the glyoxalase domain. The (*) indicates the conserved amino acids among the different FosA sequences.

Discussion

Recent studies have shown increasing rates of CR-KPN worldwide [15]. Although CR-KPN CC258/11 has been responsible for the worldwide spread of KPC, emerging STs have been reported to cause novel outbreaks [3, 16]. In addition, other CR-KPN STs carrying *bla*$_{NDM}$ have been observed [5,17]. Furthermore, there is evidence that different STs or CC vary across distinct geographical regions, raising the need for better CR-KPN characterization [15,18]. In this manner, it is important to rapidly and precisely identify CR-KPN to discriminate outbreaks from those caused by CR-KPN CC258/11, as well as to identify clinical outbreaks, prevent their spread, and evaluate possible differences in clinical outcomes [1,2].

Despite recent advances in WGS, MLST has been a technique commonly employed for microbiological characterization of KPN isolates. It involves sequencing of seven different housekeeping genes: *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*. In addition to being laborious, MLST can cost between $25 and $60 per isolate in Brazil, which is expensive for most clinical settings, especially in LMIC. In this manner, alternative typing methods to identify CR-KPN clones have been searched. Pena *et al*. (2020) recently reported the use of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) as an alternative way to type ST11 and ST101 CR-KPN, thus reducing the costs and turnaround time required for typing results [20].

In the present work, we analyzed *fosA*^{KP} sequence, a chromosomally encoded gene in *K. pneumoniae*, as an alternative typing method to distinguish CC258/11 from other STs. We observed that the *fosA*^{KP} sequence was highly conserved in ST15, ST16, ST101, ST307 and CC258/11, indicating that *fosA*^{KP} could be a surrogate marker to identify distinct STs associated with outbreaks. In contrast, it was not possible the differentiation between ST15 and ST307 because they share the same *fosA*^{KP} sequence. However, the specific *fosA*^{KP} sequence found in ST15 and ST307 could early warn about possible emergence of these two important STs. ST307 is an emergent and important *K. pneumoniae* ST related to the emergence of ceftazidime/avibactam resistance [4] and ST 15 which carries a recently identified new

adhesion system, Kpi. This adhesion system favors the strain persistence in the nosocomial environment [21].

By analyzing the FosA sequences from ST15, ST16, ST101, ST307 and CC258/11, we observed that the polymorphisms in the amino acid sequences of FosA were not associated with residues of protein active site, possibly indicating no changes in the interactions between FosA and its ligands. Klontz *et al*. (2017) studied the crystalized $FosA^{KP}$ structure and amino acid sequence, and reported residues H7, H67, E98, and E113 as important to interaction with the divalent cation; T9, K93, S97, S101, Y103, and R122 as coordinating the fosfomycin binding; and Y65 which connects the active site and the dimer loop [13]. These residues are not related to the polymorphisms observed in the present study.

Among the polymorphisms observed, only the L25Q in $FosA^{KP}$ from ST16 is supposed to affect the protein function. Even though this change is not related to the protein active site, the replacement of a charged for an uncharged amino acid could alter the protein function and consequently its efficiency [22].

Herein, we demonstrated that the $fosA^{KP}$ sequencing is a promising surrogate marker to discriminate CR-KPN CC258/11 from other clones. By sequencing only $fosA^{KP}$, the amount of work could be significantly reduced as well as the amount spent for this purpose; from \$25-\$60 to \$3-6 per isolate. In addition, the time spent to achieve the isolate's ST would be shorter, resulting in faster introduction of controlling measures to limit the CR-KPN dissemination.

It is important to highlight that even *fosA* is a fosfomycin resistance gene and typing bacteria through resistance genes is not usual, a previous study have already been successful using resistance genes to type bacteria [23].

However, our study has limitations. We included in our analysis the most frequent STs reported in high-risk KPN clones, but the number of STs evaluated was low and could be further expanded in future studies. Furthermore, although chromosomal $fosA^{KP}$ gene capture from KPN by plasmids is possible, to the best of our knowledge no report of plasmid encoded $fosA^{KP}$ (or

its variants *fosA5/fosA6/fosA10*) in this species has been documented so far. Furthermore, when located in plasmids, the *fosA* regulator, *lysR* gene, is usually truncated by an Insertion Sequence (IS), whereas in the chromosome, the *lysR* is intact. Thus, we could solve this doubt, designing primers targeting *lysR-fosA* sequence and based on the size of the amplicon suggest the *fosA* location (plasmid or chromosome). Also, it is important to highlight that the usually found plasmid-enconded *fosA* in KPN is the *fosA3* variant, which can be easily discriminated from *fosA*^{KP} by polymerase-chain reaction (PCR) [9] or sequencing, the technique chosen in the present study.

Finally, chromosomally encoded *fosA* has also been reported in other bacterial species. Ito *et al*. (2017) identified high frequencies of chromosomal *fosA* gene in *Providencia stuartii* (100 %), *Serratia marcescens* (99.7 %), *Pseudomonas aeruginosa* (98.8 %), *Klebsiella aerogenes* (98.4 %), *Klebsiella oxytoca* (96.6 %), and *Morganella morganii* (90.5 %) [8]. However, more investigation is needed to fully understand and confirm the chromosomal location of *fosA* genes in these species and to verify if the *fosA* sequence could also be applied for typing.

Therefore, new studies focusing on the analysis of *fosA* gene sequences are needed to confirm our findings and the roles of *fosA* as an alternative typing tool.

Conclusions

Our study showed that *fosA*^{KP} is a highly conserved gene in *K. pneumoniae* and its nucleotide sequence could be used as an alternative technique to discriminate CR-KPN CC258/11 clones from other clones, as well as identifying clones from ST16, ST15, ST307 and ST101. Thus, by becoming available, this alternative technique would be easier, faster, and less expensive, especially for LMIC, for typing high-risk *K. pneumoniae* clones.

Acknowledgements

We would like to thank the researchers: Dr. Willames M. B. S. Martins and Dr. Diego O. Andrey for providing the genome sequencing data. Also, we are grateful to the Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing grants to A.C.S.R. (PDS), F.F.S. (PNPD) and I.B.M (Young Talent).

References

[1]Safavi M, Bostanshirin N, Hajikhani B, Yaslianifard S, van Belkum A, Goudarzi M, et al. Global genotype distribution of human clinical isolates of New Delhi metallo-β-lactamase-producing Klebsiella pneumoniae; A systematic review. J Glob Antimicrob Resist 2020;23. https://doi.org/10.1016/j.jgar.2020.10.016.

[2]Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing Klebsiella pneumoniae: Epidemiology, genetic context, treatment options, and detection methods. Front Microbiol 2016;7:1–30. https://doi.org/10.3389/fmicb.2016.00895.

[3]Andrey DO, Dantas PP, Martins WBS, de Carvalho FM, Almeida LGP, Sands K, et al. An emerging clone, klebsiellapneumoniae carbapenemase 2-producing K. pneumoniae sequence type 16, associated with high mortality rates in a CC258-endemic setting. Clin Infect Dis 2020;71:E141–50. https://doi.org/10.1093/cid/ciz1095.

[4] Eger, E., Schwabe, M., Schulig, L., Hübner, N. O., Bohnert, J. A., Bornscheuer, U. T., et al. Extensively Drug-Resistant Klebsiella pneumoniae Counteracts Fitness and Virulence Costs That Accompanied Ceftazidime-Avibactam Resistance Acquisition. Microbiol Spectr, 2022, e0014822. https://doi.org/10.1128/spectrum.00148-22

[5] Silveira MC, Rocha-de-Souza CM, de Oliveira Santos IC, Pontes L da S, Oliveira TRT e., Tavares-Teixeira CB, et al. Genetic Basis of Antimicrobial Resistant Gram-Negative Bacteria Isolated From Bloodstream in Brazil. Front Med 2021;8. https://doi.org/10.3389/fmed.2021.635206.

[6] Raro OHF, da Silva RMC, Filho EMR, Sukiennik TCT, Stadnik C, Dias CAG, et al. Carbapenemase-Producing Klebsiella pneumoniae From Transplanted Patients in Brazil: Phylogeny, Resistome, Virulome and Mobile Genetic Elements Harboring blaKPC–2 or blaNDM–1. Front Microbiol 2020;11. https://doi.org/10.3389/fmicb.2020.01563.

[7] PAHO. Epidemiological Alert. Emergence and incrase of new combinations of carbapenemases in Enterobacterales in Latin America and the Caribbean. Https://WwwPahoOrg/En/Documents/Epidemiological-Alert-Emergence-and-Increase-New-Combinations-Carbapenemases 2021.

[8] Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, et al. Widespread Fosfomycin Resistance in Gram-Negative Bacteria Attributable to the Chromosomal fosA Gene. MBio 2017;8:1–9. https://doi.org/10.1128/mbio.00749-17.

[9] Kieffer N, Poirel L, Mueller L, Mancini S, Nordmann P. ISEcp1-mediated transposition leads to fosfomycin and broad-spectrum cephalosporin resistance in Klebsiella pneumoniae. Antimicrob Agents Chemother 2020;64(5). https://doi.org/10.1128/AAC.00150-20.

[10] Huang Y, Lin Q, Zhou Q, Lv L, Wan M, Gao X, et al. Identification of FOSA10, a novel plasmid-mediated fosfomycin resistance gene of klebsiella pneumoniae origin, in Escherichia coli. Infect Drug Resist 2020;13. https://doi.org/10.2147/IDR.S251360.

[11] Ma Y, Xu X, Guo Q, Wang P, Wang W, Wang M. Characterization of fosA5, A new plasmid-mediated fosfomycin resistance gene in Escherichia coli. Lett Appl Microbiol 2015;60. https://doi.org/10.1111/lam.12366.

[12] Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, et al. Glutathione-S-transferase FosA6 of Klebsiella pneumoniae origin conferring fosfomycin resistance in ESBL-producing Escherichia coli. J Antimicrob Chemother 2016;71. https://doi.org/10.1093/jac/dkw177.

[13] Klontz EH, Tomich AD, Günther S, Lemkul JA, Deredge D, Silverstein Z, et al. Structure and dynamics of FosA-mediated fosfomycin resistance in Klebsiella pneumoniae and Escherichia coli. Antimicrob Agents Chemother 2017;61. https://doi.org/10.1128/aac.01572-17.

[14] Mistry J, Chuguransky S, Williams L, Qureshi M, Salazar GA, Sonnhammer ELL, et al. Pfam: The protein families database in 2021. Nucleic Acids Res 2021;49. https://doi.org/10.1093/nar/gkaa913.

[15] Wang M, Earley M, Chen L, Hanson BM, Yu Y, Liu Z, et al. Clinical outcomes and bacterial characteristics of carbapenem-resistant Klebsiella pneumoniae complex among patients from different global regions (CRACKLE-2): a prospective, multicentre, cohort study. Lancet Infect Dis 2022;22(3):401-412. https://doi.org/10.1016/s1473-3099(21)00399-6.

[16] Heiden, S. E., Hübner, N. O., Bohnert, J. A., Heidecke, C. D., Kramer, A., Balau, V., et al. A Klebsiella pneumoniae ST307 outbreak clone from Germany demonstrates features of extensive drug resistance, hypermucoviscosity, and enhanced iron acquisition. Genome Med 2020; 12(1), 113. https://doi.org/10.1186/s13073-020-00814-6

[17] Cano-Martín E, Portillo-Calderón I, Pérez-Palacios P, Navarro-Marí JM, Fernández-Sierra MA, Gutiérrez-Fernández J. A study in a regional hospital of a mid-sized spanish city indicates a major increase in infection/colonization by carbapenem-resistant bacteria, coinciding with the covid-19 pandemic. Antibiotics 2021;10. https://doi.org/10.3390/antibiotics10091127.

[18] Wyres K, Holt K. Regional differences in carbapenem-resistant Klebsiella pneumoniae. Lancet Infect Dis 2021;22(3):309-310. https://doi.org/10.1016/s1473-3099(21)00425-4.

[19] Wang G, Zhao G, Chao X, Xie L, Wang H. The characteristic of virulence, biofilm and antibiotic resistance of klebsiella pneumoniae. Int J Environ Res Public Health 2020;17:1 – 17. https://doi.org/10.3390/ijerph17176278.

[20] Pena I, Pena-Vina E, Rodriguez-Avial I, Picazo JJ, Gómez-González Á, Culebras E. Comparison of performance of MALDI-TOF MS and MLST for biotyping carbapenemase-producing Klebsiella pneumoniae sequence types ST11 and ST101 isolates. Enferm Infecc Microbiol Clin 2020. https://doi.org/10.1016/j.eimc.2020.10.018.

[21] Gato, E., Vázquez-Ucha, J. C., Rumbo-Feal, S., et al. Kpi, a chaperone-usher pili system associated with the worldwide-disseminated high-risk clone Klebsiella pneumoniae ST-15. Proc Natl Acad Sci U S A 2020, 117(29), 17249–17259. https://doi.org/10.1073/pnas.1921393117

[22] Santos FF, Yamamoto D, Abe CM, Bryant JA, Hernandes RT, Kitamura FC, et al. The Type III Secretion System (T3SS)-Translocon of atypical enteropathogenic Escherichia coli (Aepec) can mediate adherence. Front Microbiol 2019;10. https://doi.org/10.3389/fmicb.2019.01527.

[23] Golparian, D., Sánchez-Busó, L., Cole, M., et al. (2021). Neisseria gonorrhoeae Sequence Typing for Antimicrobial Resistance (NG-STAR) clonal complexes are consistent with genomic phylogeny and provide simple nomenclature, rapid visualization, and antimicrobial resistance (AMR) lineage predictions. J Antimicrob Chemother 2021;76(4):940–944. https://doi.org/10.1093/jac/dkaa552

ARTIGO 2 - *In Vitro* and *In Vivo* Synergism of Fosfomycin in Combination with Meropenem or Polymyxin B against KPC-2-Producing *Klebsiella pneumoniae* Clinical Isolates

(https:// doi.org/10.3390/antibiotics12020237)

Aghata Cardoso da Silva Ribeiro [1,*], Yohanna Carvalho dos Santos Aoun Chikhani [1], Tiago Barcelos Valiatti [1], André Valêncio [1], Mariana Neri Lucas Kurihara [1], Fernanda Fernandes Santos [1], Luciene Andrade da Rocha Minarini [2] and Ana Cristina Gales [1]

[1]Laboratório Alerta, Division of Infectious Diseases, Department of Internal Medicine, Escola Paulista de Medicina/Universidade Federal de São Paulo—UNIFESP, Brazil; yohanna.carvalho@unifesp.br (Y.C.S.A.C.); tiago_valiatti@hotmail.com (T.B.V.); andrebiomedicovalencio@gmail.com (A.V.); mariana.kurihara@hotmail.com (M.N.L.K.); fernanda-fs@hotmail.com (F.F.S.); ana.gales@unifesp.br (A.C.G.)

[2]Laboratório Multidisciplinar em Saúde e Meio Ambiente, Departamento de Ciências Farmacêuticas,
Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo—UNIFESP, Brazil; luciene.minarini@unifesp.br (L.A.R.M.)

*****Correspondence: aghata.cardoso@unifesp.br (A.C.S.R.)

Abstract: Fosfomycin disodium is a potential therapeutic option to manage difficult-to-treat infections, especially when combined with other antimicrobials. In this study, we evaluated the activity of fosfomycin in combination with meropenem or polymyxin B against contemporaneous KPC-2-producing *K. pneumoniae* clinical isolates (KPC-KPN). Synergistic activity was assessed by checkerboard (CKA) and time–kill (TKA) assays. TKA was performed using serum peak and trough concentrations. The activity of these combinations was also assessed in the *Galleria mellonella* model. Biofilm disruption was assessed by the microtiter plate technique. CKA resulted in an 8- to 2048-fold decrease in meropenem MIC, restoring meropenem activity for 82.4% of the isolates when combined with fosfomycin. For the fosfomycin + polymyxin B combination, a 2- to 128-fold reduction in polymyxin B MIC was achieved, restoring polymyxin B activity for 47% of the isolates. TKA resulted in the synergism of fosfomycin + meropenem (3.0–6.7 $\log_{10}$ CFU/mL decrease) and fosfomycin + polymyxin B (6.0–6.2 $\log_{10}$ CFU/mL decrease) at peak concentrations. All larvae treated with fosfomycin + meropenem survived. Larvae survival rate was higher with fosfomycin monotherapy (95%)

than that observed for fosfomycin + polymyxin B (75%) (p-value < 0.0001). Finally, a higher biofilm disruption was observed under exposure to fosfomycin + polymyxin B (2.4–3.4-fold reduction). In summary, we observed a synergistic effect of fosfomycin + meropenem and fosfomycin + polymyxin B combinations, in vitro and in vivo, against KPC-KPN, as well as biofilm disruption.

Keywords: fosfomycin; synergism; *Klebsiella pneumoniae;* antimicrobial resistance; *Galleria mellonella*; biofilm

1. Introduction

Infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CRKP) are considered difficult-to-treat infections because treatment options are scarce. This fact led to difficulties in managing these infections, evidencing the need for treatment alternatives [1,2]. The new β-lactamase inhibitor-β-lactam combinations (IBL-BL) currently represent the main therapeutic options for the treatment of KPC-KPN infections. However, these agents are not widely available in low- and middle-income countries (LMICS) due to registration delays and costs [3]. In addition, increasing IBL-BL resistance rates have been reported [4–6].

In this sense, old antimicrobial agents such as polymyxins and fosfomycin have gained attention. Polymyxins have been used as last resort therapy, and fosfomycin has emerged as a potential option in the treatment of systemic infections [7–9]. Discovered more than 40 years ago, fosfomycin is a bactericidal drug with activity against Gram-positive and Gram-negative bacteria, including multidrug-resistant (MDR) isolates. Moreover, cross-resistance with other antibacterial agents is very uncommon since fosfomycin has a unique chemical structure and mechanism of action, inhibiting peptidoglycan synthesis at an earlier stage compared to β-lactams. Importantly, this drug demonstrates a favorable efficacy and safety profile, and it can be prescribed even for pregnant patients [10,11].

Fosfomycin trometamol is commonly used to treat urinary tract infections (UTIs), but the intravenous use of fosfomycin disodium has been prescribed for the treatment of MDR infections, usually in combination due to the risk of resistance selection [12]. Recently, the IDSA recommended against the use of fosfomycin to treat systemic infections caused by CRKP, especially because this species harbors *fosA* in their chromosome, which codifies a glutathione transferase capable of inactivating the fosfomycin molecule. Besides that, the intravenous formulation of fosfomycin is not available in the United States of America [13]. Combination therapy aims to maximize bacterial killing and minimize the emergence of MDR bacteria. Fosfomycin combination therapy has shown to be superior to fosfomycin monotherapy, as

reported by Al-Quraini and collaborators, who showed an important fosfomycin minimum inhibitory concentration (MIC) decrease in *K. pneumoniae* [14]. Nevertheless, distinct studies report conflicting results for combinations with fosfomycin. Depending on the isolate's β-lactamase content, the synergistic activity of the combination can vary, as shown by Wistrand-Yuen et al. These authors reported synergistic activity of fosfomycin + polymyxin B for 60% of KPC-, NDM-, and OXA-48-KPN producers [15]. In contrast, Bakthavatchalam et al. observed that the same combination exhibited 16% synergistic activity against a collection of NDM- and OXA-48-KPN producers [16].

In addition, there is also a discussion about which antimicrobial classes could represent the best combinations to achieve the highest synergism activity. Ideally, antimicrobials possessing distinct mechanisms of action would act at different stages of bacterial metabolism favoring bacterial killing and avoiding the selection and growth of resistant isolates. Fosfomycin and meropenem act to inhibit the bacterial cell wall synthesis, whereas in different stages: fosfomycin inhibits the MurA enzyme, blocking the murein monomers (peptidoglycan precursors) production, while meropenem, a β-lactam, acts binding transpeptidases enzymes, impairing the cross-linking of peptidoglycan subunits, blocking the cell wall synthesis [17,18]. Polymyxin, however, binds to lipopolysaccharide and phospholipids in the outer cell membrane in Gram-negative bacteria, destabilizing it [19].

Although *Galleria mellonella* larvae have a small size, it has been employed to assess pathogen virulence and the effectiveness of antimicrobial combinations because their innate immunity can recognize foreign conserved molecules on the bacteria surface and humoral (soluble) components via pattern recognition receptors [20]. In addition, *G. mellonella* has impressive evolutionary conservation close to mammals [21] and has a fast life cycle and ethical acceptance, making it an attractive low-cost model for testing survival rates [22].

Therefore, in this study, we evaluated the in vitro activity of fosfomycin alone and in combination with meropenem or polymyxin B against previously genetically characterized KPC-KPN isolates using checkerboard and time–kill assays. By testing these combinations, we observed a relevant decrease in meropenem and polymyxin B minimal inhibitory concentrations (MICs) among the tested isolates. Furthermore, we performed an in vivo assay using the *Galleria mellonella* survival model to evaluate the in vivo efficacy of these combinations. We also analyzed the effect of these antimicrobial combinations on biofilm disruption using the microtiter plate technique. Based on our results, we hypothesize that fosfomycin in combination with meropenem or polymyxin B could be a potential therapeutic option to treat difficult-to-treat infections caused by KPC-2-producing *K. pneumoniae.*

2. Results

2.1. Antimicrobial Susceptibility Profile of KPC-2-Producing K. pneumoniae Isolates

The antimicrobial susceptibility profile displayed by each isolate is shown in Table S3. Among the 17 KPC-KPN analyzed, only four were resistant to fosfomycin (MICs, 64–256 mg/L). Most isolates were susceptible to fosfomycin (82.3%) and resistant to meropenem (88.2%; MIC, 4 to 128 mg/L) and polymyxin B (70.6%; MIC, 1 to 64 mg/L). All KPC-KPN were resistant to aztreonam (MIC, 64 to >64 mg/L), ertapenem (MIC, 16 to >256 mg/L), gentamycin (MIC, 4 to >64 mg/L), ciprofloxacin, (MIC, >64 mg/L), levofloxacin (MIC, 16 to >64 mg/L), ceftazidime (MIC,128 to >256 mg/L), ceftriaxone (MIC, 256 to >256 mg/L), and cefepime (MIC,128 to >256 mg/L). The lowest resistance rate was observed for fosfomycin (23.5%), followed by amikacin (64.7%; MIC, 4 to >64 mg/L), polymyxin B (70.6%), meropenem (88.2%), and imipenem (94.1%, MIC, 4 to 256 mg/L).

2.2. In Vitro Synergism Assays

CKA test showed a synergistic effect of fosfomycin + meropenem against 58.8% of the isolates (10/17) and partial synergism against 29.4% (5/17). We observed a decrease in MICs varying from 2- to 16-fold and 8- to 2048-fold for fosfomycin and meropenem, respectively. In addition, we observed restored meropenem activity for 14 isolates (82.4%), with the remaining three isolates changing their susceptibility category from resistant (R) to susceptible, increasing the exposure (I). The meropenem MIC_{50} and MIC_{90} varied from 64 mg/L/128 mg/L to 0.25 mg/L/4 mg/L when combined with fosfomycin, while the fosfomycin MIC_{50} and MIC_{90} varied from 16 mg/L to 256 mg/L, respectively, to 8 mg/L and 32 mg/L (Table 1).

The combination of fosfomycin with polymyxin B achieved a synergism rate of 47.1% (8/17) and partial synergism of 29.4% (5/17). The MICs decreases were from two- to eight-fold for fosfomycin and from two- to 128-fold for polymyxin B. The restored polymyxin B activity was achieved for eight isolates, and for a single isolate, its susceptibility category changed from resistant (R) to susceptible, increasing the exposure (I). The polymyxin B MIC_{50} and MIC_{90} varied from 8 mg/L and 64 mg/L to 0.5 mg/L and 8 mg/L for polymyxin B in combination; for fosfomycin, MIC_{50} and MIC_{90} in combination were 8 mg/L and 64 mg/L, respectively (Table 1).

In general, we observed a higher rate of synergism between fosfomycin + meropenem (58.8%). For those isolates harboring bla_{KPC-2} and ESBL genes ($bla_{CTX-M-15}$ and/or $bla_{CTX-M-14}$ and/or bla_{OXA-1}), the synergy rates obtained for the two combinations (fosfomycin +

meropenem and fosfomycin + polymyxin B) were equal. For the four isolates, not co-harboring ESBL genes, the most active combination was fosfomycin + meropenem (Table 2).

Furthermore, no differences in the activity of both combinations were found when analyzing the polymyxin resistance determinants *mgrB, crrB, pmrA, pmrB,* and *pmrC*. We observed that mutations in these genes did not influence the activity of the determined antimicrobial combination.

Regarding the TKA, six representative isolates of KPC-2-producing *K. pneumoniae* isolates (HSP80, HSP84, HSP06, HSP29, HSP83, and P05) presenting synergism or partial synergism results in CKA, harboring different resistance genes, presenting different phenotypes and belonging to distinct STs were submitted to TKA. The bacterial colony counting at 24 h compared with the initial inoculum (t = 0) in the presence of fosfomycin, meropenem, and polymyxin B, and the respective combinations are shown in Table 3. In general, we could observe a mean decrease of 0.59 $\log_{10}$ CFU/mL for fosfomycin at peak concentration and a 3.07 $\log_{10}$ CFU/mL mean increase at trough concentration. For meropenem, we observed a mean increase of 1.57 $\log_{10}$ CFU/mL at peak and 2.85 $\log_{10}$ CFU/mL at trough concentration. Polymyxin B presented a mean decrease of -1.46 $\log_{10}$ CFU/mL at peak and 2.31 $\log_{10}$ CFU/mL increase at trough concentration (Table S1).

Peak fosfomycin concentration resulted in a mean decrease in CFU counting of 3.32 $\log_{10}$ CFU/mL among the tested isolates at 3 h. After this time, it was observed regrowth for all isolates (Table S1). For meropenem, peak concentration provided a reduction in CFU counting (mean of 0.79 CFU/mL) for four isolates at 3 h, with an observed increase from 6 h onward. Just a single isolate (HSP29) did not show growth at 24 h (Table S1). Peak polymyxin B caused a mean 3.63 $\log_{10}$ CFU/mL reduction at 6 h, being observed regrowth for five isolates. Although trough concentration decreased the colonies counting for three isolates at 3 h (mean of 0.49 $\log_{10}$ CFU/mL), regrowth was observed after this period (Table S1).

The antimicrobial combination of fosfomycin + meropenem at peak concentration was synergic against four isolates at 24 h (Table 3); these isolates also presented a decrease of 2.70–6.67 $\log_{10}$ CFU/mL at 6 h (Table S1). For the combination fosfomycin+ polymyxin B, we observed synergism against five isolates (four at peak and one at trough concentrations) at 24 h (Table 3). At peak concentrations, it was achieved a decreased range of 2.2–6.14 $\log_{10}$ CFU/mL at 3 h (Table S1). In addition, it was observed a synergistic effect of both combinations at peak concentration against three isolates (HSP80, HSP83, P05) (Table 3). The CKA and TKA results are summarized in Table 4 and Figure 1 displays the time-kill curves of two representatives isolates.

2.3. In Vivo Synergism Assay

The toxicity of antimicrobials alone or in combination, in trough and peak concentrations, was tested against 10 larvae each. No evidence of toxicity was noticed because all larvae remained alive five days after.

Five days after larvae inoculation and treatment, we observed 100% larvae survival in the groups treated with fosfomycin + meropenem. Larvae inoculated with HSP83 isolate also presented survival rates of 100% when treated with fosfomycin + polymyxin B and fosfomycin alone. For those receiving meropenem alone and polymyxin B alone, survival rates were 75% and 55%, respectively. For larvae inoculated with HSP84 isolate, the survival rates were 50% for those treated with fosfomycin + polymyxin B, 90% for fosfomycin alone, 100% for meropenem alone, and 45% for polymyxin B alone (Figure 2).

The statistical analysis revealed that the curves obtained for HSP83 indicated that fosfomycin and meropenem alone, as well as fosfomycin in combination with meropenem, presented a significative higher survival rate compared to a positive control (bacteria inoculated and not treated; p-value < 0.0001). Polymyxin B in monotherapy and in combination with fosfomycin did not achieve a significant difference. For HSP84, the curves obtained indicated that fosfomycin and meropenem alone, as well as fosfomycin in combination with meropenem or polymyxin B, presented a significative higher survival rate compared to a positive control (bacteria inoculated and not treated) (p-values < 0.005 and < 0.0001). Polymyxin B in monotherapy did not achieve a statistically significant difference.

2.4. Biofilm Assay

Among the 17 KPC-KPN, nine isolates were weakly adherent, eight were moderately adherent, and two were non-adherent isolates. The eight moderately adherent isolates (P16, P60, HSP65, HSP84, HSP83, P39, HSP29, and HSP64) were further selected for evaluation of the activity of antibiotics alone and in combination against biofilm formation. We observed a biofilm disruption in all isolates under exposure to antimicrobials alone and in combination (Table 5). The change in the ability of biofilm formation was observed from moderately to non-adherent in the presence of fosfomycin in combination with polymyxin B, fosfomycin alone, and polymyxin B alone (2.402–3.470-; 2.231–3.470-; 2.378–3.423-fold reduction on biofilm detection, respectively). For fosfomycin + meropenem and meropenem alone, six isolates changed to weakly adherent (1.481–2.724-; 1.335–2.385-fold reduction, respectively), and two isolates to non-adherent (2.016- and 2.357-; 2.052- and 2.489-fold reduction, respectively)

(Table 5). In general, higher biofilm disruption was observed under the exposure to fosfomycin + polymyxin B, followed by polymyxin B and fosfomycin alone ($p < 0.001$).

3. Discussion

Difficult-to-treat infections caused by KPC-KPN are associated with high mortality rates. Even though novel therapeutic options such as the new β-lactamases-β-lactam inhibitor combinations and cefiderocol have become available for the treatment of KPC-producing Enterobacterales infections, access to these agents is still limited in low- and middle-income countries. Delays in the approval registration and cost have been impediments to the wider use of these new agents [3]. In this manner, old antimicrobial agents such as polymyxins and fosfomycin still constitute alternative therapeutic options to treat infections caused by MDR bacteria despite the risk of toxicity and emergence of resistance [9,23]. Despite its toxicity and increasing levels of resistance, polymyxin B still remains an alternative therapeutic option for treating KPC-KPN infections because it is widely available in Brazilian hospitals [24,25]. On the other hand, fosfomycin has high safety and displays low resistance rates [26,27]. The present study was performed with contemporaneous recovered Brazilian KPC-KPN isolates from different STs, and in the time–kill assay, we used peak and trough concentrations to observe if the evaluated combinations would be synergistic. In addition, the activity of these combinations was also in vivo assessed by testing the *G. mellonella* survival model.

In our study, fosfomycin showed a high susceptibility rate (76.5%) against XDR CRKP, with all harboring KPC and ESBL encoding genes. A similar susceptibility rate was encountered by Liu and collaborators, who reported high susceptibility rates for fosfomycin against CRKP (79.0%) or ESBL-producing *K. pneumoniae* (80.6%) [28]. In contrast, Al-Quraini et al. showed that XDR *K. pneumoniae* (MIC ≤ 32 mg/L) exhibited low fosfomycin susceptibility rates (33.3%; 5/15) [14]. Co-resistance to fosfomycin and meropenem or polymyxin B was found in four and three tested isolates, respectively.

Recently, Scudeller and collaborators performed a systematic review and meta-analysis and described high and moderate synergy rates of fosfomycin + polymyxins against CRKP [29]. Other studies also showed high rates of synergy for the fosfomycin + meropenem combination [14,16,30]. In the present study, by CKA synergistic effect was observed for fosfomycin + meropenem and fosfomycin + polymyxin B against 58.8% and 47.1% of the KPC-KPN isolates, respectively (Tables 1 and 2). Furthermore, we observed meropenem and polymyxin B activity restoration with significant MIC fold decrease when meropenem or polymyxin B was combined with fosfomycin (Table 1). Our results corroborate the findings of a previous Brazilian study that tested KPC-KPN isolated before 2010. The authors showed that MIC_{50}s and MIC_{90}s were

32 and 256 mg/L for meropenem and 64 and 512 mg/L for fosfomycin, respectively. The antimicrobial combination increased bacterial susceptibility to 1/4 the MIC_{50}s and to 1/8 to 1/16 the MIC_{90}s of monotherapy. The antimicrobial combination demonstrated a synergistic effect for at least two-thirds of the isolates [30].

Among the STs evaluated in this study, STs 258 and ST437 presented higher rates of synergism for both combinations (fosfomycin + meropenem and fosfomycin + polymyxin B). This result might be extrapolated for other geographic regions because CC258 has been directly associated with *bla*$_{KPC-2}$ dissemination worldwide [23]. The synergism rates were similar among KPC-KPN isolates independent of the ESBL co-production (Table 2). The CKA results were corroborated by the TKA findings that showed a high synergistic effect against KPC-2-producing isolates, especially at peak concentrations of combinations. Moreover, we could observe that four cases of partial synergism in CKA resulted in synergism in TKA for fosfomycin + polymyxin B (HSP29, HSP80, and HSP83) and fosfomycin + meropenem (P05), as displayed in Table 4.

Also, for the combination groups that we observed to be synergistic in CKA but not in TKA at 24 h, we observed that the synergistic effect occurred earlier and was lost as time went by. For example, the fosfomycin + meropenem combination was effective at 6 h at peak concentrations and trough concentrations for the isolate HSP29 and HSP06. We also observed a similar result for the fosfomycin + polymyxin B combination against HSP84. This combination was also effective at 6 h at peak concentrations. The lack of synergistic activity at 24 h was mainly due to bacterial regrowth observed for all isolates and combinations, except for fosfomycin + meropenem and fosfomycin + polymyxin B against HSP29 and HSP84, respectively. Our results are in line with previous reports of in vitro synergism assays with fosfomycin and polymyxin B. The regrowth 6 h after suggests the emergence of resistant isolates. This is one of the reasons why some specialists recommend avoiding the prescription of these antimicrobials in monotherapy to treat severe infections [15].

In order to evaluate if our in vitro results would be corroborated by those found in animal models, we performed a *G. mellonella* in vivo model to test fosfomycin combinations against KPC-2-producing *K. pneumoniae.* In the present study, the in vivo *G. mellonella* assay further confirmed the effectiveness of antimicrobial combinations, as shown in Figure 2. Synergism with fosfomycin in the *G. mellonella* model was previously reported, but to the best of our knowledge, there is no report in the literature testing fosfomycin alone and in combination to treat larvae infected with KPC-KPN. Kussmann and collaborators also reported high synergistic in vivo activity of fosfomycin combined with cefazolin resulting in a 44–54% reduction of

larvae mortality infected with *Staphylococcus aureus,* which agreed with their in vitro data [31]. Thus, the concordance between the in vitro and in vivo (*G. mellonella)* results supports the use of this model to select the most appropriate therapeutic regimens [32–34]. Additionally, we observed fosfomycin bactericidal activity when used alone in the in vivo model (Figure 2), corroborating the in vitro data observed on TKA at 3 h at peak concentration (Table S1). However, the high activity of fosfomycin alone was observed in larvae five days after inoculation and treatment, suggesting that the immune system helped in combating the infection. Previously, it has been shown that fosfomycin has an immunomodulatory effect on cytokines production and NF-kB signaling pathway, modulating the function of B and T lymphocytes, monocytes, and neutrophils [35].

Finally, different studies have reported the fosfomycin activity on biofilm because this antimicrobial is capable of penetrating into these structures. Many studies have been performed with *Staphylococcus* spp., *Pseudomonas* spp., and *Escherichia coli* and show biofilm disruption with changes in biofilm structure leading to biofilm eradication [36–40]. In the present study, although we have observed biofilm disruption in all tested isolates under all the different exposures, greater activity was achieved by fosfomycin when combined with polymyxin B ($p < 0.001$) (Table 5 and S4). This interference on biofilm allowed the change in the isolate's biofilm categorization from moderately adherent to weakly or non-adherent (majority). Recently, synergistic activity of fosfomycin combined with colistin or tigecycline against *K. pneumoniae* biofilms was reported [36,40]. Our findings are in agreement with these results. In addition, our study also showed the antibiofilm activity of fosfomycin combined with meropenem against *K. pneumoniae*, an exposure not previously investigated. This fosfomycin activity on biofilm in *K. pneumoniae* shows the promising use of this antimicrobial against different types of infection. *K. pneumoniae* is the second most common Gram-negative pathogen in osteomyelitis, and acute bacterial skin and skin structure infections, and these isolates are related to biofilm production. These types of infection are recognized to present high levels of biofilm formation, configuring difficult-to-treat infections [41,42]. Thus, fosfomycin (alone or in combination) could be a great alternative to antimicrobial therapy in these cases.

4. Materials and Methods

4.1. Bacterial Isolates

A total of 17 KPC-2-producing *K. pneumoniae* isolates previously characterized by whole-genome sequencing were studied [43]. These KPC-KPN isolates carried different resistance-encoding genes, with all isolates possessing chromosomally encoded *fosA.* In

contrast, none harbored plasmid-encoded polymyxin resistance genes. All the isolates were classified as extensively drug-resistant (XDR) and presented different sequence types (ST): ST101 (n = 1), ST15 (n = 3), ST16 (n = 3), ST11 (n = 3), ST258 (n = 3), and ST437 (n = 4). Table S2 shows the complete resistome and the Genbank accession numbers of the isolates.

4.2. Antimicrobial Susceptibility Testing (AST)

The antimicrobial susceptibility profile of the *K. pneumoniae* isolates was determined by the agar dilution method for all antimicrobials tested, aztreonam, fosfomycin, imipenem, ertapenem, meropenem, amikacin, gentamycin, tigecycline, ciprofloxacin, levofloxacin, ceftazidime, and cefepime, except for polymyxin B, which was tested by broth microdilution following the BrCAST/EUCAST recommendations. The plates containing fosfomycin were supplemented with glucose-6-phosphate at 25 mg/L. Quality control and the interpretation of results were performed according to BrCAST/EUCAST guidelines, with results following within the expected ranges. We used as control the strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 [44,45].

4.3. Checkerboard Assay (CKA)

Synergy assessment by CKA was performed as previously described [46]. Sterile 96-well microdilution plates containing Mueller–Hinton broth with different concentrations of fosfomycin alone or in combination with meropenem and polymyxin B were used. A standard 0.5 McFarland inoculum containing approximately 1.5×10^8 CFU/mL was added to each well, and the plates were incubated at 37 °C for 24 h. *K. pneumoniae* ATCC 700603 was tested as a quality control, and the experiments were performed in duplicate. The fractional inhibitory concentration summation (ΣFIC) index indicates the efficacy of the antimicrobial combinations used. ΣFIC is calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of drug A in combination/MIC of drug A alone, and FIC B is the MIC of drug B in combination/MIC of drug B alone. Classification of the antimicrobial effect of the combination was based on ΣFIC as follows: ΣFIC ≤ 0.5 indicates synergism; 0.5 < ΣFIC ≤ 1 indicates partial synergism; 1 < ΣFIC ≤ 2 indicates indifference; and ΣFIC > 4 indicates antagonism [46].

4.4. Time–Kill Assay (TKA)

Six representatives of KPC-2-producing *K. pneumoniae* isolates that presented synergism or partial synergism in CKA, harboring different resistance genes, presenting different phenotypes, and belonging to distinct STs were submitted to TKA. Isolates were selected as follows: HSP80, HSP84, HSP06, HSP29, HSP83, and P05. Table 1, Table S2, and Table S3

summarize the isolates' CKA results, resistome, and phenotype, respectively. Free peak and trough concentrations of fosfomycin, meropenem, and polymyxin B alone and in combination were used based on the clinical doses standardized by the EUCAST/BrCAST recommendations for intravenous regimens [44] as follows: fosfomycin 8 g q8h (peak, 395 mg/L; trough, 25 mg/L) [47], meropenem 2 g single dose (peak, 40.9 mg/L; trough, 4.3 mg/L) [48,49] and polymyxin B 25,000 U/kg/day (peak, 6.44 mg/L; trough, 2.4 mg/L) [50]. The viable colony counts were determined at 0, 3, 6, and 24 h. Synergism and antagonism were defined as a ≥ 2-$\log_{10}$ CFU/mL decrease and increase, respectively, in the bacterial growth in combination when compared with the most active single agent at 24 h. Values between these ranges were considered as indifferent [46]. TKA was performed in duplicate. Briefly, a standard McFarland 0.5 inoculum was prepared using fresh colonies grown on MacConkey agar. Briefly, 100 µL of this inoculum was diluted in 5 mL of Muller Hinton Broth Cation Adjusted (CAMHB) and incubated at 37 °C until exponential growth (approximately 3 h). The inoculum was then adjusted to a McFarland 1.0 (~3 x 10^8 CFU/mL) standard with sterile NaCl 0.9%. The final inoculum was prepared using 1 mL of the McFarland 1.0 inoculum plus 4 mL of CAMHB (~6 × 10^7 CFU/mL). In each tube containing the antimicrobial to be tested as well as the combinations, 100 µL of inoculum (~6 × 10^7 CFU/mL) were added. The positive control (tube with no drug) and negative control (sterility test) were included. Then, before the incubation at 37 °C, the first culture was performed (T = 0). All tubes containing fosfomycin were supplemented with glucose-6-phosphate at 25 mg/L. A serial six-dilution was performed from the initial inoculum, and then aliquots of 10 µL from each were plated in Muller Hinton agar plates. After 24 h of incubation, the colonies counting was performed.

4.5. Galleria mellonella selection, inoculation, and treatment

The *G. mellonella* model was tested as the infection animal model for the treatment of infections caused by two different *K. pneumoniae* clones harboring distinct resistance genes. HSP83 was susceptible to fosfomycin (MIC, 16 mg/L) and resistant to polymyxin B (MIC, 64 mg/L) and meropenem (MIC, 64 mg/L), and harbored *bla*$_{\text{KPC-2}}$ and *bla*$_{\text{SHV-182}}$. HSP84 was resistant to fosfomycin (MIC, 256 mg/L), polymyxin B (MIC, 64 mg/L), and meropenem (MIC, 64 mg/L) and harbored *bla*$_{\text{KPC-2}}$, *bla*$_{\text{SHV-182}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$, and *bla*$_{\text{OXA-1}}$ (Table 1). Healthy larvae weighing 230–280 mg were selected and administered first with 10 µL of the bacteria inoculum (1.5 × 10^8 CFU/mL) and one hour later with the antimicrobial alone or in combination (10µL) at peak doses. The inoculum was delivered in the last right proleg, and the treatment was injected in the last left proleg by using an insulin syringe. Ten larvae were included in each tested group, and the experiment was performed in duplicate. We used two

control groups: one injected with saline (negative control) and the other one injected only with the bacterial inoculum (positive control). After the injection, larvae were incubated at 37 °C, and survival was observed daily for five consecutive days.

Before performing the in vivo animal model of infection, the larvae were inoculated only with the antimicrobial agents (fosfomycin, meropenem, polymyxin B) or their combinations, fosfomycin + meropenem or fosfomycin + polymyxin B, at peak concentrations to assess antimicrobial toxicity. The larvae were incubated at 37 °C, and survival rates were measured daily for five days.

4.6. Microtiter Plate Technique

The biofilm formation assay was performed using crystal violet on a polystyrene abiotic surface, and the results were interpreted as previously reported [51]. For those bacteria presenting moderate and strong adherence, these biofilms were further exposed to antimicrobials alone or in combination. Briefly, after 24 h at 37 °C of incubation, the medium was removed, and the wells were washed with PBS 1% two times. After this step, antimicrobial solution at peak concentrations was added to each well and incubated for more than 24 h at 37 °C. After incubation, the wells were washed three times with PBS 1% and fixed with formaldehyde 3%, and stained with crystal violet 1%. The dye was solubilized in ethanol 95%, and the OD was performed in a spectrophotometer at a wavelength of 570 nm. This assay was performed in triplicate.

4.7. Statistical Analysis

For *G. mellonella* analysis, the Kaplan–Meier survival curve was used, and the difference between the groups was determined by the log-rank (Mantel–Cox) test and Gehan–Breslow–Wilcoxon test. A p-value < 0.05 was considered statically significant. The analyses were performed in Prism 5.0 (GraphPad Prism Software, Inc.).

For the biofilm assay, we used the one-way ANOVA non-parametric (Kruskal–Wallis) test since the sample did not follow a normality pattern (Shapiro–Wilk test; $W = 0.715$; p-value < 0.001). In addition, we performed a comparison among the group tests using the Dwass–Steel–Critchlow–Fligner pairwise comparison. Test groups were not considered homogeneous by the Levine test ($F = 17.5$; p-value < 0.001). For this analysis, the Jamovi v. 1.6 software [52] was applied. This statistical analysis is displayed in Table S4.

5. Conclusions

Overall, we observed a high in vitro synergistic effect for both combinations, fosfomycin + meropenem and fosfomycin + polymyxin B, against CRKP isolates ST15, ST16, ST101, ST258, ST11, and ST437 despite the presence of multiple resistance genes. In addition, we could observe that when in combination with fosfomycin, meropenem, or polymyxin B presented, its susceptibility profile was restored in many isolates.

Our in vivo results are important because there is a worry about using fosfomycin (alone or in combination) to treat *K. pneumoniae* infections because of the regrowth observed in in vitro studies [15,53] and because it harbors an intrinsic *fosA* gene in its chromosome, which is usually not associated with resistance profile [54,55]. To date, just a single study reported fosfomycin resistance due to the expression of chromosomal *fosA* in *K. pneumoniae* [55].

We recognize that our work has limitations as the low number of isolates tested. Further studies testing a large number of isolates would be interesting. However, CKA and TKA are laborious and time-consuming. In addition, in our study, only isolates showing synergism or partial synergism in the CKA were selected further for TKA, which may be a cause of bias.

In conclusion, this work presents data on fosfomycin activity alone and in combination against 17 genetically characterized KPC-2-producing K. pneumoniae isolates through in vitro and in vivo assays, as well as its effect on biofilm formation. We hope that these data will help the clinician's decisions on using intravenous fosfomycin in combination with the treatment of KPC-KPN infections.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1 Table S1: TKA results in $\log_{10}$ CFU/mL for each isolate in each different time and at different concentrations; Table S2: Complementary information about the isolates.; Table S3: Antimicrobial susceptibility testing; Table S4: Statistical analysis for biofilm disruption under antimicrobials combination exposure assay.

Author Contributions: Conceptualization, A.C.S.R. and A.C.G.; methodology, A.C.S.R., A.V., Y.C.S.A.C., and M.N.L.K.; validation, A.C.S.R. and Y.C.S.A.C.; formal analysis, A.C.S.R.; investigation, A.C.S.R., F.F.S., and T.B.V.; resources, A.C.G.; data curation, A.C.S.R., F.F.S., and T.B.V.; writing—original draft preparation, A.C.S.R., F.F.S., T.B.V., A.V., and M.N.L.K.; writing—review and editing, A.C.S.R., L.A.R.M., and A.C.G.; visualization, A.C.S.R. and M.N.L.K.; supervision, L.A.R.M. and A.C.G.; project administration, A.C.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We acknowledge CAPES for the scholarships granted to A.C.S.R., T.B.V., F.F.S., Y.C.S.A.C., M.N.L.K., and A.V.

Conflicts of Interest: A.C.G. has received honoraria and/or consultation fees from Entasis Therapeutics, BioMerieux, Eurofarma, MSD, Pfizer, Roche, Sandoz, and United Medical, and research funding from Eurofarma.

References

1. WORLD HEALTH ORGANIZATION WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed. Available online: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed on 1 December 2022).
2. Yang, P.; Liu, C.; Wu, Z.; Zheng, J.; Yi, J.; Wu, N.; Wu, Z.; Lu, M.; Cui, L.; Shen, N. Clinical Outcomes, Microbiological Characteristics and Risk Factors for Difficult-to-Treat Resistance to Klebsiella Pneumoniae Infection. Infect. Drug Resist. **2022**, 15, 5959–5969, doi:10.2147/IDR.S377064.
3. Antimicrobial Resistance Research Programme. Lack of Access to Medicine Is a Major Driver of Drug Resistance. How Can Pharma Take Action? *Access Med. Found.* **2022**. Available online: https://accesstomedicinefoundation.org/medialibrary/62c2f0dcda565_atmf_appropriate_access_to_antimicrobials_2022-1666595298.pdf (accessed on 2 December 2022).
4. Ho, S.; Nguyen, L.; Trinh, T.; MacDougall, C. Recognizing and Overcoming Resistance to New Beta-Lactam/Beta-Lactamase Inhibitor Combinations. *Curr. Infect. Dis. Rep.* **2019**, *21*, 39. https://doi.org/10.1007/S11908-019-0690-9.
5. Lee, J.-A.; Du, S.-H.; Lee, T.; Huang, Y.-S.; Liao, C.-H.; Huang, Y.-T.; Hsueh, P.-R. Rapid Emergence of Ceftazidime-Avibactam Resistance among Carbapenem-Resistant Enterobacterales in a Tertiary-Care Hospital in Taiwan. *J. Infect.* **2022**, *86*, 66–117. https://doi.org/10.1016/j.jinf.2022.10.003.
6. Castanheira, M.; Doyle, T.B.; Collingsworth, T.D.; Sader, H.S.; Mendes, R.E. Increasing Frequency of OXA-48-Producing Enterobacterales World- Wide and Activity of Ceftazidime/Avibactam, Meropenem/Vaborbactam and Comparators against These Isolates. *J. Antimicrob. Chemother.* **2021**, *76*, 3125–3134. https://doi.org/10.1093/jac/dkab306.
7. Theuretzbacher, U.; Van Bambeke, F.V.; Cantón, R.; Giske, C.G.; Mouton, J.W.; Nation, R.L.; Paul, M.; Turnidge, J.D.; Kahlmeter, G. Reviving Old Antibiotics. *J. Antimicrob. Chemother.* **2015**, *70*, 2177–2181. https://doi.org/10.1093/jac/dkv157.
8. Falagas, M.E.; Vouloumanou, E.K.; Samonis, G.; Vardakas, K.Z.; Matthew, E. Fosfomycin. *Clin. Microbiol. Rev.* **2016**, *29*, 321–347. https://doi.org/10.1128/CMR.00068-15.Address.
9. Kaye, K.S.; Gales, A.C.; Dubourg, G. Old Antibiotics for Multidrug-Resistant Pathogens: From in Vitro Activity to Clinical Outcomes. *Int. J. Antimicrob. Agents* **2017**, *49*, 542–548. https://doi.org/10.1016/j.ijantimicag.2016.11.020.

10. Falagas, M.E.; Giannopoulou, K.P.; Kokolakis, G.N.; Rafailidis, P.I. Fosfomycin: Use beyond Urinary Tract and Gastrointestinal Infections. *Chin. J. Infect. Chemother.* **2010**, *10*, 103. https://doi.org/10.1086/527442.

11. Perdigão Neto, L.V.; Oliveira, M.S.; Orsi, T.D.A.; do Prado, G.V.B.; Martins, R.C.R.; Leite, G.C.; Marchi, A.P.; de Lira, E.S.A.; Côrtes, M.F.; Espinoza, E.P.S.; et al. Alternative Drugs against Multiresistant Gram-Negative Bacteria. *J. Glob. Antimicrob. Resist.* **2020**, *23*, 33–37. https://doi.org/10.1016/j.jgar.2020.07.025.

12. EMA. Recommendations to Restrict Use of Fosfomycin Antibiotics. Available online: https://www.ema.europa.eu/en/documents/referral/fosfomycin-article-31-referral-recommendations-restrict-use-fosfomycin-antibiotics_en.pdf (accessed on 1 December 2022).

13. Tamma, P.D.; Aitken, S.L.; Bonomo, R.A.; Mathers, A.J.; Van Duin, D.; Clancy, C.J. Infectious Diseases Society of America 2022 Guidance on the Treatment of Extended-Spectrum β-Lactamase Producing Enterobacterales (ESBL-E), Carbapenem-Resistant Enterobacterales (CRE), and Pseudomonas Aeruginosa with Difficult-to-Treat Resistance (DTR-P). *Clin. Infect. Dis.* **2022**, *75*, 187–212. https://doi.org/10.1093/cid/ciac268.

14. Al-Quraini, M.; Rizvi, M.; Al-Jabri, Z.; Sami, H.; Al-Muzahmi, M.; Al-Muharrmi, Z.; Taneja, N.; Al-Busaidi, I.; Soman, R. Assessment of In-Vitro Synergy of Fosfomycin with Meropenem, Amikacin and Tigecycline in Whole Genome Sequenced Extended and Pan Drug Resistant Klebsiella Pneumoniae: Exploring A Colistin Sparing Protocol. *Antibiotics* **2022**, *11 (2)*: 153. https://doi.org/10.3390/antibiotics11020153.

15. Wistrand-Yuen, P.; Olsson, A.; Skarp, K.P.; Friberg, L.E.; Nielsen, E.I.; Lagerbäck, P.; Tängdén, T. Evaluation of Polymyxin B in Combination with 13 Other Antibiotics against Carbapenemase-Producing Klebsiella Pneumoniae in Time-Lapse Microscopy and Time-Kill Experiments. *Clin. Microbiol. Infect.* **2020**, *26*, 1214–1221. https://doi.org/10.1016/j.cmi.2020.03.007.

16. Bakthavatchalam, Y.D.; Shankar, A.; Muthuirulandi Sethuvel, D.P.; Asokan, K.; Kanthan, K.; Veeraraghavan, B. Synergistic Activity of Fosfomycin-Meropenem and Fosfomycin-Colistin against Carbapenem Resistant Klebsiella Pneumoniae: An in Vitro Evidence. *Future Sci. OA* **2020**, *6 (4)*: FSO461. https://doi.org/10.2144/fsoa-2019-0074.

17. Díez-aguilar, M. Current Key Topics in Fosfomycin. *Span. J. Chemother.* **2019**, *32*, 8–18.

18. Golan, D.E.; Junior, A.H.T.; Armstrong, E.J.; Armstrong, A.W. *Príncipios de Farmacologia*; 2009. Ed. Guanabara Koogan. Rio de Janeiro. 2 ed; ISBN 9788527715201.

19. Zavascki, A.P.; Goldani, L.Z.; Li, J.; Nation, R.L. Polymyxin B for the Treatment of Multidrug-Resistant Pathogens: A Critical Review. *J. Antimicrob. Chemother.* **2007**, *60*, 1206–1215. https://doi.org/10.1093/jac/dkm357.

20. Wright, C.L.; Kavanagh, O. Galleria Mellonella as a Novel In Vivo Model to Screen Natural Product-Derived Modulators of Innate Immunity. *Appl. Sci.* **2022**, *12*, 6587. https://doi.org/10.3390/APP12136587.

21. Benthall, G.; Touzel, R.E.; Hind, C.K.; Titball, R.W.; Sutton, J.M.; Thomas, R.J.; Wand, M.E. Evaluation of Antibiotic Efficacy against Infections Caused by Planktonic or Biofilm Cultures of Pseudomonas Aeruginosa and Klebsiella Pneumoniae in Galleria Mellonella. *Int. J. Antimicrob. Agents* **2015**, *46*, 538–545. https://doi.org/10.1016/J.IJANTIMICAG.2015.07.014.

22. Cutuli, M.A.; Petronio Petronio, G.; Vergalito, F.; Magnifico, I.; Pietrangelo, L.; Venditti, N.; Di Marco, R. Galleria Mellonella as a Consolidated in Vivo Model Hosts: New Developments in Antibacterial Strategies and Novel Drug Testing. *Virulence* **2019**, *10*, 527–541. https://doi.org/10.1080/21505594.2019.1621649.

23. Wang, M.; Earley, M.; Chen, L.; Hanson, B.M.; Yu, Y.; Liu, Z.; Salcedo, S.; Cober, E.; Li, L.; Kanj, S.S.; et al. Clinical Outcomes and Bacterial Characteristics of Carbapenem-Resistant Klebsiella Pneumoniae Complex among Patients from Different Global Regions

(CRACKLE-2): A Prospective, Multicentre, Cohort Study. *Lancet Infect. Dis.* **2021**, *22 (3)*, 401–412. https://doi.org/10.1016/s1473-3099(21)00399-6.

24. ANVISA. Boletim Segurança Do Paciente e Qualidade Em Serviços de Saúde N°22: Avaliação Dos Indicadores Nacionais Das Infecções Relacionadas à Assistência à Saúde (IRAS) e Resistência Microbiana Do Ano de. 2019. Available online: https://www.gov.br/anvisa/pt-br/centraisdeconteudo/publicacoes/servicosdesaude/publicacoes (accessed on 1 November 2022).

25. Falagas, M.E.; Kasiakou, S.K. Toxicity of Polymyxins: A Systematic Review of the Evidence from Old and Recent Studies. *Crit. Care* **2006**, *10 (1)*: R27. https://doi.org/10.1186/CC3995/TABLES/2.

26. Falagas, M.E.; Athanasaki, F.; Voulgaris, G.L.; Triarides, N.A.; Vardakas, K.Z. Resistance to Fosfomycin: Mechanisms, Frequency and Clinical Consequences. *Int. J. Antimicrob. Agents* **2019**, *53*, 22–28. https://doi.org/10.1016/j.ijantimicag.2018.09.013.

27. Fransen, F.; Hermans, K.; Melchers, M.J.B.; Lagarde, C.C.M.; Meletiadis, J.; Mouton, J.W. Pharmacodynamics of Fosfomycin against ESBL- and/or Carbapenemase-Producing Enterobacteriaceae. *J. Antimicrob. Chemother.* **2017**, *72*, 3374–3381. https://doi.org/10.1093/JAC/DKX328.

28. Liu, P.; Chen, S.; Wu, Z.Y.; Qi, M.; Li, X.Y.; Liu, C.X. Mechanisms of Fosfomycin Resistance in Clinical Isolates of Carbapenem-Resistant Klebsiella Pneumoniae. *J. Glob. Antimicrob. Resist.* **2020**, *22*, 238–243. https://doi.org/10.1016/j.jgar.2019.12.019.

29. Scudeller, L.; Righi, E.; Chiamenti, M.; Bragantini, D.; Menchinelli, G.; Cattaneo, P.; Giske, C.G.; Lodise, T.; Sanguinetti, M.; Piddock, L.J.V.; et al. Systematic Review and Meta-Analysis of in Vitro Efficacy of Antibiotic Combination Therapy against Carbapenem-Resistant Gram-Negative Bacilli. *Int. J. Antimicrob. Agents* **2021**, *57*, 106344. https://doi.org/10.1016/j.ijantimicag.2021.106344.

30. Albiero, J.; Sy, S.K.B.; Mazucheli, J.; Caparroz-Assef, S.M.; Costa, B.B.; Alves, J.L.B.; Gales, A.C.; Tognim, M.C.B. Pharmacodynamic Evaluation of the Potential Clinical Utility of Fosfomycin and Meropenem in Combination Therapy against KPC-2-Producing Klebsiella Pneumoniae. *Antimicrob. Agents Chemother.* **2016**, *60*, 4128–4139. https://doi.org/10.1128/AAC.03099-15.

31. Kussmann, M.; Obermueller, M.; Karer, M.; Kriz, R.; Chen, R.Y.; Hohl, L.; Schneider, L.; Burgmann, H.; Traby, L.; Vossen, M.G. Synergistic Effect of Cefazolin Plus Fosfomycin Against Staphylococcus Aureus in Vitro and in Vivo in an Experimental Galleria Mellonella Model. *Front. Pharmacol.* **2021**, *12*, 1095. https://doi.org/10.3389/FPHAR.2021.685807/BIBTEX.

32. Skinner, K.; Sandoe, J.A.T.; Rajendran, R.; Ramage, G.; Lang, S. Efficacy of Rifampicin Combination Therapy for the Treatment of Enterococcal Infections Assessed in Vivo Using a Galleria Mellonella Infection Model. *Int. J. Antimicrob. Agents* **2017**, *49*, 507–511. https://doi.org/10.1016/J.IJANTIMICAG.2016.12.006.

33. Borjan, J.; Meyer, K.A.; Shields, R.K.; Wenzler, E. Activity of Ceftazidime-Avibactam Alone and in Combination with Polymyxin B against Carbapenem-Resistant Klebsiella Pneumoniae in a Tandem in Vitro Time-Kill/in Vivo Galleria Mellonella Survival Model Analysis. *Int. J. Antimicrob. Agents* **2020**, *55*, 105852. https://doi.org/10.1016/j.ijantimicag.2019.11.009.

34. Lázár, V.; Snitser, O.; Barkan, D.; Kishony, R. Antibiotic Combinations Reduce Staphylococcus Aureus Clearance. *Nature* **2022**, *610 (7932)*: 540–546. https://doi.org/10.1038/S41586-022-05260-5.

35. Matsumoto, T.; Tateda, K.; Miyazaki, S.; Furuya, N.; Ohno, A.; Ishii, Y.; Hirakata, Y.; Yamaguchi, K. Fosfomycin Alters Lipopolysaccharide-Induced Inflammatory Cytokine Production in Mice. *Antimicrob. Agents Chemother.* **1999**, *43*, 697–698. https://doi.org/10.1128/AAC.43.3.697.

36. Ruiz, J.; Sanjuan, E.; Amaro, C.; Gordon, M.; Villarreal, E.; Castellanos-Ortega, Á.; Ramirez, P. In Vitro Study of Antimicrobial Activity on Klebsiella Pneumoniae Biofilms in Endotracheal Tubes. *J. Chemother.* **2019**, *31*, 202–208. https://doi.org/10.1080/1120009X.2019.1601801.

37. Nakamura, G.; Wachino, J.I.; Sato, N.; Kimura, K.; Yamada, K.; Jin, W.; Shibayama, K.; Yagi, T.; Kawamura, K.; Arakawa, Y. Practical Agar-Based Disk Potentiation Test for Detection of Fosfomycin-Nonsusceptible Escherichia Coli Clinical Isolates Producing Glutathione S-Transferases. *J. Clin. Microbiol.* **2014**, *52*, 3175–3179. https://doi.org/10.1128/JCM.01094-14.

38. Skarzynski, T.; Mistry, A.; Wonacott, A.; Hutchinson, S.E.; Kelly, V.A.; Duncan, K. Structure of UDP-N-Acetylglucosamine Enolpyruvyl Transferase, an Enzyme Essential for the Synthesis of Bacterial Peptidoglycan, Complexed with Substrate UDP-N-Acetylglucosamine and the Drug Fosfomycin. *Structure* **1996**, *4*, 1465–1474. https://doi.org/10.1016/S0969-2126(96)00153-0.

39. Mihailescu, R.; Tafin, U.F.; Corvec, S.; Oliva, A.; Betrisey, B.; Borens, O.; Trampuza, A. High Activity of Fosfomycin and Rifampin against Methicillin-Resistant Staphylococcus Aureus Biofilm in Vitro and in an Experimental Foreign-Body Infection Model. *Antimicrob. Agents Chemother.* **2014**, *58*, 2547–2553. https://doi.org/10.1128/AAC.02420-12.

40. Boncompagni, S.R.; Micieli, M.; Di Maggio, T.; Aiezza, N.; Antonelli, A.; Giani, T.; Padoani, G.; Vailati, S.; Pallecchi, L.; Rossolini, G.M. Activity of Fosfomycin/Colistin Combinations against Planktonic and Biofilm Gram-Negative Pathogens. *J. Antimicrob. Chemother.* **2022**, *77*, 2199–2208. https://doi.org/10.1093/JAC/DKAC142.

41. Ribeiro, Á.C.S.; Santos, F.F.; Valiatti, T.B.; Lenzi, M.H.; Meneses, J.P.; Sessa, R.G.G.; Salles, M.J.; Gales, A.C. Avaliação Da Atividade Antimicrobiana de Delafloxacino Contra Patógenos de Importância Clínica Causadores de Infecções d Epele e Partes Moles Complicadas e Osteomielite. *Braz. J. Infect. Dis.* **2022**, *26*, 102428. https://doi.org/10.1016/J.BJID.2022.102428.

42. Jabbour, J.F.; Sharara, S.L.; Kanj, S.S. Treatment of Multidrug-Resistant Gram-Negative Skin and Soft Tissue Infections. *Curr. Opin. Infect. Dis.* **2020**, *33*, 146–154. https://doi.org/10.1097/QCO.0000000000000635.

43. Ribeiro, C.S.Á.; Santos, F.F.; Moses, I.B.; Andrade Da, L.; Minarini, R.; Gales, A.C. Sequencing of FosA: A Rapid and Inexpensive Method for Discriminating Klebsiella Pneumoniae CC258 from Other Clones. *Microb. Drug Resist.* **2022**, *28 (11)*: 1037–1042. https://doi.org/10.1089/MDR.2022.0081.

44. BRCast—Brazilian Committee on Antimicrobial Susceptibility Testing, Brc. Tabela-Pontos-de-Corte-Clinicos-BrCAST. **2022**, 88.

45. CLSI. *M100. Performance Standards for Antimicrobial Susceptibility Testing: 31st Informational Supplement*, 32nd ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2022.

46. Leber, A.L. *Clinical Microbiology Procedures Handbook*, 4th ed.; ASM Press: Washington, DC, USA, 2016; ISBN 9781555818814.

47. Frossard, M.; Joukhadar, C.; Erovic, B.M.; Dittrich, P.; Mrass, P.E.; Van Houte, M.; Burgmann, H.; Georgopoulos, A.; Muller, M. Distribution and Antimicrobial Activity of Fosfomycin in the Interstitial Fluid of Human Soft Tissues. *Antimicrob. Agents Chemother.* **2000**, *44*, 2728–2732. https://doi.org/10.1128/AAC.44.10.2728-2732.2000/ASSET/58B0BB7B-BB8E-4F5D-9CC4-4C92D4F5DD1A/ASSETS/GRAPHIC/AC1000076003.JPEG.

48. Rubino, C.M.; Bhavnani, S.M.; Loutit, J.S.; Morgan, E.E.; White, D.; Dudley, M.N.; Griffith, D.C. Phase 1 Study of the Safety, Tolerability, and Pharmacokinetics of Vaborbactam and Meropenem Alone and in Combination Following Single and Multiple Doses in Healthy

Adult Subjects. *Antimicrob. Agents Chemother.* **2018**, *62 (4)*: e02228-17. https://doi.org/10.1128/AAC.02228-17.

49. Jaruratanasirikul, S.; Sriwiriyajan, S.; Punyo, J. Comparison of the Pharmacodynamics of Meropenem in Patients with Ventilator-Associated Pneumonia Following Administration by 3-Hour Infusion or Bolus Injection. *Antimicrob. Agents Chemother.* **2005**, *49*, 1337–1339. https://doi.org/10.1128/AAC.49.4.1337-1339.2005.

50. Han, L.; Xu, F.M.; Zhang, X.S.; Zhang, C.H.; Dai, Y.; Zhou, Z.Y.; Wang, Y.X.; Chen, F.; Shi, D.W.; Lin, G.Y.; et al. Trough Polymyxin B Plasma Concentration Is an Independent Risk Factor for Its Nephrotoxicity. *Br. J. Clin. Pharmacol.* **2022**, *88*, 1202–1210. https://doi.org/10.1111/BCP.15061.

51. Stepanović, S.; Vuković, D.; Dakić, I.; Savić, B.; Švabić-Vlahović, M. A Modified Microtiter-Plate Test for Quantification of Staphylococcal Biofilm Formation. *J. Microbiol. Methods* **2000**, *40*, 175–179. https://doi.org/10.1016/S0167-7012(00)00122-6.

52. The jamovi project (2022). Jamovi (Version 2.3) [Computer Software]. Retrieved from https://www.jamovi.org

53. Falagas, M.E.; Kastoris, A.C.; Kapaskelis, A.M.; Karageorgopoulos, D.E. Fosfomycin for the Treatment of Multidrug-Resistant, Including Extended-Spectrum β-Lactamase Producing, Enterobacteriaceae Infections: A Systematic Review. *Lancet Infect. Dis.* **2010**, *10 (1)*: 43–50.

54. Ito, R.; Mustapha, M.M.; Tomich, A.D.; Callaghan, J.D.; McElheny, C.L.; Mettus, R.T.; Shanks, R.M.Q.; Sluis-Cremer, N.; Doi, Y. Widespread Fosfomycin Resistance in Gram-Negative Bacteria Attributable to the Chromosomal FosA Gene. *MBio* **2017**, *8* (4): e00749-17. https://doi.org/10.1128/mbio.00749-17.

55. Kieffer, N.; Poirel, L.; Mueller, L.; Mancini, S.; Nordmann, P. ISEcp1-Mediated Transposition Leads to Fosfomycin and Broad-Spectrum Cephalosporin Resistance in Klebsiella Pneumoniae. *Antimicrob. Agents Chemother.* **2020**, *64 (5)*: e00150-20. https://doi.org/10.1128/AAC.00150-20.

Table 1. CKA results and fosfomycin, β-lactams, and polymyxin resistance determinants for the isolates studied.

Isolate	ST	β-Lactamase Content	Polymyxin Resistance Determinants	*fosA* *	MIC (mg/L) and MIC Category of Antimicrobials in Monotherapy						MIC (mg/L) and MIC Category of Antimicrobials in Combination								MIC Fold Decrease		ΣFIC/Interpretation	
					FOS		MEM		POLB		FOS + MEM				FOS + POL				FOS + MEM	FOS + POL	FOS + MEM	FOS + POL
											FOS		MEM		FOS		POLB					
					MIC	C	MIC	C	MIC	C	MIC	C	MIC	C	MIC	C	MIC	C				
P60	ST101	bla_{KPC-2}; bla_{SHV-28}; bla_{TEM-1B}; $bla_{CTX-M-15}$		+	16	S	128	R	2	S	8	S	**0.25**	**S**	8	S	0.5	S	2; 512	2; 4	0.5/SYN	0.75/PSYN
HSP29	ST11	bla_{KPC-2}; $bla_{SHV-182}$		+	64	R	128	R	1	S	**4**	**S**	**0.125**	**S**	**4**	**S**	0.5	S	16; 1024	16; 2	0.06/SYN	0.53/PSYN
HSP64	ST11	bla_{KPC-2}; $bla_{SHV-182}$; bla_{TEM-1B}	Deletion in *mgrB* (aa46)*;* mutation in *crrB* (D189E)*; pmrB* (R256G); mutation in *pmrC* (Q319R)	+	16	S	128	R	8	R	32	S	**1**	**S**	64	R	**0.5**	**S**	0.5; 128	0.25; 16	2.01/IND	2.03/IND
HSP73	ST11	bla_{KPC-2}; $bla_{SHV-182}$	*mgrB* disruption; mutation in *crrB* (D189E); *pmrB* (R256G); mutation in *pmrC* (Q319R)	+	8	S	64	R	8	R	4	S	4	I	8	S	**0.5**	**S**	2; 16	1; 16	0.56/PSYN	1.06/IND
P16	ST15	bla_{KPC-2}; bla_{SHV-28}; $bla_{CTX-M-15}$		+	16	S	32	R	2	S	16	S	**0.125**	**S**	4	S	0.25	S	1; 256	4; 8	1.0/IND	0.375/SYN
P35	ST15	bla_{KPC-2}; bla_{SHV-28}; bla_{SHV-98}; bla_{TEM-1B}; $bla_{CTX-M-15}$	Mutation in *pmrB* (A246T)*;* mutation in *pmrC* (F27C; S257L)	+	16	S	64	R	16	R	1	S	4	I	4	S	**2**	**S**	16; 16	4; 8	0.13/SYN	0.375/SYN
P51	ST15	bla_{KPC-2}; bla_{SHV-28}; $bla_{CTX-M-15}$	Mutation in *pmrB* (A246T)*;* mutation in *pmrC* (F27C; S257L)	+	4	S	32	R	16	R	2	S	4	I	4	S	**2**	**S**	2; 8	1; 8	0.63/PSYN	1.125/IND
HSP80	ST16	bla_{KPC-2}; bla_{SHV-1}; bla_{TEM-1A}; $bla_{CTX-M-15}$; bla_{OXA-1}	Deletion in *mgrB* (aa3)*;* mutation in *crrB* (D189E; V237I)*;* mutation in *pmrC* (F27C)	+	256	R	128	R	32	R	**32**	**S**	**0.06**	**S**	128	R	8	R	8; 2048	2; 4	0.13/SYN	0.75/PSYN
HSP83	ST16	bla_{KPC-2}; $bla_{SHV-182}$	*mgrB* disruption; mutation in *crrB* (D189E; V237I); *pmrB* (R256G); mutation in *pmrC* (Q319R)	+	16	S	64	R	64	R	4	S	**0.25**	**S**	8	S	**0.5**	**S**	4; 256	2; 128	0.25/SYN	0.508/PSYN
P05	ST16	bla_{KPC-2}; bla_{SHV-1}; bla_{TEM-1B}; $bla_{CTX-M-14}$	Mutation in *crrB* (D189E; V237I); mutation in *pmrA* (E57G); mutation in *pmrC* (F27C)	+	16	S	16	R	16	R	16	S	**0.06**	**S**	4	S	**0.125**	**S**	1; 256	4; 128	1/PSYN	0.258/SYN

P86	ST258	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-14}$	Mutation in *mgrB* (W36S), *crrB* (D189E; V237I; Q296L)*; pmrB* (R256G)*;* mutation in *pmrC* (Q319R)	+	16	S	4	I	16	R	1	S	**0.5**	**S**	4	S	4	R	16; 8	4; 4	0.19/SYN	0.50/SYN
P39	ST258	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-14}$	Mutation in *crrB* (D189E; V237I; Q296L)*; pmrB* (R256G)*;* mutation in *pmrC (Q319R)*	+	16	S	64	R	4	R	8	S	**0.25**	**S**	8	S	**0.25**	**S**	2; 256	2; 16	0.5/SYN	0.56/PSYN
P71	ST258	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-14}$	Mutation in *mgrB* (W36S), *crrB* (D189E; V237I; Q296L)*; pmrB* (R256G)*;* mutation in *pmrC* (Q319R)	+	8	S	4	I	4	R	0.5	S	**0.25**	**S**	2	S	**1**	**S**	16; 16	4; 4	0.13/SYN	0.5/SYN
HSP06	ST437	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{OXA-1}$		+	128	R	128	R	2	S	**8**	**S**	**0.25**	**S**	**16**	**S**	0.5	S	16; 512	8; 4	0.03/SYN	0.375/SYN
HSP17	ST437	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-15}$	Mutation in *pmrB* (R256G)*;* mutation in *pmrC (Q319R)*	+	16	S	128	R	32	R	16	S	**0.5**	**S**	16	S	8	R	1; 256	1; 4	1.00/PSYN	1.25/IND
HSP84	ST437	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$	Mutation in *pmrB* (R256G)*;* mutation in *pmrC (Q319R)*	+	256	R	64	R	64	R	64	R	**0.06**	**S**	**32**	**S**	**0.5**	**S**	4; 1024	8; 128	0.25/SYN	0.13/SYN
P29	ST437	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$		+	16	S	64	R	2	S	8	S	**0.5**	**S**	4	S	0.5	S	2; 128	4; 4	0.51/PSYN	0.5/SYN
MIC_{50}					16		64		8		8		0.25		8		0.5					
MIC_{90}					256		128		64		32		4		64		8					

The MICs in combination, which changed the classification from resistant, R, or susceptible, increasing the exposure, I, to susceptible, S, are highlighted in bold. FOS, fosfomycin; MEM, meropenem; POLB polymyxin; C, category; SYN, synergism; PSYN, partial synergism; IND, indifferent.

Table 2. CKA results split by β-lactamase content.

β-Lactamases Genes	FOS + MEM			FOS + POL		
	Synergy, *n* (%)	**Partial Synergy, *n* (%)**	**Indifference, *n* (%)**	**Synergy, *n* (%)**	**Partial Synergy, *n* (%)**	**Indifference, *n* (%)**
bla_{KPC-2} (*n* = 17)	10 (58.8)	5 (29.4)	2 (11.8)	8 (47.1)	5 (29.4)	4 (23.5)
bla_{KPC-2}+ $bla_{CTX-M-15}$ + bla_{OXA-1} (*n* = 3)	2 (66.6)	1 (33.3)	0	2 (66.6)	1 (33.3)	0
bla_{KPC-2} + $bla_{CTX-M-15}$ (*n* = 5)	2 (40.0)	2 (40.0)	1 (20.0)	2 (40.0)	1 (20.0)	2 (40.0)
bla_{KPC-2} + $bla_{CTX-M-14}$ (*n* = 4)	3 (75.0)	1 (25.0)	0	3 (75.0)	1 (25.0)	0
bla_{KPC-2}+ bla_{OXA-1} (*n* = 1)	1 (100)	0	0	1 (100)	0	0
No ESBL (*n* = 4)	2 (50)	1 (25)	1 (25)	0	2 (50)	2 (50)

FOS, fosfomycin; MEM, meropenem; POL, polymyxin.

Table 3. TKA results showing the growth rates for six representative isolates between time zero and 24 h.

	Change from Time Zero to 24 h (log_{10} CFU/mL)					
	HSP80	**HSP84**	**HSP83**	**P05**	**HSP29**	**HSP06**
FOS peak	0.56	2.38	−3.78	1.27	−6.60	2.71
FOS trough	3.04	2,46	2.90	2.93	3.09	3.24
MEM peak	2.66	3.37	2.60	3.06	−6.00	3.74
FOS + MEM peak	**−3.78**	**−3.00**	**−6.50**	**−6.00**	−6.00	1.40
MEM trough	3.32	3.74	2.95	2.99	0.84	3.24
FOS + MEM trough	2.78	3.27	3.43	3.27	2.84	3.74
POL peak	1.70	−6.50	−1.98	1.53	−6.00	2.51
FOS + POL peak	**−6.21**	−6.23	**−6.00**	**−6.14**	−6.00	**−6.00**
POL trough	3.24	3.02	1.72	2.08	1.74	2.05
FOS + POL trough	3.74	3.13	3.36	2.39	**−6.00**	0.36

The values indicate the change in log_{10} CFU/mL for each isolate, comparing the time zero and the time 24 h. Synergism was highlighted in bold. Synergism was defined as a ≥2-log_{10} CFU/mL decrease in bacterial growth in combination when compared with the most active single agent at 24 h. FOS, fosfomycin; MEM, meropenem; POL, polymyxin.

Table 4. Comparison between CKA and TKA.

Isolate	Sequence Type	β-Lactamase Content	FOS + MEM	FOS + POLB	FOS + MEM	FOS + POLB
HSP 29	ST11	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$	0.06/SYN	0.53/PSYN	-	SYN/trough
HSP 80	ST16	*bla*$_{KPC-2}$; *bla*$_{SHV-1}$; *bla*$_{TEM-1A}$; *bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$	0.13/SYN	0.75/PSYN	SYN/peak	SYN/peak
HSP 83	ST16	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$	0.25/SYN	0.508/PSYN	SYN/peak	SYN/peak
P05	ST16	*bla*$_{KPC-2}$; *bla*$_{SHV-1}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-14}$	1/PSYN	0.258/SYN	SYN/peak	SYN/peak
HSP 06	ST437	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{OXA-1}$	0.03/SYN	0.375/SYN	-	SYN/peak
HSP 84	ST437	*bla*$_{KPC-2}$; *bla*$_{SHV-182}$; *bla*$_{TEM-1B}$; *bla*$_{CTX-M-15}$; *bla*$_{OXA-1}$	0.25/SYN	0.13/SYN	SYN/peak	-

FOS, fosfomycin; MEM, meropenem; POLB, polymyxin; SYN, synergism; PSYN, partial synergism; IND, indifferent.

Table 5. Biofilm assay results showing the optical densities values for each isolate at each different exposure to antimicrobials and combinations.

ISOLATE	NO DRUG	Classification	FOS	Classification	Biofilm Fold Reduction	FOS + MEM	Classification	Biofilm Fold Reduction	POLB	Classification	Biofilm Fold Reduction	FOS + POLB	Classification	Biofilm Fold Reduction	MEM	Classification	Biofilm Fold Reduction
P16	0.124	M	0.054	NA	2.313	0.078	W	1.604	0.048	NA	2.610	0.048	NA	2.574	0.093	W	1.335
P60	0.167	M	0.048	NA	3.470	0.061	W	2.724	0.049	NA	3.423	0.048	NA	3.470	0.070	W	2.385
HSP65	0.117	M	0.049	NA	2.397	0.075	W	1.575	0.048	NA	2.455	0.049	NA	2.405	0.064	W	1.830
HSP84	0.127	M	0.048	NA	2.633	0.054	NA	2.357	0.047	NA	2.681	0.048	NA	2.626	0.051	NA	2.489
HSP83	0.116	M	0.052	NA	2.231	0.072	W	1.611	0.048	NA	2.442	0.048	NA	2.408	0.080	W	1.453
HSP29	0.115	M	0.050	NA	2.298	0.057	NA	2.016	0.048	NA	2.378	0.048	NA	2.402	0.056	NA	2.052
HSP64	0.132	M	0.052	NA	2.516	0.089	W	1.481	0.048	NA	2.728	0.048	NA	2.728	0.098	W	1.350
P39	0.124	M	0.049	NA	2.551	0.059	W	2.092	0.047	NA	2.667	0.049	NA	2.522	0.058	W	2.132

Values indicate the optical density obtained in the spectrophotometer. FOS, fosfomycin; MEM, meropenem; POLB, polymyxin; M, moderately adherent; W, weakly adherent; NA, non-adherent.

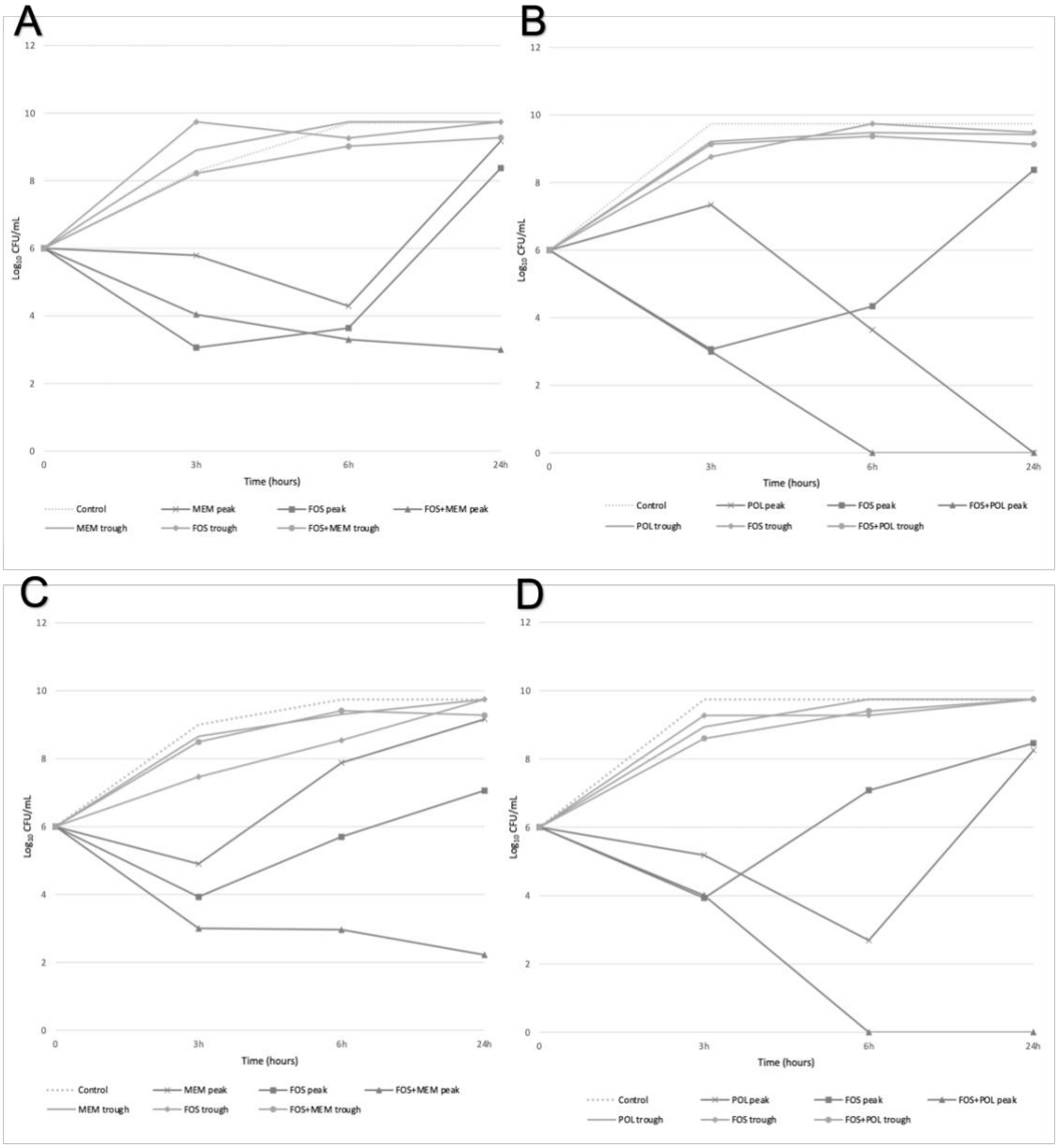

Figure 1. Representative time–kill curves under exposure to peak and trough concentrations of antimicrobials. This figure shows the time–kill curves of two representative isolates (HSP84 and HSP80) exposed to fosfomycin alone, meropenem alone, polymyxin B alone, fosfomycin + meropenem combination, and fosfomycin + polymyxin B combination. (**A**) Time–kill curves for isolate HSP84 under fosfomycin alone, meropenem alone, and fosfomycin + meropenem combination; (**B**) time–kill curves for isolate HSP84 under fosfomycin alone, polymyxin B alone, and fosfomycin + polymyxin B combination; (**C**) time–kill curves for isolate HSP80 under fosfomycin alone, meropenem alone and fosfomycin + meropenem combination; (**D**) time–kill curves for isolate HSP80 under fosfomycin alone, polymyxin B alone and fosfomycin + polymyxin B combination.

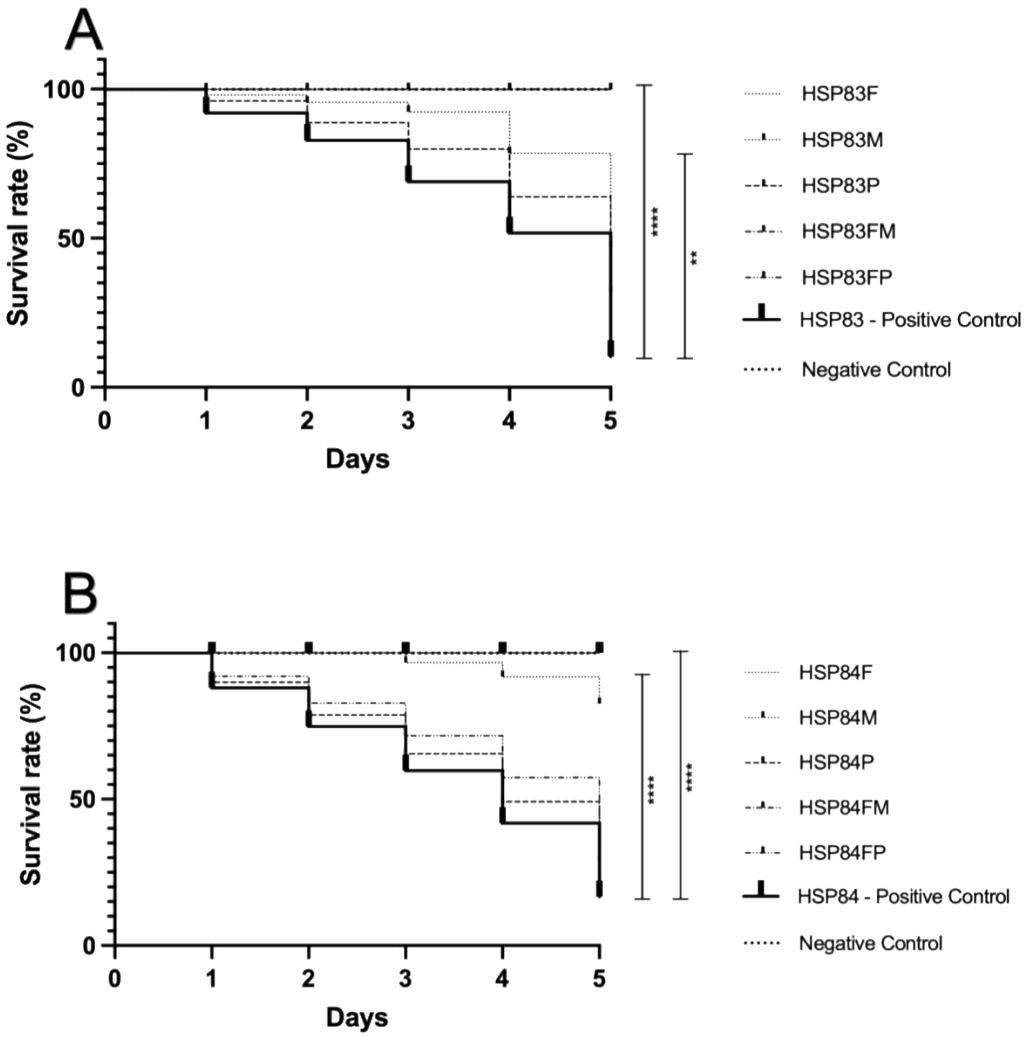

Figure 2. ***Galleria mellonella*** **infection model.** (**A**) Kaplan–Meier plots for larvae inoculated with HSP83 isolate. The curves obtained indicate that fosfomycin and meropenem alone, as well as the fosfomycin + meropenem combination, presented a significative higher survival rate compared to a positive control (bacteria inoculated and not treated). Polymyxin B in monotherapy and fosfomycin + polymyxin B combination did not achieve a significant difference. (**B**) Kaplan–Meier plots for larvae inoculated with HSP84 isolate. The curves obtained indicate that fosfomycin and meropenem alone, as well as fosfomycin in combination with meropenem or polymyxin B, presented a significative higher survival rate compared to a positive control (bacteria inoculated and not treated). Polymyxin B in monotherapy did not achieve a significant difference. F, fosfomycin alone; M, meropenem alone; P, polymyxin B alone; FM, fosfomycin + meropenem combination; FP, fosfomycin + polymyxin B combination; HSP83- or HSP84-positive control, larvae not treated; negative control, larvae inoculated with saline. (*p*-value: ** < 0.005; **** < 0.0001). The assay was performed with ten larvae per group and in duplicate.

ARTIGO 3 - Fosfomycin resistance associated with chromosomic *fosA* gene overexpression in *Klebsiella pneumoniae* Clinical Isolates

Aghata Cardoso da Silva Ribeiro[1]*, Fernanda Fernandes Santos[1], Tiago Barcelos Valiatti[1], Yohanna Carvalho dos Santos Aoun Chikhani[1], André Valêncio[1], Josiane Trevisol[1], Luciene Andrade da Rocha Minarini[2], Ana Cristina Gales[1]

[1]Laboratório Alerta. Division of Infectious Diseases, Department of Internal Medicine. Escola Paulista de Medicina/Universidade Federal de São Paulo - UNIFESP, Rua Pedro de Toledo, 781, São Paulo-SP, Brazil.

[2]Universidade Federal de São Paulo - UNIFESP, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Laboratório Multidisciplinar em Saúde e Meio Ambiente, Departamento de Ciências Farmacêuticas, Rua São Nicolau, 210, Diadema - SP, Brazil.

**Corresponding Author*
e-mail address: aghata.cardoso@unifesp.br
Universidade Federal de São Paulo
Rua Pedro de Toledo, 781 - 6º andar
Vila Clementino - 04025-010 - São Paulo – SP

Abstract

The emergence of fosfomycin resistance due to chromosomic *fosA* during treatment of *Klebsiella pneumoniae* infections has been a cause of concern. Here, we investigate the transcriptional levels of *fosA* in *K. pneumoniae* isolates co-resistant to carbapenems and fosfomycin. For comparison reasons, *K. pneumoniae* isolates susceptible to fosfomycin were also studied. No differences in the chromosomic *fosA* genetic context were observed when fosfomycin-resistant and fosfomycin-susceptible *K. pneumoniae* clinical isolates were compared. The fosfomycin-resistant *K. pneumoniae* isolates showed increased expression of chromosomic *fosA* without mutations in other known genes encoding fosfomycin resistance determinants. We also observed that the chromosomic *fosA* gene overexpression was induced in the presence of fosfomycin. However, additional studies are necessary to clarify the clinical significance of *fosA* expression in selecting fosfomycin-resistant *K. pneumoniae* isolates.

Overall, our results showed fosfomycin resistance in *K. pneumoniae* clinical isolates associated with chromosomic *fosA* overexpression and pointed it as a possible inducible mechanism of resistance to fosfomycin.

Brief Report

In the last years, fosfomycin was revisited due to the high and increasing bacterial resistance rates. Fosfomycin disodium is currently considered a promising combination option to treat systemic difficult-to-treat infections caused by multidrug-resistant bacteria [1]. However, there is a concern about using this antimicrobial to treat infections caused by pathogens, which harbor the *fosA* gene intrinsically in its chromosome, such as *Klebsiella pneumoniae* [2,3].

Plasmid mediated *fosA* has been associated with high fosfomycin MICs leading resistance to this agent. For example, the most common and the most recent *fosA* variants, *fosA3* and *fosA10,* were associated with high fosfomycin, MICs, > 256mg/L and > 128mg/L, respectively [4,5]. On the other hand, the chromosomic *fosA* gene is usually harbored by *K. pneumoniae* isolates categorized as susceptible to fosfomycin with MICs, < 16mg/L [6,7]. This fact suggests that the chromosomic *fosA* gene is weakly or not expressed in this species. To date, just one study has reported fosfomycin resistance in a *K. pneumoniae* strain directly attributed to overexpression of the chromosomic *fosA* gene [8].

In *K. pneumoniae,* the chromosomic *fosA* gene is located downstream of the *lysR* regulator, overlapping its end. This tandem is downstream followed by a DNA-binding transcriptional regulator *mocR* and is upstream preceded by the *mdaB* gene, a NAD(P)H oxidoreductase [9] (Figure 1). The *fosA5, fosA6* and the recently described *fosA10*, which were all encountered in *Escherichia coli* plasmids [9, 10, 11], were recognized to be originated from *K. pneumoniae* since these genes present high nucleotide identity with chromosomic *fosA of K. pneumoniae* [7].

The Infectious Diseases Society of America has not recommended the use of fosfomycin to treat infectious caused by *K. pneumoniae* based on the risk of possible emergence of fosfomycin resistance during treatment due to chromosomic *fosA* gene [2]. Furthermore, different *in vitro* studies have observed *K. pneumoniae* regrowth under fosfomycin exposure after 24h, which may favour treatment failure [3,12].

Despite the increasing numbers of research involving fosfomycin resistance and the *fosA* genes, there is yet scarce information about the characterization of chromosomic *fosA*. Recently, our group showed that this gene is highly conserved in *K. pneumoniae,* with each sequence type (ST) or clonal complex (CC) presenting the same *fosA* gene sequence [13].

In the present work, we aimed to compare the transcriptional level of *fosA* gene between fosfomycin-resistant and -susceptible *K. pneumoniae* clinical isolates as well as analyze and characterize the *fosA* genetic context in fosfomycin-resistant isolates. These bacterial strains were previously genetically characterized through whole genome sequencing [12, 13, 14].

Fosfomycin resistance was confirmed by agar-dilution method supplemented with glucose-6-phosphate (G6P) following BRCAST/EUCAST recommendations [15]. The fosfomycin MICs obtained were as follows: HSP06 (128 mg/L), HSP29 (64 mg/L), HSP80 and HSP84 (both 256 mg/L). For comparison purposes, we analyzed fosfomycin-susceptible isolates (HSP73, MIC of 8 mg/L; P05, MIC of 16 mg/L; P86, MIC of 16mg/L; HSP64, MIC of 16 mg/L) as well as the *K. pneumoniae* strain ATCC 700603 (MIC, 256 mg/L).

In order to determine the possible impact of other fosfomycin resistance determinants leading to fosfomycin resistance in these isolates, we also analyzed the nucleotide and amino acids sequences of different determinants which have been associated with fosfomycin resistance: *murA, glpT, uhpT, uhpA, uhpB, uhpC, ptsI, cyaA* and *galU* using the Clustal Omega tool. None of these determinants presented nucleotide mutations. According to the previously reported sequencing data [12], these isolates did not harbor any plasmid-mediated fosfomycin resistance *fosA* gene, but as expected all had chromosomic *fosA* gene.

Thus, we performed a phenotypic test based on the use of phosphonoformate (PPF), a recognized FosA inhibitor to evaluate the activity of FosA enzymes [16]. The disk potentiation testing with PPF was performed as described previously by Ito et al. [17]. Briefly, cultures of each isolate were plated on Mueller-Hinton agar with a 50-mg/mL sodium phosphonoformate solution added to a 200 μg fosfomycin disk supplemented with 50 μg of G6P. The plates were incubated overnight at 37°C and the inhibition zones were recorded. The obtained inhibition zones in the presence of PPF were then compared to the inhibition zones obtained with the disks of fosfomycin without supplementation with PPF. For each isolate tested, a blank disk with PPF was also placed on the agar plate as a negative control.

We observed that the fosfomycin-resistant isolates presented the inhibition zone in the presence of PPF, varying from 3 to 8 mm of difference (Table 1) when comparing with those

inhibition zones obtained by testing fosfomycin disks without PFF. For the fosfomycin-susceptible isolates, we observed zero or one millimeter of change of inhibition zone diameter when disks of fofomycin were tested on Mueller-Hinton agar supplemented with PPF. Also, we observed the growth of many colonies inside the inhibitory zone on the isolates tested on plates with no PPF supplementation. Most of these colonies were inhibited under the exposure to PPF (Figure 2). Elliot and colleagues in a previous study postulated that these colonies frequently seen in some non-*E. coli* Enterobacterales possibly were driven by the presence of chromosomic *fosA* rather than channel or transporter mutations as observed for *E. coli* mutants [18].

To confirm the *fosA* expression, we performed a quantitative real-time PCR, aiming to analyze the *fosA* transcriptional levels in these isolates. The isolates were then cultured overnight in Mueller Hinton broth medium in two different conditions: i. under fosfomycin exposure (8mg/L); and ii. without fosfomycin exposure. After this period, the inoculum was prepared based on a 1:20 dilution and the absorbance of each sample was measured until the bacterial log phase of growth was reached. Then, the RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) from the exponentially growing cells. The cDNA synthesis was performed by reverse transcriptase reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Foster, US). The cDNA obtained was used to quantify the level of *fosA* gene using the oligonucleotide previously described [19]. The quantitative RT-PCR was conducted in thermocycler AppliedTwo ABI 7500 Fast Sequence Detection Instruments (Applied Biosystems Foster, US) using Platinum SYBR Green Supermix (Invitrogen, Carlsbad, USA). The *16S* gene was used as endogenous control and the data was analyzed using the comparative cycle threshold (ΔΔCT) method [20]. All data on gene expression are the average result of triplicate analysis.

All isolates after being exposed to fosfomycin presented an increased *fosA* transcriptional rate (Figure 3). Among the four fosfomycin-resistant clinical isolates we observed a 5.99- to 7.73-fold increase on *fosA* transcriptional rate under fosfomycin exposure when comparing with each isolate without fosfomycin exposure. The fosfomycin-susceptible isolates presented a 1.27 to 1.44 *fosA* -fold increase. On average, after fosfomycin exposure, we observed that the relative expression of chromosomic *fosA* gene in fosfomycin-resistant clinical isolates was 4.9-fold higher than those observed for fosfomycin-susceptible isolates (Figure 3). The ATCC 700603 presented 1.66-fold increase on *fosA* expression after fosfomycin exposure.

Furthermore, by comparing the *fosA* transcriptional level of each sample with the same level obtained for the ATCC 700603 after fosfomycin exposure, we observed that all the isolates (fosfomycin-resistant and -susceptible) presented *fosA* overexpression. Among the fosfomycin-resistant it varied from 5.393 to 186.744-fold and among the fosfomycin-susceptible, from 2.148 to 14.704-fold (Figure 4). Yet, we analyzed the *fosA* expression of each isolate compared to the ATCC 700603 without fosfomycin exposure. Overall, we noted that without fosfomycin exposure, the transcriptional level of *fosA* decreased for all isolates (1.153 - 45.665-fold), but still remained higher than that observed for the ATCC 700603 (Figure 4).

These increases in the *fosA* transcriptional level, especially with the resistant isolates when exposed to fosfomycin, and its respective decrease without fosfomycin exposure suggests that the chromosomic *fosA* overexpression can be an inducible mechanism of resistance. However, more studies are needed to confirm this hypothesis.

Aiming to understand if the increased in the *fosA* transcriptional level could have been influenced by other genetic determinantes, we analyzed the *lysR* gene sequences as well as the promoters surrounding the genetic context of *fosA* gene by using the BPROM software (http://www.softberry.com/berry.phtml?topic=bprom). We observed that all the fosfomycin-resistant as well as the fosfomycin-susceptible clinical isolates presented the same *lysR* and promoter sequences (P1, P2 and P3) with the exact same position (Figure 1). Interestingly, we found that the ATCC 700603 which is resistant to fosfomycin (MIC, 256 mg/L) and showed no chromosomic *fosA* overexpression, presented two different promoters, P1', overlapping the beginning of *lysR* gene and P4 (Figure 1). These differences on promoter's sequences and positions could explain why some *K. pneumoniae* overexpresses chromosomic *fosA* gene and others do not, but more studies are needed to confirm this observation. For example, in this study we analyzed fosfomycin-susceptible isolates which presented increased *fosA* transcriptional level after fosfomycin exposure compared to the ATCC 700603, and these isolates also presented the same *fosA* genetic context of the fosfomycin-resistant isolates. In this manner, emergence of resistance due to *fosA* overexpression could occur in *K. pneumoniae* susceptible to fosfomycin when exposed to fosfomycin.

To date, this is the second study which reported fosfomycin resistance in *K. pneumoniae* clinical isolates associated with chromosomic *fosA* overexpression. The first report occurred in 2020, in a study conducted by Kieffer et al. with an urinary *K. pneumoniae* isolate. In that study, the chromosomic *fosA* overexpression was associated with a hybrid promoter present in a IS*Ecp1-bla*$_{\text{CTX-M-14}}$ tandem located between the *lysR* regulator and the *mdaB* gene. This

promoter caused an overexpression of *fosA* gene 340-fold compared to the recombinant without this hybrid promoter [8]. In our work, we did not observe this tandem or any other similar within the *fosA* gene. Also, the *fosA* expression level was lower (5.99 – 7.73-fold increase) than that observed by Kieffer et al. These results were supported by the differences found in the fosfomycin MICs. While the isolate characterized by Kieffer et al. showed a higher fosfomycin MIC (>1024 mg/L), our isolates displayed MICs ranging from 64 to 256 mg/L [8].

Thus, our work showed that despite being uncommon, fosfomycin resistance in *K. pneumoniae* associated with overexpression of chromosomic *fosA* can occur. We showed that the four *K. pneumoniae* fosfomycin-resistant clinical isolates presented no plasmidial *fosA* gene and no mutations in other known genes encoding fosfomycin resistance determinants. On the other hand, these isolates presented increased *fosA* transcriptional levels and, phenotypically, FosA production possibly justifying fosfomycin resistance. However, we could not identify a genetic factor influencing the increase in chromosomic *fosA* expression. Lastly, we hypothesize that chromosomic *fosA* overexpression can be an inducible mechanism of fosfomycin resistance because after fosfomycin exposure all isolates showed some level of *fosA* transcriptional level increase. Additional studies are necessary to evaluate the clinical significance of this finding as well as if the emergence of inducible fosfomycin resistance would impact the *K. pneumoniae* fitness.

Data availability. The whole genome of the studied isolates can be encountered in GenBank under accession numbers SAMEA7556444, SAMN10592704, SAMEA7556454, ASM1103817v1, ASM1103836v1, ASM1103821v1, SAMEA7556485, SAMN10592697.

References

1. Falagas, M.E.; Kastoris, A.C.; Kapaskelis, A.M.; Karageorgopoulos, D.E. Fosfomycin for the Treatment of Multidrug-Resistant, Including Extended-Spectrum β-Lactamase Producing, Enterobacteriaceae Infections: A Systematic Review. *Lancet Infect. Dis.* 2010.
2. Tamma, P.D.; Aitken, S.L.; Bonomo, R.A.; Mathers, A.J.; Van Duin, D.; Clancy, C.J. Infectious Diseases Society of America 2022 Guidance on the Treatment of Extended-Spectrum β-Lactamase Producing Enterobacterales (ESBL-E), Carbapenem-Resistant Enterobacterales (CRE), and Pseudomonas Aeruginosa with Difficult-to-Treat Resistance (DTR-P. *Clin. Infect. Dis.* **2022**, doi:10.1093/cid/ciac268.

3. Wistrand-Yuen, P.; Olsson, A.; Skarp, K.P.; Friberg, L.E.; Nielsen, E.I.; Lagerbäck, P.; Tängdén, T. Evaluation of Polymyxin B in Combination with 13 Other Antibiotics against Carbapenemase-Producing Klebsiella Pneumoniae in Time-Lapse Microscopy and Time-Kill Experiments. *Clin. Microbiol. Infect.* **2020**, *26*, 1214–1221, doi:10.1016/j.cmi.2020.03.007.

4. Wachino, J.I.; Yamane, K.; Suzuki, S.; Kimura, K.; Arakawa, Y. Prevalence of Fosfomycin Resistance among CTX-M-Producing Escherichia Coli Clinical Isolates in Japan and Identification of Novel Plasmid-Mediated Fosfomycin-Modifying Enzymes. *Antimicrob. Agents Chemother.* **2010**, *54*, 3061–3064, doi:10.1128/AAC.01834-09/ASSET/DE0C64BC-F8F4-4C6B-AC78-75A71D92FD10/ASSETS/GRAPHIC/ZAC9991090760003.JPEG.

5. Huang, Y.; Lin, Q.; Zhou, Q.; Lv, L.; Wan, M.; Gao, X.; Wang, C.; Liu, J.H. Identification of FOSA10, a Novel Plasmid-Mediated Fosfomycin Resistance Gene of Klebsiella Pneumoniae Origin, in Escherichia Coli. *Infect. Drug Resist.* **2020**, *13*, 1273–1279, doi:10.2147/IDR.S251360.

6. Endimiani A, Patel G, Hujer KM, et al. In vitro activity of fosfomycin against bla_{KPC}-containing *Klebsiella pneumoniae* isolates, including those nonsusceptible to tigecycline and/or colistin. *Antimicrob Agents Chemother*. **2010**;54(1):526-529. doi:10.1128/AAC.01235-09

7. Ito, R.; Mustapha, M.M.; Tomich, A.D.; Callaghan, J.D.; McElheny, C.L.; Mettus, R.T.; Shanks, R.M.Q.; Sluis-Cremer, N.; Doi, Y. Widespread Fosfomycin Resistance in Gram-Negative Bacteria Attributable to the Chromosomal *fosA* Gene. *MBio* **2017**, *8*, 1–9, doi:10.1128/mbio.00749-17.

8. N.; Poirel, L.; Mueller, L.; Mancini, S.; Nordmann, P. ISEcp1-Mediated Transposition Leads to Fosfomycin and Broad-Spectrum Cephalosporin Resistance in Klebsiella Pneumoniae. *Antimicrob. Agents Chemother.* **2020**, *64*, doi:10.1128/AAC.00150-20.

9. Ma, Y.; Xu, X.; Guo, Q.; Wang, P.; Wang, W.; Wang, M. Characterization of FosA5, A New Plasmid-Mediated Fosfomycin Resistance Gene in Escherichia Coli. *Lett. Appl. Microbiol.* **2015**, *60*, 259–264, doi:10.1111/lam.12366.

10. Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, et al. Glutathione-S-transferase FosA6 of Klebsiella pneumoniae origin conferring fosfomycin resistance in ESBL-producing Escherichia coli. J Antimicrob Chemother 2016;71.

https://doi.org/10.1093/jac/dkw177.

11. Huang Y, Lin Q, Zhou Q, Lv L, Wan M, Gao X, et al. Identification of FOSA10, a novel plasmid-mediated fosfomycin resistance gene of klebsiella pneumoniae origin, in Escherichia coli. Infect Drug Resist 2020;13. https://doi.org/10.2147/IDR.S251360.

12. Ribeiro, C. da S.Á.; Santos, F.F.; Moses, I.B.; Andrade Da, L.; Minarini, R.; Gales, A.C. Sequencing of FosA: A Rapid and Inexpensive Method for Discriminating Klebsiella Pneumoniae CC258 from Other Clones. *https://home.liebertpub.com/mdr* **2022**, doi:10.1089/MDR.2022.0081.

13. Ribeiro, A.C. da S.; Chikhani, Y.C. dos S.A.; Valiatti, T.B.; Valêncio, A.; Kurihara, M.N.L.; Santos, F.F.; Minarini, L.A. da R.; Gales, A.C. In Vitro and In Vivo Synergism of Fosfomycin in Combination with Meropenem or Polymyxin B against KPC - 2 - Producing Klebsiella Pneumoniae Clinical Isolates. *Antibiotics* **2023**, *12*, 237, doi:https://doi.org/10.3390/antibiotics12020237.

14. Andrey, D.O.; Dantas, P.P.; Martins, W.B.S.; de Carvalho, F.M.; Almeida, L.G.P.; Sands, K.; Portal, E.; Sauser, J.; Cayô, R.; Nicolas, M.F.; et al. An Emerging Clone, Klebsiellapneumoniae Carbapenemase 2-Producing K. Pneumoniae Sequence Type 16, Associated with High Mortality Rates in a CC258-Endemic Setting. *Clin. Infect. Dis.* **2020**, *71*, E141–E150, doi:10.1093/cid/ciz1095.

15. European, C. European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters. *Eucast* **2013**, 0–99.

16. Tomich, A.D.; Klontz, E.H.; Deredge, D.; Barnard, J.P.; Mcelheny, C.L.; Eshbach, M.L. Crossm Small-Molecule Inhibitor of FosA Expands Fosfomycin Activity. **2019**, *63*, 1–14.

17. Ito, R.; Adam D. Tomich; McElheny, C.L.; Mettus, R.T.; Sluis-Cremer, N.; Doi, Y. Inhibition of Fosfomycin Resistance Protein FosA by Phosphonoformate (Foscarnet) in Multidrug-Resistant Gram-Negative Pathogens. *Antimicrob. Agents Chemother.* **2017**, *61*.

18. Elliott ZS, Barry KE, Cox HL, Stoesser N, Carroll J, Vegesana K, et al. The Role of fosA in challenges with fosfomycin susceptibility testing of multispecies Klebsiella pneumoniae carbapenemase-producing clinical isolates. J Clin Microbiol 2019;57. https://doi.org/10.1128/JCM.00634-19.

19. Nordmann, P.; Poirel, L.; Mueller, L. Rapid Detection of Fosfomycin Resistance in Escherichia Coli. *J. Clin. Microbiol.* **2019**, *57*, 16–20, doi:10.1128/JCM.01531-18.

20. Livak, K. J., and Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔ-CT Method. *Methods* **2001**, *25*, 402–408, doi:doi: 10.1006/METH.2001.1262.

Table 1. Fosfomycin minimal inhibitory concentrations (MICs), diameters of inhibition zone obtained through disk potentiation test using phosphonoformate (PPF) and the *fosA* transcriptional levels for each isolate

Isolate	Fosfomycin MIC (mg/L)	Inhibitory zone (mm)			*fosA transcriptional level*[c]
		Fos	Fos+PPF	Difference	*fosA*
HSP06	128	20	23	3	7.73
HSP80[a]	256	20	28	8	7.92
HSP29	64	20	25	5	4.69
HSP84	256	16	20	4	6.36
HSP73	8	21	21	0	1.59
P05[b]	16	21	22	1	1.26
P86	16	23	24	1	1.39
HSP64	16	25	26	1	1.43
ATCC 700603	256	12	12	0	1.66

a. Without PPF supplementation many colonies grew within the inhibition zone. With PPF supplementation no colonies were observed (Figure 2a).

b. Without PPF supplementation few colonies grew within the inhibition zone. With PPF supplementation = few colonies grew, showing no PPF activity (Figure 2b).

c. Diferences in the *fosA* transcriptional levels comparing the same isolate grown with and without selective pressure (fosfomycin)

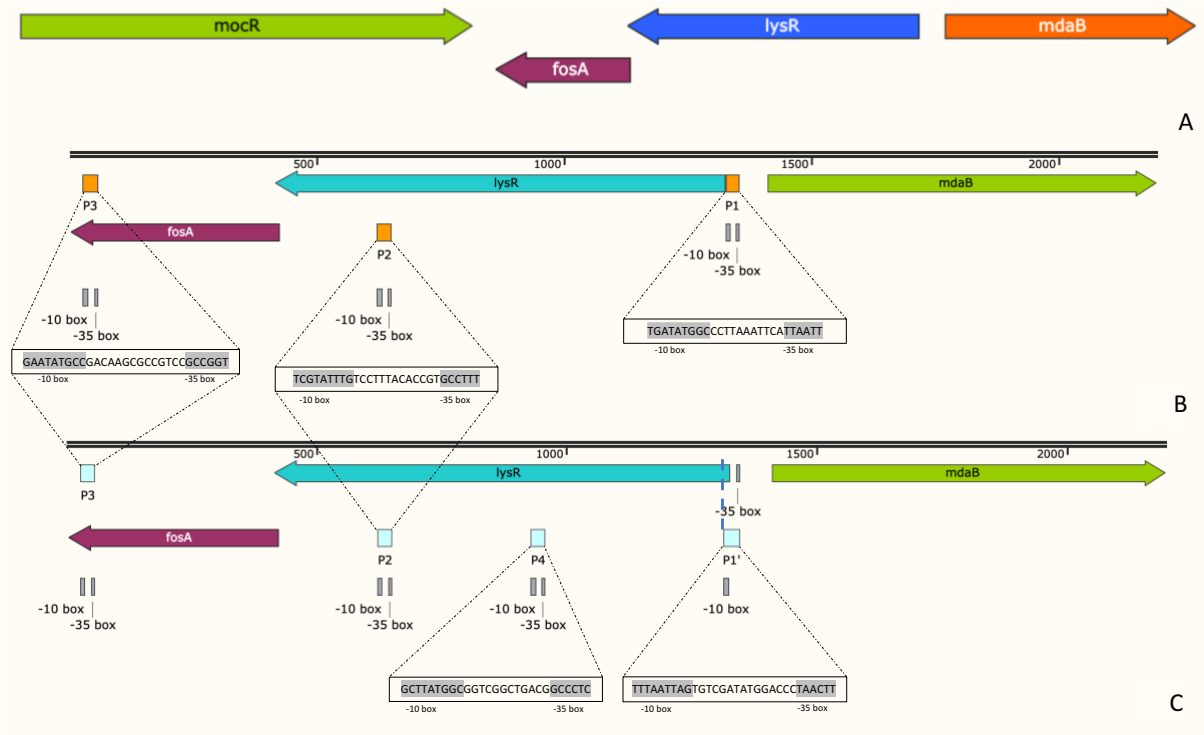

Figure 1. Genetic context of chromosomic *fosA* gene encountered in *Klebsiella pneumoniae* (A) and the promoters within the *fosA* genetic context. (B) Fosfomycin-resistant and -susceptible *K. pneumoniae* clinical isolates presented three promoters in the *fosA* genetic context. (C) The ATCC 700603 showed four promoters, sharing P2 and P3 sequences and positions with clinical isolates. This strain also presented two different promoters named P1'and P4 which are represented in the figure. P1'overlaps the begin of *lysR* gene as highlighted by the dashed blue line.

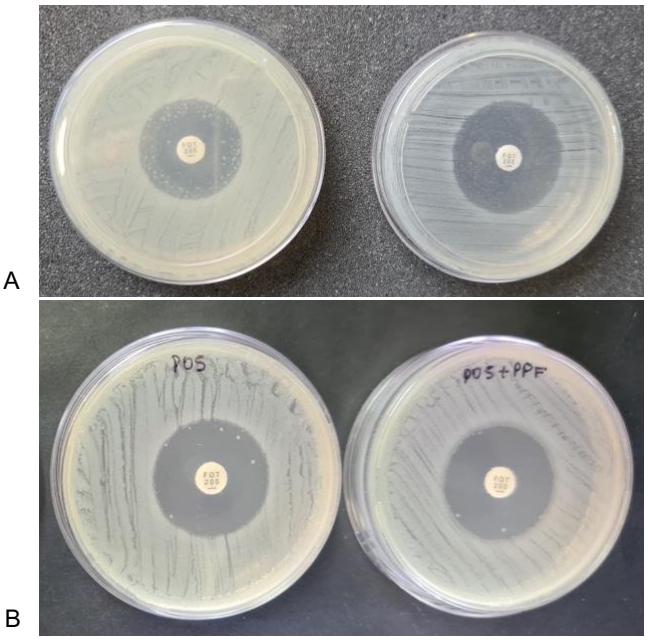

Figure 2. Phenotypic test for *fosA* detection in Mueller-Hinton plates supplemented with 50-mg/mL sodium phosphonoformate (PPF). Panel A, a fosfomycin-resistant isolate (HSP80); and Panel B, a fosfomycin-susceptible isolate (P05).

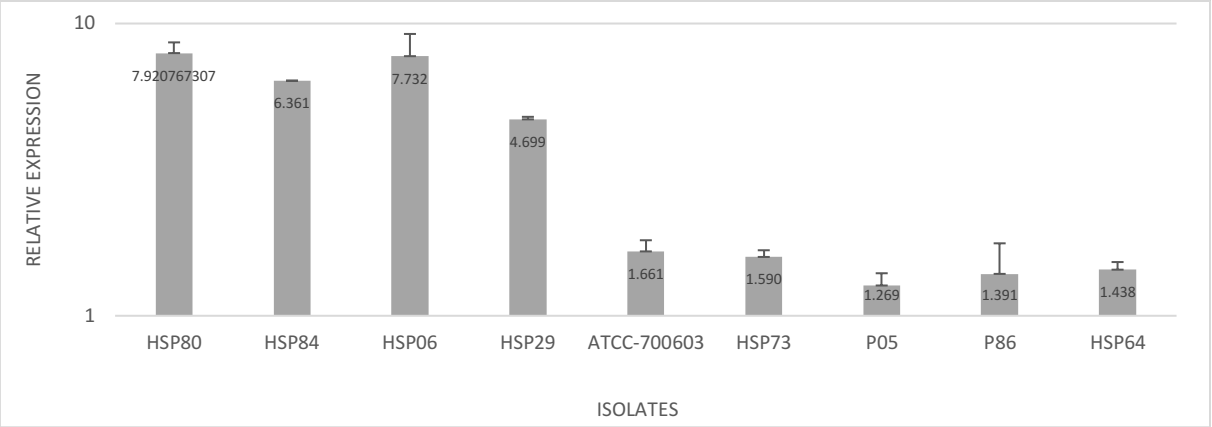

Figure 3. Relative expression of the *fosA* gene in fosfomycin-resistant and -susceptible *K. pneumoniae* clinical isolates grown under fosfomycin exposure. The relative expression was calculated for each isolate based on the CTs obtained under and without fosfomycin exposure. Experiments were performed triplicates.

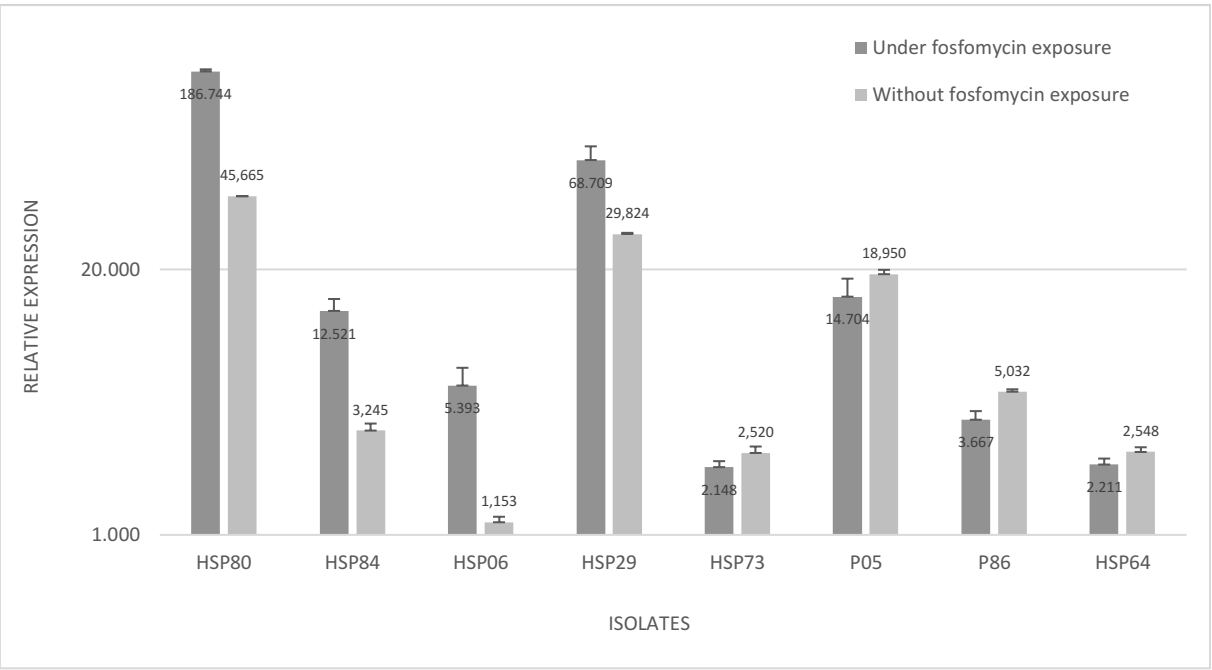

Figure 4. Relative expression of the *fosA* gene in fosfomycin-resistant and -susceptible *K. pneumoniae* clinical isolates under and without fosfomycin exposure. The relative expression was calculated for each isolate based on the CTs obtained under and without fosfomycin exposure compared to the CTs obtained for the ATCC 700603. This analysis considered the ATCC as the control. Experiments were performed triplicates.

5. DISCUSSÃO

Infecções de difícil tratamento causadas por *K. pneumoniae* produtora de KPC são associadas a altos índices de mortalidade (WANG et al., 2021; WORLD HEALTH ORGANIZATION, 2017). Embora novos antimicrobianos tenham sido desenvolvidos nos últimos anos, como as novas combinações de novos inibidores de β-lactamases com β-lactâmicos e o cefiderocol, o acesso a estes antimicrobianos ainda é restrito em muito países, uma vez que o processo de aprovação e registro pode ser moroso e quando disponíveis, apresentam alto valor agregado (ANTIMICROBIAL RESISTANCE RESEARCH PROGRAMME, 2022).

Alternativas terapêuticas tornaram-se, então, necessárias. É nesse contexto que o uso de clínico da bacteriofagoterapia foi retomado no ocidente (CISEK et al., 2017), bem como o uso de diferentes combinações de antimicrobianos já usados na clínica passou a ser explorado (SCUDELLER et al., 2021). Além disso, retomou-se o uso de antimicrobianos antigos, que haviam caído em desuso (KARAISKOS et al., 2019). A fosfomicina, que foi descoberta no século XX, é considerada segura e associada a baixos índices de resistência (FALAGAS et al., 2019; FRANSEN et al., 2017). Inclusive, diferentes estudos têm demonstrado boa atividade de fosfomicina contra patógenos MDR, como *E. coli* produtora de ESBL e *K. pneumoniae* produtora de KPC (AL-QURAINI et al., 2022; LIU et al., 2020). Entretanto, existe uma preocupação quanto ao uso de fosfomicina para tratamento de infecções causadas por *K. pneumoniae,* uma vez que essa espécie bacteriana é considerada reservatório natural do *fosA,* um gene que confere resistência à fosfomicina (ITO et al., 2017c; TAMMA et al., 2022).

No presente estudo, buscou-se investigar e caracterizar o gene *fosA* em *K. pneumoniae,* bem como avaliar a atividade *in vitro* e *in vivo* de fosfomicina em combinação com meropenem ou polimixina B contra isolados clínicos de *K. pneumoniae* produtoras de KPC.

Ao analisarmos as sequências do gene *fosA* cromossômico nos isolados clínicos de *K. pneumoniae*, bem como seu contexto genético, observamos que *fosA* é um gene altamente conservado no cromossomo dessa espécie bacteriana, uma vez que bactérias pertencentes a um mesmo ST ou complexo clonal apresentaram a mesma sequência de *fosA*. Com base nessa evidência, nós analisamos a sequência de *fosA* cromossômico em *K. pneumoniae* como um método alternativo de tipagem molecular dessa espécie bacteriana, objetivando principalmente distinguir isolados pertencentes ao CC258/11 de outros STs.

Sabe-se que a tipagem molecular é importante para identificar precisamente os microrganismos causadores de surtos, prevenir sua disseminação e avaliar/predizer os

desfechos clínicos (WANG et al., 2021; WYRES; HOLT, 2021). Estudos recentes conduzidos com *K. pneumoniae* resistente aos carbapenêmicos evidenciaram que STs e CCs variam de acordo com a região geográfica, aumentando ainda mais a necessidade de uma caracterização mais ágil desses isolados (WANG et al., 2021; WYRES; HOLT, 2021). Atualmente, as principais metodologias que permitem a tipagem molecular bacteriana são o *Multilocus Sequence Typing* (MLST) e o sequenciamento de genoma completo (WGS, do inglês *Whole Genome Sequencing)*. O WGS tem se tornado mais acessível nos últimos anos, embora ainda seja considerada uma metodologia cara para a maioria dos países em desenvolvimento. Além disso, existe a complexidade relacionada à análise dos dados provenientes do WGS fator que é desafiador (LAM et al., 2021). No Brasil, especificamente, esses custos variam de $25 a $60. Nos últimos anos, tem-se buscado alternativas para simplificar e baratear a tipagem molecular. O estudo conduzido por Pena e colaboradores (2020), por exemplo, reportou o uso do MALDI-TOF MS como alternativa para tipagem de *K. pneumoniae* resistente aos carbapenêmicos pertencentes aos STs 11 e 101 (PENA et al., 2020). No presente estudo, conseguiu-se diferenciar clones de *K. pneumoniae* do CC258/11 de outros STs, bem como identificar clones dos STs 16, 15, 307 e 101 por meio da análise da sequência do gene *fosA* cromossômico. Portanto, por meio de uma técnica simples, rápida e barata, seria possível realizar a tipagem de clones de *K. pneumoniae* de alto risco.

K. pneumoniae resistente aos carbapenêmicos é um dos principais patógenos causadores de infecções graves. Essas infecções são desafiadoras aos clínicos, devido ao limitado espectro de alternativas terapêuticas disponíveis (WANG et al., 2021). O uso de fosfomicina como monoterapia contra *K. pneumoniae* não tem sido recomendado principalmente por conta do gene *fosA* intrínseco que essa espécie carreia (ITO et al., 2017c). Entretanto, buscando potencializar e otimizar seu uso clínico, diferentes combinações de antimicrobianos com fosfomicina têm sido exploradas (OLIVA et al., 2022, 2021; SCUDELLER et al., 2021). Nesse estudo analisamos o uso de fosfomicina em combinação com meropenem ou polimixina B por metodologias *in vitro* (*checkerboard* e *time-kill*) e *in vivo* (modelo de sobrevivência em *Galleria mellonella)*.

Os resultados obtidos *in vitro* apontaram para a recuperação da atividade de meropenem e de polimixina B em 82,4% e em 47% dos isolados, respectivamente, quando combinados com fosfomicina. Além disso, observou-se sinergismo entre fosfomicina e meropenem contra 58,8% dos isolados, com redução do crescimento bacteriano variando de 3,0 a 6,7 $\log_{10}$ CFU/mL; e entre fosfomicina e polimixina B contra 47,1% dos isolados, com redução de 6,0 a 6,2 $\log_{10}$ CFU/mL quando usadas as concentrações de pico (fosfomicina, 395 mg/L; meropenem, 40.9

mg/L; polimixina B 6.44mg/L. Um estudo brasileiro realizado com *K. pneumoniae* produtora de KPC isoladas antes de 2010, demonstrou também sinergismo entre fosfomicina e meropenem, com redução importante da CIM de meropenem (1/4MIC$_{50}$) (ALBIERO et al., 2016). Scudeller et al. (2021) analisaram dois estudos que avaliaram o sinergismo entre fosfomicina e polimixina B, e observaram um alto índice de sinergismo (p-valor = 0,05) (SCUDELLER et al., 2021).

O ensaio de sinergismo conduzido em *G. mellonella* demonstrou atividade de fosfomicina em combinação com meropenem, uma vez que todas as larvas tratadas com essa combinação sobreviveram. A análise estatística dos resultados demonstrou que os grupos das larvas tratadas com fosfomicina ou meropenem isoladamente, bem como com a combinação de fosfomicina e meropenem ou polimixina B apresentaram maior e significativo índice de sobrevivência quando comparado com o controle positivo (larvas infectadas e não tratadas). Diferentes estudos têm usado o modelo de sobrevivência em *G. mellonella* para avaliar sinergismo entre drogas (BORJAN et al., 2020; KUSSMANN et al., 2021), obtendo importantes resultados com concordância entre os resultados *in vitro* e *in vivo (G. mellonella)* (BORJAN et al., 2020; LÁZÁR et al., 2022; SKINNER et al., 2017). Esses estudos, bem como o presente estudo corroboram o uso desse modelo *in vivo* para sugerir possíveis regimes terapêuticos.

Observou-se também a atividade sinérgica de fosfomicina com meropenem ou polimixina B na desestruturação do biofilme produzido por *K. pneumoniae* produtora de KPC. Uma redução de 1,481 a 3,470 vezes na detecção do biofilme foi observada quando comparadas as densidades ópticas obtidas sob exposição aos antimicrobianos com as obtidas sem exposição. A desestruturação do biofilme permitiu a mudança nas densidades ópticas obtidas para todos os isolados, com variação da categoria “moderadamente aderentes” para “fracamente ou não aderentes”. A atividade antibiofilme de fosfomicina foi previamente explorada em outros estudos, avaliando atividade de fosfomicina combinada com outros fármacos (CAI et al., 2018; CHAI et al., 2016), como por exemplo, a N-acetilcisteína (MARCHESE et al., 2003). Entretanto, a ação da combinação de fosfomicina com meropenem na desestruturação de biofilme em *K. pneumoniae* nunca fora reportada, e o presente estudo demonstrou importante atividade dessa combinação contra o biofilme formado por *K. pneumoniae*. Este estudo aponta para o potencial uso de fosfomicina em combinação para o tratamento de diferentes tipos de infecções, inclusive aquelas caracterizadas por importante formação de biofilme, como as infecções de pele e partes moles.

Por fim, dentre os 17 isolados de *K. pneumoniae* produtores de KPC analisados neste estudo, quatro apresentaram-se resistentes à fosfomicina. Buscou-se, portanto, detectar os

determinantes de resistência à fosfomicina nesses isolados. Com essa finalidade, foram investigadas as mutações nos genes *murA, glpT, uhpT, uhpA, uhpB, uhpC, ptsI, cya, lysR* e *galU,* e a detecção do gene *fosA* plasmideal, genes estes previamente reportados como relacionados à resistência à fosfomicina (CASTAÑEDA-GARCÍA; BLÁZQUEZ; RODRÍGUEZ-ROJAS, 2013b; FALAGAS et al., 2019). Nenhuma mutação nesses determinantes, bem como *fosA* plasmideal foram detectados entre os isolados caracterizados nesse estudo.

Baseados em um estudo anterior que reportou aumento da expressão de *fosA* cromossômico em *K. pneumoniae,* investigou-se a expressão desse gene nos isolados do presente estudo. Observou-se um aumento de 4,69 a 7,73 vezes na expressão de *fosA* nos isolados resistentes à fosfomicina. Em média, os isolados resistentes à fosfomicina apresentaram expressão de *fosA* cerca de 4,9 vezes maior do que os isolados sensíveis à fosfomicina quando expostos à fosfomicina. Entretanto, por meio das análises realizadas *in silico* não se encontrou o fator influenciador desse aumento da expressão de *fosA* cromossômico. *K. pneumoniae* é considerada uma espécie reservatório do gene *fosA* (ITO et al., 2017c), sendo que as variantes *fosA5, fosA6* e *fosA10*, descobertas em plasmídeos em *E. coli,* são consideradas originárias do cromossomo de *K. pneumoniae* (GUO et al., 2016; HUANG et al., 2020a; MA et al., 2015). O gene *fosA* cromossômico é habitualmente associado a valores de CIMs compatíveis com sensibilidade à fosfomicina. Entretanto, este é o segundo estudo que relaciona resistência à fosfomicina em *K. pneumoniae* com aumento da expressão de *fosA* cromossômico (KIEFFER et al., 2020a). Recentemente, Wang et al. (2022) analisou a expressão de *fosA* cromossômico em 14 isolados de *K. pneumoniae* buscando identificar o determinante de resistência à fosfomicina nestes patógenos. Entretanto, não obtiveram diferença na expressão de *fosA* entre isolados sensíveis e resistentes (WANG et al., 2022a).

Os resultados obtidos por meio da análise dos dados de expressão do gene *fosA* nos permitem sugerir que é possível que a hiperexpressão desse gene cromossômico em *K. pneumoniae* seja um mecanismo de resistência induzível. Entretanto, mais estudos são necessários para confirmar essa hipótese.

6. CONCLUSÃO

A partir dos resultados desse trabalho, podemos concluir que:

a. O gene *fosA* cromossômico em *K. pneumoiae* apresenta uma sequência conservada e pode ser uma ferramenta simples, rápida e barata para realizar a tipagem de clones de *K. pneumoniae* de alto risco;
b. Os bons resultados *in vitro* e *in vivo* da atividade de fosfomicina isolada e em combinação com meropenem ou polimixina B demonstraram o seu promissor uso como terapia contra infecções de difícil tratamento;
c. Fosfomicina isolada e em combinação apresentou-se ativa na desestruturação do biofilme produzido por *K. pneumoniae;*
d. Nos testes com fosfonoformato, observou-se uma maior alteração no diâmetro da zona inibitória para os isolados clínicos resistentes à fosfomicina;
e. A aumento da expressão do gene *fosA* observado especialmente sob exposição de fosfomicina nos leva a hipotetizar que esse talvez seja um mecanismo de resistência induzível à fosfomicina;
f. O aumento da expressão de *fosA* cromossômico observado nos isolados resistentes à fosfomicina neste estudo corroboraram a preocupação com o uso de fosfomicina para tratamento de infecções causadas por *K. pneumoniae*, necessitando de permanente avaliação da possibilidade de surgimento de resistência à fosfomicina.

REFERÊNCIAS BIBLIOGRÁFICAS

AL-QURAINI, Manawr; RIZVI, Meher; AL-JABRI, Zaaima; SAMI, Hiba; AL-MUZAHMI, Muna; AL-MUHARRMI, Zakariya; TANEJA, Neelam; AL-BUSAIDI, Ibrahim; SOMAN, Rajeev. Assessment of In-Vitro Synergy of Fosfomycin with Meropenem, Amikacin and Tigecycline in Whole Genome Sequenced Extended and Pan Drug Resistant Klebsiella Pneumoniae: Exploring A Colistin Sparing Protocol. **Antibiotics**, *[S. l.]*, v. 11, n. 2, p. 1–17, 2022. DOI: 10.3390/antibiotics11020153.

ALBIERO, James et al. Pharmacodynamic attainment of the synergism of meropenem and fosfomycin combination against pseudomonas aeruginosa producing metallo-lactamase. **Antimicrobial Agents and Chemotherapy**, *[S. l.]*, v. 63, n. 6, p. 1–16, 2019. DOI: 10.1128/AAC.00126-19.

ALBIERO, James; SY, Sherwin K. B.; MAZUCHELI, Josmar; CAPARROZ-ASSEF, Silvana Martins; COSTA, Bruno Buranello; ALVES, Janio Leal Borges; GALES, Ana Cristina; TOGNIM, Maria Cristina Bronharo. Pharmacodynamic evaluation of the potential clinical utility of fosfomycin and meropenem in combination therapy against KPC-2-producing Klebsiella pneumoniae. **Antimicrobial Agents and Chemotherapy**, *[S. l.]*, v. 60, n. 7, p. 4128–4139, 2016. DOI: 10.1128/AAC.03099-15.

ANDREY, Diego O. et al. An emerging clone, klebsiellapneumoniae carbapenemase 2-producing K. pneumoniae sequence type 16, associated with high mortality rates in a CC258-endemic setting. **Clinical Infectious Diseases**, *[S. l.]*, v. 71, n. 7, p. E141–E150, 2020. DOI: 10.1093/cid/ciz1095.

ANTIMICROBIAL RESISTANCE RESEARCH PROGRAMME. Lack of access to medicine is a major driver of drug resistance. How can pharma take action? **ACCESS TO MEDICINE FOUNDATION**, *[S. l.]*, 2022. Disponível em: https://accesstomedicinefoundation.org/medialibrary/62c2f0dcda565_atmf_appropriate_access_to_antimicrobials_2022-1666595298.pdf.

ANTONELLO, Roberta Maria et al. Fosfomycin as partner drug for systemic infection management. a systematic review of its synergistic properties from in vitro and in vivo studies. **Antibiotics**, *[S. l.]*, v. 9, n. 8, p. 1–74, 2020. DOI: 10.3390/antibiotics9080500.

ANVISA. **Boletim Segurança do Paciente e Qualidade em serviços de Saúde n°22: Avaliação dos indicadores nacionais das Infecções Relacionadas à Assistência à Saúde (IRAS) e Resistência Microbiana do ano de 2019.** 2021. Disponível em: https://www.gov.br/anvisa/pt-br/centraisdeconteudo/publicacoes/servicosdesaude/publicacoes. Acesso em: 1 nov. 2022.

BONCOMPAGNI, Selene Rebecca et al. Activity of fosfomycin/colistin combinations against planktonic and biofilm Gram-negative pathogens. **Journal of Antimicrobial Chemotherapy**, *[S. l.]*, v. 77, n. 8, p. 2199–2208, 2022. DOI: 10.1093/JAC/DKAC142. Disponível em: https://academic.oup.com/jac/article/77/8/2199/6580675.

BORJAN, Jovan; MEYER, Kevin A.; SHIELDS, Ryan K.; WENZLER, Eric. Activity of ceftazidime-avibactam alone and in combination with polymyxin B against carbapenem-resistant Klebsiella pneumoniae in a tandem in vitro time-kill/in vivo Galleria mellonella survival model analysis. **International Journal of Antimicrobial Agents**, *[S. l.]*, v. 55, n. 1, 2020. DOI: 10.1016/j.ijantimicag.2019.11.009. Disponível em:

https://doi.org/10.1016/j.ijantimicag.2019.11.009.

BRASIL. **Nota Técnica Nº 74/2022 - CGLAB/DAEVS/SVS/MS**. [s.l: s.n.]. Disponível em: https://brcast.org.br/wp-content/uploads/2022/09/SEI_MS-0028220258-Nota-Tecnica-NDM-e-coproducao-carbapenemase.pdf.

BRCAST - BRAZILIAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING, BRCast. Tabela-pontos-de-corte-clinicos-BrCAST. *[S. l.]*, p. 88, 2022. Disponível em: http://brcast.org.br/documentos/.

BURASTERO, Giulia Jole et al. Ceftazidime/Avibactam in Ventilator-Associated Pneumonia Due to Difficult-to-Treat Non-Fermenter Gram-Negative Bacteria in COVID-19 Patients: A Case Series and Review of the Literature. **Antibiotics**, *[S. l.]*, v. 11, n. 8, p. 1007, 2022. DOI: 10.3390/ANTIBIOTICS11081007. Disponível em: file:///pmc/articles/PMC9330655/.

CAI, Yun; YANG, Deqing; WANG, Jin; WANG, Rui. Amikacin and cefoperazone/sulbactam alone or in combination against carbapenem-resistant Pseudomonas aeruginosa. **Diagnostic Microbiology and Infectious Disease**, *[S. l.]*, v. 91, n. 2, p. 186–190, 2018. DOI: 10.1016/j.diagmicrobio.2018.01.023. Disponível em: https://doi.org/10.1016/j.diagmicrobio.2018.01.023.

CARCIONE, Davide; SIRACUSA, Claudia; SULEJMANI, Adela; LEONI, Valerio; INTRA, Jari. Old and New Beta-Lactamase Inhibitors: Molecular Structure, Mechanism of Action, and Clinical Use. **Antibiotics 2021, Vol. 10, Page 995**, *[S. l.]*, v. 10, n. 8, p. 995, 2021. DOI: 10.3390/ANTIBIOTICS10080995. Disponível em: https://www.mdpi.com/2079-6382/10/8/995/htm.

CASTAÑEDA-GARCÍA, Alfredo; BLÁZQUEZ, Jesús; RODRÍGUEZ-ROJAS, Alexandro. Molecular mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. **Antibiotics**, *[S. l.]*, v. 2, n. 2, p. 217–236, 2013. a. DOI: 10.3390/antibiotics2020217.

CASTAÑEDA-GARCÍA, Alfredo; BLÁZQUEZ, Jesús; RODRÍGUEZ-ROJAS, Alexandro. Molecular Mechanisms and Clinical Impact of Acquired and Intrinsic Fosfomycin Resistance. **Antibiotics**, *[S. l.]*, v. 2, n. 2, p. 217–236, 2013. b. DOI: 10.3390/antibiotics2020217.

CEJAS, Daniela; MAGARIÑOS, Francisco; ELENA, Alan; FERRARA, Micaela; ORMAZÁBAL, Cecilia; YERNAZIAN, María Valeria; GUTKIND, Gabriel; RADICE, Marcela. Emergence and clonal expansion of Klebsiella pneumoniae ST307, simultaneously producing KPC-3 and NDM-1. **Revista Argentina de Microbiologia**, *[S. l.]*, 2022. DOI: 10.1016/J.RAM.2022.04.002.

CHAI, Dong; LIU, Xu; WANG, Rui; BAI, Yan; CAI, Yun. Efficacy of Linezolid and Fosfomycin in Catheter-Related Biofilm Infection Caused by Methicillin-Resistant Staphylococcus aureus. *[S. l.]*, 2016. DOI: 10.1155/2016/6413982. Disponível em: http://dx.doi.org/10.1155/2016/6413982.

CISEK, Agata Anna; DA ˛BROWSKA, Iwona; KAROLINA, •; GREGORCZYK, Paulina; ZBIGNIEW, •; ZEWSKI, Wy_. Phage Therapy in Bacterial Infections Treatment: One Hundred Years After the Discovery of Bacteriophages. **Current Microbiology**, *[S. l.]*, v. 74, n. 2, p. 277–283, 2017. DOI: 10.1007/s00284-016-1166-x.

CUBA, Gabriel T.; ROCHA-SANTOS, Gerlan; CAYÔ, Rodrigo; STRELING, Ana Paula; NODARI, Carolina S.; GALES, Ana C.; PIGNATARI, Antonio C. C.; NICOLAU, David P.; KIFFER, Carlos R. V. In vitro synergy of ceftolozane/tazobactam in combination with fosfomycin or aztreonam against MDR Pseudomonas aeruginosa. **Journal of Antimicrobial Chemotherapy**, *[S. l.]*, v. 75, n. 7, p. 1874–1878, 2020. DOI: 10.1093/jac/dkaa095.

DAVIDO, Benjamin; SENARD, Olivia; DE TRUCHIS, Pierre; SALOMON, Jérôme; DINH,

Aurélien. Monotherapy of ceftazidime-avibactam and ceftolozane-tazobactam: two effective antimicrobial agents against multidrug-resistant organisms except for NDM-1 isolates. **International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases**, *[S. l.]*, v. 62, p. 124–125, 2017. DOI: 10.1016/J.IJID.2017.06.021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28676347/.

DE ROSSI, Patricia et al. Joint report of SBI (Brazilian Society of Infectious Diseases), FEBRASGO (Brazilian Federation of Gynecology and Obstetrics Associations), SBU (Brazilian Society of Urology) and SBPC/ML (Brazilian Society of Clinical Pathology/Laboratory Medicine): recomm. **Brazilian Journal of Infectious Diseases**, *[S. l.]*, v. 24, n. 2, p. 110–119, 2020. DOI: 10.1016/j.bjid.2020.04.002. Disponível em: https://doi.org/10.1016/j.bjid.2020.04.002.

DÍEZ-AGUILAR, María. Current key topics in fosfomycin. **Spanish Journal of Chemotherapy**, *[S. l.]*, v. 32, n. 1, p. 8–18, 2019.

EMA. **Disabling and potentially permanent side effects lead to suspension or restrictions of quinolone and fluoroquinolone antibioticsEuropean Medicines Agency-Science Medicines Agency**. [s.l: s.n.]. Disponível em: https://www.ema.europa.eu/en/documents/press-release/disabling-potentially-permanent-side-effects-lead-suspension-restrictions-quinolone-fluoroquinolone_en.pdf.

ESCHENBURG, Susanne; PRIESTMAN, Melanie; SCHÖNBRUNN, Ernst. Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamine Enolpyruvyl Transferase (MurA) is essential for product release. **Journal of Biological Chemistry**, *[S. l.]*, v. 280, n. 5, p. 3757–3763, 2005. DOI: 10.1074/jbc.M411325200. Disponível em: http://www.jbc.org/article/S0021925820762460/fulltext.

EUROPEAN, Committee. European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters. **Eucast**, *[S. l.]*, p. 0–99, 2013.

FALAGAS, Matthew E.; ATHANASAKI, Florentia; VOULGARIS, Georgios L.; TRIARIDES, Nikolaos A.; VARDAKAS, Konstantinos Z. Resistance to fosfomycin: Mechanisms, Frequency and Clinical Consequences. **International Journal of Antimicrobial Agents**, *[S. l.]*, v. 53, n. 1, p. 22–28, 2019. DOI: 10.1016/j.ijantimicag.2018.09.013. Disponível em: https://doi.org/10.1016/j.ijantimicag.2018.09.013.

FALAGAS, Matthew E.; GIANNOPOULOU, Konstantina P.; KOKOLAKIS, George N.; RAFAILIDIS, Petros I. Fosfomycin: Use beyond urinary tract and gastrointestinal infections. **Chinese Journal of Infection and Chemotherapy**, *[S. l.]*, v. 10, n. 2, p. 103, 2010. a. DOI: 10.1086/527442.

FALAGAS, Matthew E.; KASTORIS, Antonia C.; KAPASKELIS, Anastasios M.; KARAGEORGOPOULOS, Drosos E. **Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum β-lactamase producing, Enterobacteriaceae infections: a systematic review**. **The Lancet Infectious Diseases**, 2010. b. DOI: 10.1016/S1473-3099(09)70325-1.

FALAGAS, Matthew E.; VOULOUMANOU, Evridiki K.; SAMONIS, George; VARDAKAS, Konstantinos Z. Matthew E. Falagas,. **Clinical microbiology reviews**, *[S. l.]*, v. 29, n. 2, p. 321–347, 2016. DOI: 10.1128/CMR.00068-15.Address.

FRANSEN, Fiona; HERMANS, Kelly; MELCHERS, Maria J. B.; LAGARDE, Claudia C. M.; MELETIADIS, Joseph; MOUTON, Johan W. Pharmacodynamics of fosfomycin against ESBL- and/or carbapenemase-producing Enterobacteriaceae. **Journal of Antimicrobial Chemotherapy**, *[S. l.]*, v. 72, n. 12, p. 3374–3381, 2017. DOI: 10.1093/JAC/DKX328. Disponível em: https://academic.oup.com/jac/article/72/12/3374/4339347.

GOLLA, Vinaya Kumar; SANS-SERRAMITJANA, Eulàlia; POTHULA, Karunakar Reddy; BENIER, Lorraine; BAFNA, Jayesh Arun; WINTERHALTER, Mathias; KLEINEKATHÖFER, Ulrich. Fosfomycin Permeation through the Outer Membrane Porin OmpF. **Biophysical Journal**, *[S. l.]*, v. 116, n. 2, p. 258–269, 2019. DOI: 10.1016/j.bpj.2018.12.002.

GU, Danxia et al. A fatal outbreak of ST11 carbapenem-resistant hypervirulent Klebsiella pneumoniae in a Chinese hospital: a molecular epidemiological study. **The Lancet Infectious Diseases**, *[S. l.]*, v. 18, n. 1, p. 37–46, 2018. DOI: 10.1016/S1473-3099(17)30489-9. Disponível em: http://www.thelancet.com/article/S1473309917304899/fulltext.

GUO, Qinglan; TOMICH, Adam D.; MCELHENY, Christi L.; COOPER, Vaughn S.; STOESSER, Nicole; WANG, Minggui; SLUIS-CREMER, Nicolas; DOI, Yohei. Glutathione-S-transferase FosA6 of Klebsiella pneumoniae origin conferring fosfomycin resistance in ESBL-producing Escherichia coli. **Journal of Antimicrobial Chemotherapy**, *[S. l.]*, v. 71, n. 9, 2016. DOI: 10.1093/jac/dkw177.

HASHEMIAN, Seyed MohammadReza; FARHADI, Zinat; FARHADI, Tayebeh. Fosfomycin: the characteristics, activity, and use in critical care . **Therapeutics and Clinical Risk Management**, *[S. l.]*, v. Volume 15, p. 525–530, 2019. DOI: 10.2147/tcrm.s199119.

HENDLIN, D. et al. Phosphonomycin, a New Antibiotic Produced by Strains of Streptomyces. **Science**, *[S. l.]*, v. 166, n. 3901, p. 122–123, 1969. DOI: 10.1126/SCIENCE.166.3901.122. Disponível em: https://www.science.org/doi/10.1126/science.166.3901.122.

HUANG, Ying; LIN, Qingqing; ZHOU, Qiaoli; LV, Luchao; WAN, Miao; GAO, Xun; WANG, Chengzhen; LIU, Jian Hua. Identification of FOSA10, a novel plasmid-mediated fosfomycin resistance gene of klebsiella pneumoniae origin, in Escherichia coli. **Infection and Drug Resistance**, *[S. l.]*, v. 13, 2020. a. DOI: 10.2147/IDR.S251360.

HUANG, Ying; LIN, Qingqing; ZHOU, Qiaoli; LV, Luchao; WAN, Miao; GAO, Xun; WANG, Chengzhen; LIU, Jian Hua. Identification of FOSA10, a novel plasmid-mediated fosfomycin resistance gene of klebsiella pneumoniae origin, in Escherichia coli. **Infection and Drug Resistance**, *[S. l.]*, v. 13, p. 1273–1279, 2020. b. DOI: 10.2147/IDR.S251360.

ITO, Ryota; ADAM D. TOMICH; MCELHENY, Christi L.; METTUS, Roberta T.; SLUIS-CREMER, Nicolas; DOI, Yohei. Inhibition of fosfomycin resistance protein FosA by phosphonoformate (Foscarnet) in multidrug-resistant Gram-negative pathogens. **Antimicrobial agents and chemotherapy**, *[S. l.]*, v. 61, n. pii:e01424-17., 2017. a.

ITO, Ryota; MUSTAPHA, Mustapha M.; TOMICH, Adam D.; CALLAGHAN, Jake D.; MCELHENY, Christi L.; METTUS, Roberta T.; SHANKS, Robert M. Q.; SLUIS-CREMER, Nicolas; DOI, Yohei. Widespread Fosfomycin Resistance in Gram-Negative Bacteria Attributable to the Chromosomal fosA Gene. **mBio**, *[S. l.]*, v. 8, n. 4, p. 1–9, 2017. b. DOI: 10.1128/mbio.00749-17.

ITO, Ryota; MUSTAPHA, Mustapha M.; TOMICH, Adam D.; CALLAGHAN, Jake D.; MCELHENY, Christi L.; METTUS, Roberta T.; SHANKS, Robert M. Q.; SLUIS-CREMER, Nicolas; DOI, Yohei. Widespread fosfomycin resistance in gram-negative bacteria attributable to the chromosomal fosA gene. **mBio**, *[S. l.]*, v. 8, n. 4, p. 1–9, 2017. c. DOI: 10.1128/mBio.00749-17.

jamovi - open statistical software for the desktop and cloud. , [s.d.]. Disponível em: https://www.jamovi.org/.

KAHAN, Frederick M.; KAHAN, Jean S.; CASSIDY, Patrick J.; KROPP, Helmut. THE

MECHANISM OF ACTION OF FOSFOMYCIN (PHOSPHONOMYCIN). **Annals of the New York Academy of Sciences**, *[S. l.]*, 1974. DOI: 10.1111/j.1749-6632.1974.tb43277.x.

KARAISKOS, Ilias; LAGOU, Styliani; PONTIKIS, Konstantinos; RAPTI, Vasiliki; POULAKOU, Garyphallia. The "Old" and the "New" antibiotics for MDR Gram-negative pathogens: For whom, when, and how. **Frontiers in Public Health**, *[S. l.]*, v. 7, n. JUN, p. 151, 2019. DOI: 10.3389/FPUBH.2019.00151/BIBTEX.

KAYE, Keith S.; GALES, Ana C.; DUBOURG, Grégory. Old antibiotics for multidrug-resistant pathogens: from in vitro activity to clinical outcomes. **International Journal of Antimicrobial Agents**, *[S. l.]*, v. 49, n. 5, p. 542–548, 2017. DOI: 10.1016/j.ijantimicag.2016.11.020. Disponível em: http://dx.doi.org/10.1016/j.ijantimicag.2016.11.020.

KIEFFER, Nicolas; POIREL, Laurent; MUELLER, Linda; MANCINI, Stefano; NORDMANN, Patrice. ISEcp1-mediated transposition leads to fosfomycin and broad-spectrum cephalosporin resistance in klebsiella pneumoniae. **Antimicrobial Agents and Chemotherapy**, *[S. l.]*, 2020. a. DOI: 10.1128/AAC.00150-20.

KIEFFER, Nicolas; POIREL, Laurent; MUELLER, Linda; MANCINI, Stefano; NORDMANN, Patrice. ISEcp1-mediated transposition leads to fosfomycin and broad-spectrum cephalosporin resistance in klebsiella pneumoniae. **Antimicrobial Agents and Chemotherapy**, *[S. l.]*, v. 64, n. 5, 2020. b. DOI: 10.1128/AAC.00150-20.

KUSSMANN, Manuel et al. Synergistic Effect of Cefazolin Plus Fosfomycin Against Staphylococcus aureus in vitro and in vivo in an Experimental Galleria mellonella Model. **Frontiers in Pharmacology**, *[S. l.]*, v. 12, p. 1095, 2021. DOI: 10.3389/FPHAR.2021.685807/BIBTEX.

LAM, Margaret M. C.; WICK, Ryan R.; WATTS, Stephen C.; CERDEIRA, Louise T.; WYRES, Kelly L.; HOLT, Kathryn E. A genomic surveillance framework and genotyping tool for Klebsiella pneumoniae and its related species complex. **Nature Communications**, *[S. l.]*, v. 12, n. 1, p. 4188, 2021. DOI: 10.1038/s41467-021-24448-3. Disponível em: http://dx.doi.org/10.1038/s41467-021-24448-3.

LÁZÁR, Viktória; SNITSER, Olga; BARKAN, Daniel; KISHONY, Roy. Antibiotic combinations reduce Staphylococcus aureus clearance. **Nature**, *[S. l.]*, v. 610, n. 7932, 2022. DOI: 10.1038/S41586-022-05260-5. Disponível em: https://pubmed.ncbi.nlm.nih.gov/36198788/.

LEE, Chang Ro; LEE, Jung Hun; PARK, Kwang Seung; KIM, Young Bae; JEONG, Byeong Chul; LEE, Sang Hee. Global dissemination of carbapenemase-producing Klebsiella pneumoniae: Epidemiology, genetic context, treatment options, and detection methods. **Frontiers in Microbiology**, *[S. l.]*, v. 7, n. JUN, p. 1–30, 2016. DOI: 10.3389/fmicb.2016.00895.

LIU, Peng; CHEN, Shuo; WU, Zhuo ying; QI, Meng; LI, Xiang yang; LIU, Cai xia. Mechanisms of fosfomycin resistance in clinical isolates of carbapenem-resistant Klebsiella pneumoniae. **Journal of Global Antimicrobial Resistance**, *[S. l.]*, v. 22, p. 238–243, 2020. DOI: 10.1016/j.jgar.2019.12.019. Disponível em: https://doi.org/10.1016/j.jgar.2019.12.019.

LIVAK, K. J., AND SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔ-CT Method. **Methods**, *[S. l.]*, v. 25, n. 4, p. 402–408, 2001. DOI: doi: 10.1006/METH.2001.1262.

MA, Y.; XU, X.; GUO, Q.; WANG, P.; WANG, W.; WANG, M. Characterization of fosA5, A new plasmid-mediated fosfomycin resistance gene in Escherichia coli. **Letters in Applied**

Microbiology, *[S. l.]*, v. 60, n. 3, p. 259–264, 2015. DOI: 10.1111/lam.12366.

MARCHESE, Anna; BOZZOLASCO, Micaela; GUALCO, Laura; DEBBIA, Eugenio A.; SCHITO, Gian Carlo; SCHITO, Anna Maria. Effect of fosfomycin alone and in combination with N-acetylcysteine on E. coli biofilms. **International Journal of Antimicrobial Agents**, *[S. l.]*, v. 22, n. SUPPL. 2, p. 95–100, 2003. DOI: 10.1016/S0924-8579(03)00232-2.

MENDOZA, C.; GARCIA, J. M.; LLANEZA, J.; MENDEZ, F. J.; HARDISSON, C.; ORTIZ, J. M. Plasmid-determined resistance to fosfomycin in Serratia marcescens. **Antimicrobial Agents and Chemotherapy**, *[S. l.]*, v. 18, n. 2, p. 215–219, 1980. DOI: 10.1128/AAC.18.2.215.

MURRAY, Christopher JL et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. **The Lancet**, *[S. l.]*, v. 399, n. 10325, p. 629–655, 2022. DOI: 10.1016/S0140-6736(21)02724-0.

NILSSON, Annika I.; BERG, Otto G.; ASPEVALL, Olle; KAHLMETER, Gunnar; ANDERSSON, Dan I. Biological costs and mechanisms of fosfomycin resistance in Escherichia coli. **Antimicrobial agents and chemotherapy**, *[S. l.]*, v. 47, n. 9, p. 2850–8, 2003. DOI: 10.1128/AAC.47.9.2850. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/12936984%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC182645.

NORDMANN, Patrice; POIREL, Laurent; MUELLER, Linda. Rapid Detection of Fosfomycin Resistance in Escherichia coli. **Journal of Clinical Microbiology**, *[S. l.]*, v. 57, n. 1, p. 16–20, 2019. DOI: 10.1128/JCM.01531-18.

OLIVA, A. et al. JAC-Antimicrobial Resistance Effect of ceftazidime/avibactam plus fosfomycin combination on 30 day mortality in patients with bloodstream infections caused by KPC-producing Klebsiella pneumoniae: results from a multicentre retrospective study. *[S. l.]*, 2022. DOI: 10.1093/jacamr/dlac121. Disponível em: https://doi.org/10.1093/jacamr/dlac121.

OLIVA, Alessandra et al. Synergistic meropenem/vaborbactam plus fosfomycin treatment of kpc producing k. Pneumoniae septic thrombosis unresponsive to ceftazidime/avibactam: From the bench to the bedside. **Antibiotics**, *[S. l.]*, v. 10, n. 7, 2021. DOI: 10.3390/antibiotics10070781.

PENA, Irene; PENA-VINA, Eduardo; RODRIGUEZ-AVIAL, Iciar; PICAZO, Juan J.; GÓMEZ-GONZÁLEZ, Álvaro; CULEBRAS, Esther. Comparison of performance of MALDI-TOF MS and MLST for biotyping carbapenemase-producing Klebsiella pneumoniae sequence types ST11 and ST101 isolates. **Enfermedades Infecciosas y Microbiologia Clinica**, *[S. l.]*, 2020. DOI: 10.1016/j.eimc.2020.10.018.

PERDIGÃO NETO, Lauro Vieira et al. Alternative drugs against multiresistant Gram-negative bacteria. **Journal of Global Antimicrobial Resistance**, *[S. l.]*, v. 23, p. 33–37, 2020. DOI: 10.1016/j.jgar.2020.07.025.

PERETZ, Avi; NAAMNEH, Basel; TKHAWKHO, Linda; NITZAN, Orna. High Rates of Fosfomycin Resistance in Gram-Negative Urinary Isolates from Israel. **Microbial Drug Resistance**, *[S. l.]*, v. 25, n. 3, p. 408–412, 2019. DOI: 10.1089/mdr.2018.0393.

RIBEIRO, Aghata Cardoso da Silva; CHIKHANI, Yohanna Carvalho dos Santos Aoun; VALIATTI, Tiago Barcelos; VALÊNCIO, André; KURIHARA, Mariana Neri Lucas; SANTOS, Fernanda Fernandes; MINARINI, Luciene Andrade da Rocha; GALES, Ana Cristina. In Vitro and In Vivo Synergism of Fosfomycin in Combination with Meropenem or Polymyxin B against KPC - 2 - Producing Klebsiella pneumoniae Clinical Isolates. **Antibiotics**, *[S. l.]*, v. 12, n. 2, p. 237, 2023. DOI: https://doi.org/10.3390/antibiotics12020237.

RIBEIRO, Cardoso da Silva Ághata; SANTOS, Fernanda Fernandes; MOSES, Ikechukwu Benjamin; ANDRADE DA, Luciene; MINARINI, Rocha; GALES, Ana Cristina. Sequencing of fosA: A Rapid and Inexpensive Method for Discriminating Klebsiella pneumoniae CC258 from Other Clones. **https://home.liebertpub.com/mdr**, *[S. l.]*, 2022. DOI: 10.1089/MDR.2022.0081. Disponível em: https://www.liebertpub.com/doi/10.1089/mdr.2022.0081.

RODRÍGUEZ, Cecilia et al. Successful management with fosfomycin + ceftazidime of an infection caused by multiple highly-related subtypes of multidrug-resistant and extensively drug-resistant KPC-producing Serratia marcescens. **International Journal of Antimicrobial Agents**, *[S. l.]*, v. 52, n. 5, p. 737–739, 2018. DOI: 10.1016/J.IJANTIMICAG.2018.07.020.

SCUDELLER, Luigia et al. Systematic review and meta-analysis of in vitro efficacy of antibiotic combination therapy against carbapenem-resistant Gram-negative bacilli. **International Journal of Antimicrobial Agents**, *[S. l.]*, v. 57, n. 5, p. 106344, 2021. DOI: 10.1016/j.ijantimicag.2021.106344. Disponível em: https://doi.org/10.1016/j.ijantimicag.2021.106344.

SHORTRIDGE, D.; RHOMBERG, PR; BRADFORD, P.; ELLIS-GROSSE, EJ; FLAMM, RK. **Fosfomycin Activity against Gram-Negative Isolates from Eastern Europe Collected by the SENTRY Antimicrobial Surveillance Program**. Poster session presented at: ASM Microbe 2018, Jun 7-11; Atlanta, GA, , 2018.

SILVER, Lynn L. Fosfomycin: Mechanism and resistance. **Cold Spring Harbor Perspectives in Medicine**, *[S. l.]*, v. 7, n. 2, p. 1–11, 2017. DOI: 10.1101/cshperspect.a025262.

SKINNER, Kirsty; SANDOE, Jonathan A. T.; RAJENDRAN, Ranjith; RAMAGE, Gordon; LANG, Sue. Efficacy of rifampicin combination therapy for the treatment of enterococcal infections assessed in vivo using a Galleria mellonella infection model. **International journal of antimicrobial agents**, *[S. l.]*, v. 49, n. 4, p. 507–511, 2017. DOI: 10.1016/J.IJANTIMICAG.2016.12.006. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28235571/.

SUGATHAN, Sugalya; MANDAL, Jharna. An invitro experimental study of the effect of fosfomycin in combination with amikacin, ciprofloxacin or meropenem on biofilm formation by multidrug-resistant urinary isolates of Escherichia coli. **Journal of medical microbiology**, *[S. l.]*, v. 68, n. 12, p. 1699–1706, 2019. DOI: 10.1099/jmm.0.001061.

TAMMA, Pranita D.; AITKEN, Samuel L.; BONOMO, Robert A.; MATHERS, Amy J.; VAN DUIN, David; CLANCY, Cornelius J. Infectious Diseases Society of America 2022 Guidance on the Treatment of Extended-Spectrum β-lactamase Producing Enterobacterales (ESBL-E), Carbapenem-Resistant Enterobacterales (CRE), and Pseudomonas aeruginosa with Difficult-to-Treat Resistance (DTR-P. **Clinical infectious diseases : an official publication of the Infectious Diseases Society of America**, *[S. l.]*, 2022. DOI: 10.1093/cid/ciac268. Disponível em: https://doi.org/10.1093/cid/ciac268.

TOMICH, Adam D.; KLONTZ, Erik H.; DEREDGE, Daniel; BARNARD, John P.; MCELHENY, Christi L.; ESHBACH, Megan L. crossm Small-Molecule Inhibitor of FosA Expands Fosfomycin Activity. *[S. l.]*, v. 63, n. 3, p. 1–14, 2019.

TSENG, Sung Pin et al. The plasmid-mediated fosfomycin resistance determinants and synergy of fosfomycin and meropenem in carbapenem-resistant Klebsiella pneumoniae isolates in Taiwan. **Journal of Microbiology, Immunology and Infection**, *[S. l.]*, v. 50, n. 5, p. 653–661, 2017. DOI: 10.1016/j.jmii.2017.03.003. Disponível em: https://doi.org/10.1016/j.jmii.2017.03.003.

VÁSQUEZ-PONCE, Felipe et al. Detecting KPC-2 and NDM-1 Coexpression in Klebsiella

pneumoniae Complex from Human and Animal Hosts in South America. **Microbiology Spectrum**, *[S. l.]*, 2022. DOI: 10.1128/SPECTRUM.01159-22/SUPPL_FILE/SPECTRUM.01159-22-S0003.XLSX. Disponível em: https://journals.asm.org/doi/10.1128/spectrum.01159-22.

WACHINO, Jun Ichi; YAMANE, Kunikazu; SUZUKI, Satowa; KIMURA, Kouji; ARAKAWA, Yoshichika. Prevalence of fosfomycin resistance among CTX-M-producing Escherichia coli clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. **Antimicrobial Agents and Chemotherapy**, *[S. l.]*, v. 54, n. 7, p. 3061–3064, 2010. DOI: 10.1128/AAC.01834-09/ASSET/DE0C64BC-F8F4-4C6B-AC78-75A71D92FD10/ASSETS/GRAPHIC/ZAC9991090760003.JPEG. Disponível em: https://journals.asm.org/doi/10.1128/AAC.01834-09.

WANG, Minggui et al. Clinical outcomes and bacterial characteristics of carbapenem-resistant Klebsiella pneumoniae complex among patients from different global regions (CRACKLE-2): a prospective, multicentre, cohort study. **The Lancet Infectious Diseases**, *[S. l.]*, 2021. DOI: 10.1016/s1473-3099(21)00399-6.

WANG, Yu-ping; CHEN, Yen-hao; HUNG, I-cheng; CHU, Po-hsun; CHANG, Yu-han; WANG, Jin-town. Transporter Genes and fosA Associated With Fosfomycin Resistance in Carbapenem-Resistant Klebsiella pneumoniae. **Frontiers in Microbiology**, *[S. l.]*, v. 13, n. January, p. 1–9, 2022. a. DOI: 10.3389/fmicb.2022.816806.

WANG, Zongqiang; KOIRALA, Bimal; HERNANDEZ, Yozen; ZIMMERMAN, Matthew; PARK, Steven; PERLIN, David S.; BRADY, Sean F. A naturally inspired antibiotic to target multidrug-resistant pathogens. **Nature**, *[S. l.]*, v. 601, n. 7894, p. 606–611, 2022. b. DOI: 10.1038/s41586-021-04264-x. Disponível em: https://doi.org/10.1038/s41586-021-04264-x.

WINK, Priscila Lamb; MARTINS, Amanda Silva; VOLPATO, Fabiana; ZAVASCKI, Alexandre P.; BARTH, Afonso L. Increased frequency of blaNDM in a tertiary care hospital in southern Brazil. **Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]**, *[S. l.]*, v. 52, n. 1, p. 299–301, 2021. DOI: 10.1007/S42770-020-00412-Z. Disponível em: https://pubmed.ncbi.nlm.nih.gov/33392936/.

WISTRAND-YUEN, P.; OLSSON, A.; SKARP, K. P.; FRIBERG, L. E.; NIELSEN, E. I.; LAGERBÄCK, P.; TÄNGDÉN, T. Evaluation of polymyxin B in combination with 13 other antibiotics against carbapenemase-producing Klebsiella pneumoniae in time-lapse microscopy and time-kill experiments. **Clinical Microbiology and Infection**, *[S. l.]*, v. 26, n. 9, p. 1214–1221, 2020. DOI: 10.1016/j.cmi.2020.03.007.

WORLD HEALTH ORGANIZATION. **WHO publishes list of bacteria for which new antibiotics are urgently needed**. 2017. Disponível em: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Acesso em: 1 dez. 2022.

WYRES, Kelly; HOLT, Kathryn. Regional differences in carbapenem-resistant Klebsiella pneumoniae. **The Lancet Infectious Diseases**, *[S. l.]*, 2021. DOI: 10.1016/s1473-3099(21)00425-4.

YANG, Tsung Ying; LU, Po Liang; TSENG, Sung Pin. Update on fosfomycin-modified genes in Enterobacteriaceae. **Journal of Microbiology, Immunology and Infection**, *[S. l.]*, v. 52, n. 1, p. 9–21, 2019. DOI: 10.1016/j.jmii.2017.10.006. Disponível em: https://doi.org/10.1016/j.jmii.2017.10.006.