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Prospection of *Klebsiella quasipneumoniae* phages and their efficacy on multidrug-resistant strains of *Klebsiella* spp.

Uberlândia
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Área de concentração: Microbiologia

Orientador: Rosineide Marques Ribas

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Dedico este trabalho a todos que um dia se sentiram insuficientes para a área da pesquisa acadêmica. Gostaria de lembrar que ninguém nasceu para ser nada, todos nos tornamos algo a partir de nossa vivência e experiências únicas.

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''Well, can you see me? I cannot see you
Everything I thought I knew has fallen out of view
In this blindness I'm condemned to
Well, can you hear me? I cannot hear you
Every song I thought I knew, I've been deafened to
And there's no one left to sing to''

Cassandra – Florence Welch

RESUMO

A crescente resistência bacteriana aos antibióticos representa um dos maiores desafios da saúde pública global, especialmente no que se refere a patógenos que foram listados como críticos pela Organização Mundial da Saúde como a *Klebsiella pneumoniae* multirresistente. Neste contexto, os bacteriófagos (ou fagos) surgem como uma alternativa promissora à antibioticoterapia tradicional. Este trabalho teve como objetivo o isolamento e a caracterização de fagos com atividade lítica contra *K. pneumoniae* (ATCC 700603) e outras cepas de interesse clínico, visando seu potencial uso terapêutico. Amostras ambientais foram coletadas e submetidas a protocolos para triagem e purificação para a seleção de fagos capazes de provocar lise. Os isolados foram posteriormente avaliados quanto ao espectro de ação, estabilidade físico-química e morfologia, com ênfase em sua eficácia frente a cepas produtoras de β-lactamases de espectro estendido (ESBL) e carbapenemases. Além disso, foram realizados ensaios de inibição da formação de biofilme e desestruturação de biofilmes maduros. Na cepa ATCC 700603, apenas KqP1 foi capaz de reduzir significativamente biofilmes estabelecidos, efeito potencializado pelo uso do coquetel de fagos. Para a cepa K2010, os fagos K2010P2 e K2010P4 apresentaram os maiores níveis de desestruturação, enquanto K2010P5 foi o mais eficaz na inibição da formação de biofilmes; o coquetel também se mostrou ativo em ambos os testes. Os resultados demonstram a viabilidade do isolamento de fagos com perfil lítico promissor e atividade antibiofilme, reforçando a base para implementação da fagoterapia local e auxilia no enfrentamento do problema da resistência antimicrobiana, contribuindo para o avanço do conhecimento sobre fagos de ação lítica contra *K. pneumoniae*.

Palavras-chave: Fago, fagoterapia, *Klebsiella pneumoniae*¸ resistência antimicrobiana, biofilme.

ABSTRACT

The growing bacterial resistance to antibiotics represents one of the greatest challenges to global public health, particularly with regard to pathogens listed as critical by the World Health Organization, such as multidrug-resistant *Klebsiella pneumoniae*. In this context, bacteriophages (or phages) emerge as a promising alternative to traditional antibiotic therapy. This study aimed to isolate and characterize phages with lytic activity against *K. pneumoniae* (ATCC 700603) and other clinically relevant strains, focusing on their potential therapeutic use. Environmental samples were collected and subjected to screening and purification protocols to select phages capable of inducing lysis. The isolates were subsequently evaluated for their host range, physicochemical stability, and morphology, with emphasis on their effectiveness against extended-spectrum β-lactamase (ESBL) and carbapenemase-producing strains. Additionally, assays for biofilm formation inhibition and mature biofilm disruption were performed. In the ATCC 700603 strain, only phage KqP1 significantly reduced established biofilms, with an enhanced effect observed when using a phage cocktail. For strain K2010, phages K2010P2 and K2010P4 showed the highest levels of biofilm disruption, while K2010P5 was the most effective in preventing biofilm formation; the cocktail was also active in both assays. The results demonstrate the feasibility of isolating phages with a promising lytic profile and antibiofilm activity, reinforcing the basis for local implementation of phage therapy and contributing to the fight against antimicrobial resistance, while advancing knowledge on lytic phages active against *K. pneumoniae*.

Keywords: Phage, phage therapy, *Klebsiella pneumoniae*, antimicrobial resistance. biofilm.

LISTA DE ABREVIATURAS E SIGLAS

ABNT	Associação Brasileira de Normas Técnicas
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
UFU	Universidade Federal de Uberlândia
ICTV	Comitê Internacional de Taxonomia de Vírus
RNA	Ácido ribonucleico
THG	Transferência Horizontal de Genes
OMS	Organização Mundial da Saúde
ESBL	β-lactamases de amplo espectro

SUMÁRIO

1 APRESENTAÇÃO DA ESTRUTURA DO TRABALHO

Essa dissertação foi realizada de acordo com o documento ‘’Formato Alternativo de Dissertação e/ ou tese:’’, que preconiza que a estrutura possa ser feita da seguinte forma: Folha de rosto, ficha catalográfica, resumo e abstract, lista de abreviaturas, sumário, fundamentação teórica, objetivos, referências bibliográficas – desta parte do trabalho, manuscrito oriundo da pesquisa a publicar e considerações finais.

Desta forma, este trabalho se inicia por esta apresentação seguida de uma fundamentação teórica acerca do tema, que são bacteriófagos e fagoterapia relacionados à *Klebsiella pneumoniae*, os objetivos do trabalho e as referências bibliográficas deste capítulo. Em seguida, o manuscrito do artigo científico oriundo da pesquisa realizada durante o mestrado é apresentado, seguido das normas da revista selecionada. Por fim, algumas palavras para as considerações finais, resumindo os resultados do trabalho, limitações, possíveis contribuições e perspectivas futuras.

2 REVISÃO BIBLIOGRÁFICA

2.1 Bacteriófagos

Os bacteriófagos, vírus que infectam bactérias, foram descobertos de forma independente por Twort e d'Herelle, em 1915 pelo primeiro e depois publicado em 1917 pelo segundo, sem conhecimento prévio da descoberta anterior (Duckworth, 1976). Esses vírus também são denominados "fagos". Com a descoberta, logo se pensou que seu uso no tratamento de infecções bacterianas seria um florescente campo de pesquisa. Entretanto, os fagos perderam um pouco sua importância com a descoberta dos antibióticos, inicialmente a penicilina, em 1929, pelo inglês Alexander Fleming (Nossa capa [...], 2009). Assim, apenas em alguns locais, como na antiga União Soviética e na atual Geórgia, o uso terapêutico de bacteriófagos continuou sendo explorado ao longo do século XX, estudados em institutos de pesquisa como o Eliava Institute of Bacteriophage, Microbiology and Virology (Sulakvelidze *et al*., 2001; Clockie et al., 2011).

Os vírus têm papeis importantes na natureza. Eles estão presentes em praticamente todos os ambientes nos quais bactérias são encontradas, como solos, rios, esgotos, oceanos e em simbiose com humanos e os outros animais (Clokie *et al*., 2011). Estima-se que existam aproximadamente 10^{31} fagos nos oceanos, o que os coloca como sendo as entidades biológicas mais abundantes nos oceanos e tendo importante papel nas interações ecológicas, na regulação populacional microbiana (Suttle, 2005). Como exemplo, estudos sobre ecologia viral marinha têm demonstrado que vírus, incluindo os bacteriófagos, são os principais agentes de mortalidade de plânctons e outros microrganismos marinhos. Devido a essa atividade, têm papel fundamental nos ciclos de nutrientes e de energia nesse ecossistema, contribuindo para a reciclagem de matéria orgânica e a regulação das populações microbianas (Suttle, 2005; Weinbauer, 2004). Os fagos fazem parte desse balanço ao serem responsáveis por parte dos ciclos que envolvem as bactérias (Wilhelm & Suttle, 1999).

Os fagos em geral possuem uma morfologia parecida, todos possuem um capsídeo que envolve o material genético, podendo ou não possuir uma cauda proteica e por fim uma placa

basal que pode ou não conter fibras (Trun; Trempy, 2004). A estrutura geral está ilustrada na figura 1.

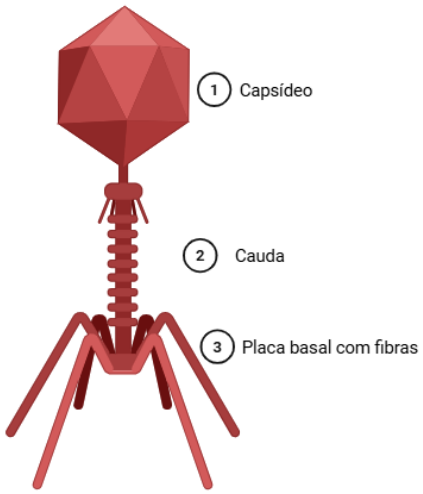

Figura 1: Estrutura geral de um bacteriófago, composta por (1) um capsídeo que envolve o material genético, (2) uma cauda que pode estar presente ou não e (3) uma placa basal com fibras que também podem estar ausentes.

Apesar dessa estrutura relativamente conservada, os fagos apresentam uma diversidade morfológica que foi historicamente agrupada em três famílias principais: *Myoviridae*, *Siphoviridae* e *Podoviridae*, classificadas de acordo com a morfologia da cauda. No entanto, essa classificação foi revista pelo Comitê Internacional de Taxonomia de Vírus (ICTV), que a partir de 2022 passou a adotar uma taxonomia baseada em genômica comparativa e filogenia, de forma que as três famílias clássicas foram reagrupadas, e os fagos com cauda agora estão sob a classe *Caudoviricetes*, com novas ordens e famílias em desenvolvimento contínuo (Turner *et al*., 2023; ICTV, 2022). Essa mudança demonstra a grande diversidade genômica observada entre os fagos, que não podia mais ser explicada apenas pela morfologia da cauda.

No que diz respeito a como são produzidos, os fagos possuem um ciclo relativamente simples (Figura 2). Inicialmente, ocorre a adsorção do fago à parede celular da bactéria, seguida da injeção do seu material genético no citosol da célula onde é replicado e, a partir dele, ocorre a síntese de RNAs mensageiros, ocasionando a síntese das proteínas virais. Assim, as novas partículas virais são montadas e a bactéria é lisada, ou seja, a membrana e a parede celular são rompidas, ocasionando a liberação dos novos vírus (Abedon *et al*., 2008).

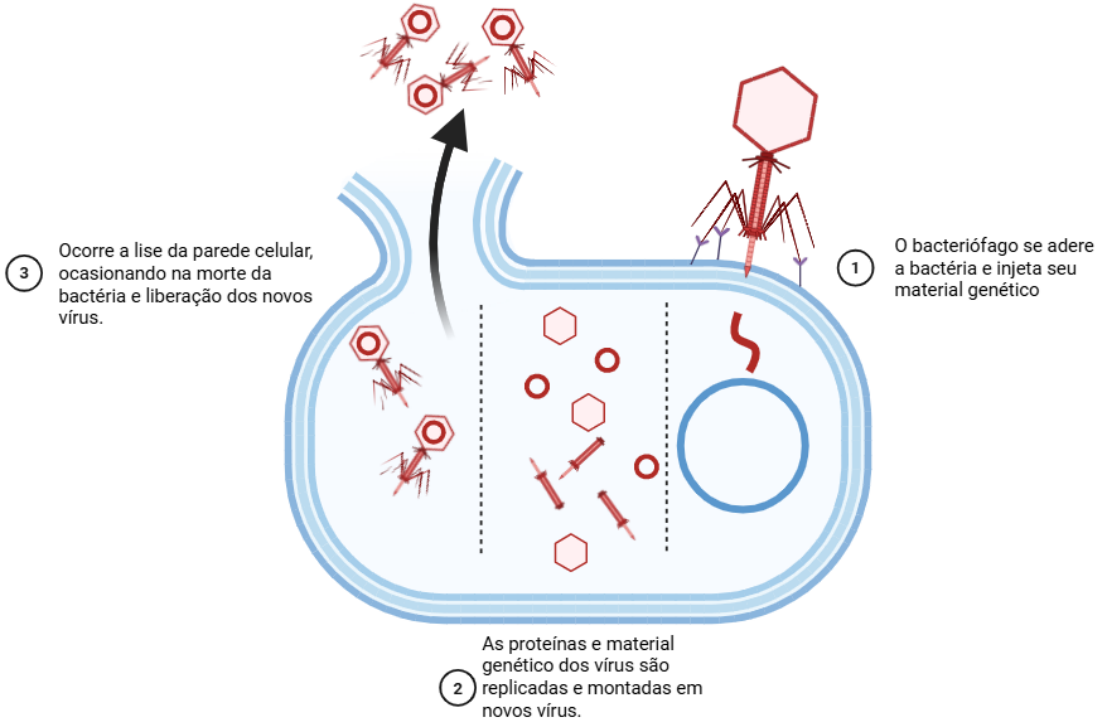

Figura 2: Ciclo lítico dos bacteriófagos, esquema representativo.

Esse processo é característico do chamado ciclo lítico, típico de fagos virulentos. Um exemplo clássico é o fago T4, que infecta a enterobactéria *Escherichia coli* e é considerado um fago lítico estrito (Hyman; Abedon, 2019). Fagos líticos são preferidos em aplicações terapêuticas, pois garantem a destruição da bactéria hospedeira sem risco de integração genética, o que reduz a possibilidade de transferência horizontal de genes indesejáveis, como os de resistência a antibióticos (Brüssow, 2012).

No ciclo lisogênico, em vez de ocorrer replicação do material genético e síntese das proteínas virais, o genoma do fago é integrado ao da bactéria hospedeira, permanecendo num estado dormente. Quando ocorre a divisão celular, o material genético do fago também é replicado, sendo passado para as células filhas (Howard-Varona *et al*., 2017). Quando estão dessa forma, são denominados pró-fagos, que, em decorrência de influências ambientais, como

exposição a raios UV, antimicrobianos ou estresse nutricional, podem se tornar ativos, ocasionando em uma mudança para o ciclo lítico (Nanda *et al.*, 2015; Feiner *et al.*, 2015).

Esses fagos também são denominados “temperados”, vírus que ao infectar a célula podem iniciar tanto um ciclo lítico quanto lisogênico (Howard-Varona *et al*., 2017). Um exemplo bem estudado é o fago lambda (λ), que infecta *E. coli* e pode alternar entre os diferentes ciclos dependendo das condições ambientais e da regulação de proteínas específicas, como o repressor lambda, que reprime os fatores de ativação do ciclo lítico deste fago e, quando esta proteína deixa de ser expressa, o fago adentra o ciclo lítico (Bednarz *et al.*, 2014).

Uma questão de importância clínica no que diz respeito ao possível uso de fagos para combater infecções bacterianas é a de que os fagos lisogênicos podem carrear genes de resistência e virulência, desenvolvidos a partir de ferramentas como a recombinação, passando essas características para as bactérias que infectam (Vale *et al*., 2024). Essa transferência gênica pode incluir toxinas, como a da cólera, fatores de adesão e sistemas de secreção, tornando as bactérias mais patogênicas (Waldor; Mekalanos, 1996; Feiner *et al.*, 2015).

Além de seu potencial terapêutico, os fagos têm sido amplamente estudados em outras áreas da biotecnologia, principalmente devido a sua especificidade, diversidade e capacidade de interagir com as bactérias e comunidades bacterianas e de expressão genética. Um exemplo relevante é o uso de fagos como ferramentas de detecção bacteriana em ambientes clínicos, alimentares e ambientais. Estudos demonstram que fagos modificados geneticamente podem ser empregados como biossensores, por meio de emissão de sinais fluorescentes ou luminescentes ao infectarem células-alvo, permitindo a identificação de patógenos como *Listeria* e *Yersinia* (Loessner *et al*., 1996; Schofield et al., 2009).

Outra aplicação envolve o uso dos fagos no controle de contaminações bacterianas na indústria alimentícia. Vários estudos demonstraram a eficácia de preparações comerciais à base de fagos na redução de bactérias patogênicas em carnes, vegetais frescos e laticínios, aumentando a segurança microbiológica dos produtos sem afetar suas propriedades sensoriais e nutricionais (Moye *et al*., 2018). Além disso, há interesse crescente no uso de fagos na agroindústria, tendo em vista que o uso indiscriminado de antimicrobianos nos animais de produção contribui para o problema da resistência antimicrobiana nas infecções em humanos. Também podem ser utilizados como uma forma de modular a microbiota intestinal de animais de produção, visando reduzir infecções e diminuir o uso de antibióticos na pecuária (Kutter; Sulakvelidze, 2004; Loc-Carrillo; Abedon, 2011).

Nessa esteira, foi desenvolvido o conceito de “Saúde Única”, um conceito que compreende que todas as categorias da saúde no planeta estão interrelacionadas, como a saúde

humana, ambiental e animal. Devido a isso, quando uma esfera é impactada, todas as outras também são. Isso pode ser evidenciado pelas discussões e pesquisas realizadas por cientistas, que demonstram o aparecimento de "reservatórios naturais" de resistência aos antimicrobianos nos animais e plantas, ao utilizar estes medicamentos de forma indiscriminada na agropecuária (MCEWEN; COLLIGNON, 2018; GONÇALVES DA SILVA *et al.*, 2023).

Os fagos também podem ser utilizados em uma técnica denominada *Phage display*, em a sequência codificadora de um determinado peptídeo é adicionada ao material genético do fago, fazendo com que o vírus expresse o peptídeo desejado. Essa abordagem pode ser aplicada por exemplo para estudar e expressar epítopos específicos, desenvolvimento de vacinas e até mesmo é possível seu uso em materiais não biológicos, como metais. O fago M13, por exemplo, é frequentemente usado para esses fins devido à sua estrutura filamentosa e a facilidade de ser manipulado geneticamente (Fadaie *et al.*, 2023).

Atualmente, os fagos estão passando por um período de renascença no âmbito científico, especialmente frente ao problema atual enfrentado pela humanidade de aumento do número e frequência da resistência bacteriana a antibióticos. Estudos têm explorado seu uso não apenas na medicina humana, mas também na agropecuária, na indústria de alimentos e em outras aplicações, como por exemplo, no controle de biofilmes em ambientes hospitalares (Chan *et al*., 2013; Kortright *et al*., 2019). A engenharia genética também tem sido empregada para modificar fagos, como a remoção dos fatores e genes que causam a repressão do ciclo lítico, ampliação do seu espectro de ação e melhoramento de sua estabilidade (Dedrick *et al*., 2019).

2.2 O problema da multirresistência bacteriana

As bactérias são organismos que, como todo ser vivo, estão sujeitas ao processo de evolução. Com isso, a resistência aos antimicrobianos é de certa forma previsível. Entretanto, quando se trata de importância clínica, o que está em evidência são as bactérias que adquirem resistência a múltiplos antibióticos, principalmente no ambiente hospitalar (Munita; Arias, 2016).

A resistência antimicrobiana pode ser adquirida pelas bactérias de diferentes formas: por mutação em genes específicos, que, por sua vez, podem ser passados para outra célula bacteriana por meio do processo denominado de Transferência Horizontal de Genes (THG). A THG também pode acontecer pela incorporação de DNA presente no ambiente por transdução, quando ocorre a incorporação de material genético viral, por conjugação, um processo em que uma bactéria ativamente troca informações genéticas com outra, e por meio de plasmídeos ou de *integrons*, que são elementos genéticos móveis que podem ter genes novos integrados a eles,

podendo ser os que conferem resistência e serem disseminados entre bactérias (Munita; Arias, 2016).

Além de genes que conferem resistência direta aos antimicrobianos, também existe o biofilme, uma estrutura composta por várias bactérias em uma matriz polimérica de substâncias produzidas por elas. Essa substância funciona como uma camada protetora, e pode aderir em inúmeros locais, desde feridas cirúrgicas a equipamentos hospitalares (Paczosa; Mecsas, 2016).

Neste contexto, muitos problemas relacionados à multirresistência têm surgido. Em 2017, a Organização Mundial da Saúde (OMS) publicou um documento enumerando a prioridade para a descoberta e produção de novos antimicrobianos em relação às espécies e o tipo de resistência desses patógenos, tendo como prioridade máxima *Acinetobacter baumannii*, *Pseudomonas aeruginosa* e Enterobacteriaceae, que inclui *Klebsiella pneumoniae* e *Escherichia coli*, dentre outras bactérias (Tacconelli *et al.*, 2017).

Rice (2007) publicou um comentário editorial sobre a resistência antimicrobiana em patógenos nosocomiais, uma ameaça crescente que, quase vinte anos depois, provou ser mais do que verdadeira, urgente. O autor lista as seguintes bactérias como de extrema importância clínica: *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* e espécies do gênero *Enterobacter*, que formam o acrônimo ESKAPE, aludindo ao fato de que não há escapatória desses patógenos, devido a isso a urgência e importância de se pesquisar novas alternativas aos antibióticos existentes.

2.3 O gênero *Klebsiella*

Presentes no grupo ESKAPE as bactérias do gênero *Klebsiella* são gram-negativas e encapsuladas, tendo várias espécies como a *K. pneumoniae*, *K. quasipneumoniae*, *K. similipneumoniae*, entre várias outras. A *Klebsiella* pode fazer parte da microbiota normal dos seres humanos, principalmente colonizando o trato gastrointestinal e, em menor escala, as vias respiratórias. Contudo, trata-se de um patógeno oportunista, capaz de ocasionar infecções como sepse, pneumonia e infecções do trato urinário quando o organismo se encontra vulnerabilizado (Dong et al., 2022).

Ainda que a imunossupressão seja um fator importante nesse processo, outros mecanismos também contribuem para a transição da *Klebsiella* de comensal a patógeno. Dentre eles, destacam-se a capacidade de formação de biofilme em superfícies inúmeras superfícies, a produção de cápsulas polissacarídicas que dificultam a fagocitose, além da expressão de sideróforos que aumentam a captação de ferro em ambientes limitados, favorecendo sua sobrevivência e proliferação (Paczosa; Mecsas, 2016; Martin; Bachman, 2018). A presença de genes associados à resistência antimicrobiana, especialmente em cepas hipervirulentas, também

contribui para um perfil infeccioso mais agressivo mesmo em indivíduos previamente saudáveis (Choby; Howard-Anderson; Weiss, 2020).

A classificação tradicional do gênero *Klebsiella* dividia as cepas em duas categorias principais: multirresistentes e hipervirulentas. No entanto, essa distinção tem se tornado progressivamente limitada devido à elevada plasticidade genética desse gênero, que possibilita a assimilação simultânea de genes relacionados tanto à resistência quanto à virulência, resultando no surgimento de cepas que são multirresistentes e hipervirulentas (Chen; Kreiswirth, 2018; Dong et al., 2022). Ao longo dos anos, o grupo *Klebsiella* spp. acumulou diversos marcadores de resistência, dentre os quais se destacam o gene *armA*, que confere resistência a aminoglicosídeos; mutações nas subunidades *gyrA-gyrB* da DNA girase e *parC-parE* da topoisomerase IV; e os genes *acrAB*, *qnr* e *aac(6')-Ib-cr*, que promovem resistência às quinolonas. Além disso, variações nos genes blaSHV, *bla*TEM, *bla*CTX-M, *bla*OXA, *bla*GES, *bla*SFO, *bla*PER, *bla*TLA, *bla*VEB e *bla*KLUC promovem resistência aos β-lactâmicos, caracterizando cepas produtoras de β-lactamases de amplo espectro - Extended Spectrum β-Lactamases (ESBL). A resistência à polimixina é mediada por genes que alteram a estrutura dos lipopolissacarídeos, como *lpxM*, *pmrC* e *mcr-1*, entre outros (Navon-Venezia et al., 2017; Dong et al., 2022).

Um estudo realizado em Manaus teve como objetivo isolar cepas multirresistentes de *Klebsiella* presentes na cidade. Dos 21 isolados analisados, 17 foram classificados como multirresistentes, apresentando genes associados à resistência a antibióticos (Nakamura-Silva et al., 2022). Adicionalmente, uma pesquisa conduzida no Rio de Janeiro identificou cepas de *K. quasipneumoniae* portadoras dos genes *bla*NDM-1 e *bla*CTXM-15, responsáveis pelo fenótipo ESBL, em moscas, evidenciando o potencial de disseminação dessas bactérias no ambiente (Carramaschi et al., 2020).

Estimativas de 2022 indicaram que, em 2019, aproximadamente 1,2 milhão de mortes em todo o mundo foram causadas diretamente pela resistência a antimicrobianos, enquanto cerca de 3,75 milhões de óbitos estiveram associados a esse problema. O grupo de patógenos ESKAPE, adicionando-se o *Streptococcus pneumoniae*, foi responsável por 929 mil mortes diretamente atribuídas à resistência, representando cerca de 73% do total de mortes relacionadas à resistência antimicrobiana, sem considerar as espécies do gênero *Enterobacter* (Strathdee et al., 2023).

Entretanto, a resistência a antimicrobianos não constitui o único desafio que ameaça o futuro da humanidade no contexto das infecções bacterianas. Paralelamente, observa-se uma crise significativa relacionada à descoberta de novos antibióticos. Desde a década de 1970, não foram

identificados novos antimicrobianos naturais, e os compostos atualmente em desenvolvimento consistem, em sua maioria, em derivados de classes naturais já conhecidas ou em modificações sintéticas dessas moléculas. Isso também se dá, devido ao possível desinteresse da indústria farmacêutica, resultado dos grandes custos de produção de novos tratamentos. Esse cenário evidencia a necessidade urgente de inovação na pesquisa e desenvolvimento de agentes antimicrobianos, a fim de enfrentar a crescente ameaça das bactérias resistentes (Hutchings *et al.*, 2019).

Devido a isso, se destaca a importância de se pesquisar alternativas aos antibióticos convencionais, incluindo aquelas não derivadas de antimicrobianos já existentes, como dito anteriormente. Um importante recurso natural a ser estudado para esse fim são os fagos, dentre eles os líticos, que lisam bactérias, mesmo aquelas que apresentam resistência, e por isso a terapia com fagos, ou fagoterapia é considerada um potencial novo tratamento antibacteriano.

2.4 Fagoterapia

A fagoterapia surge como uma estratégia promissora no combate à resistência antimicrobiana, com estudos recentes demonstrando sua eficácia clínica. O isolamento de fagos a partir de esgotos e efluentes contaminados reduz custos comparados ao desenvolvimento de novos antimicrobianos (Martins *et al.*, 2020). Pesquisas com terapia inalatória personalizada em pacientes com fibrose cística e infecções pulmonares por *P. aeruginosa* multirresistente observaram redução significativa na carga bacteriana e melhora da função pulmonar (Chan *et al.*, 2025). Além disso, a fagoterapia tem sido aplicada com sucesso em casos clínicos complexos, como em pacientes com fibrose cística submetidos a transplante pulmonar, nos quais coquetéis personalizados de fagos administrados via inalatória promoveram redução significativa da carga bacteriana e melhora clínica progressiva (Lebeaux *et al*., 2021; Armanhi *et al.*, 2023).

A associação de fagos com antimicrobianos, como já mencionado anteriormente, é uma abordagem terapêutica promissora no combate a infecções causadas por bactérias multirresistentes, que pode ser efetivo ocasionando em uma ação sinérgica, ou seja, associados tem ação melhor que separados e uma ação aditiva, onde os agentes em conjunto atual de forma isolada, mas juntos tem um efeito potencializado (Liu *et al*., 2020).

Bao *et al.* (2020) descreve um caso clínico de uma paciente de 63 anos com infecção urinária recorrente por *K. pneumoniae* sensível apenas para tigeciclina e polimixina não obteve melhora somente com antibioticoterapia. Então, foi realizada fagoterapia com administração de três coqueteis de fagos - cada um com uma diferente combinação de fagos - suplementada com

antibióticos, em uma terapia personalizada para a cepa da paciente, aplicado via irrigação da bexiga urinaria por cinco dias, combinado com antibióticos. E mesmo com a eliminação da infecção na paciente, este estudo relata diversos problemas enfrentados devido ao surgimento de clones resistentes aos fagos, exaltando a importância do cuidado e atenção necessários para cada caso estudado, e mesmo assim, a fagoterapia foi bem-sucedida.

Em um estudo realizado por Schooley *et al*. (2017), foi realizado o uso de um coquetel personalizado de fagos – ou seja, fagos específicos para a cepa infectando o paciente - para tratar uma infecção sistêmica por *A. baumannii* resistente a múltiplas drogas, em um paciente que não apresentava melhora com antibióticos. A administração intravenosa de fagos isolados em posterior associação com antibióticos resultou em melhora do quadro infeccioso e alta hospitalar. Já em outro estudo, conduzido por Dedrick *et al*. (2022), foi demonstrado o sucesso da fagoterapia no tratamento de um paciente com fibrose cística e infecção pulmonar crônica por uma cepa de *Mycobacterium abscessus* resistente. Após a seleção de fagos compatíveis e modificações genéticas para otimizar sua ação, observou-se regressão da infecção e estabilização do quadro clínico. Esses exemplos reforçam o potencial da fagoterapia como ferramenta complementar ou alternativa em cenários de resistência antimicrobiana, além do seu uso efetivo em bactérias pertencentes ao grupo ESKAPE.

Outros estudos demonstraram que bacteriófagos específicos para *K. pneumoniae* apresentaram eficiente atividade lítica, tanto em formas planctônicas quanto em biofilmes bacterianos, o que é fundamental para o sucesso terapêutico, dado o papel dos biofilmes na persistência das infecções e na resistência aos tratamentos convencionais. Como exemplo, o fago HS106, reduziu significativamente biofilmes maduros de *K. pneumoniae*, além de aumentar a susceptibilidade à gentamicina, além de melhorar a sobrevida de peixe-zebra infectado por cepas multirresistentes (Wang et al., 2025). Além desse, em outro estudo, o bacteriófago UPM2146 demonstrou alta eficiência na lise de diversas cepas de *K. pneumoniae* em modelos experimentais, incluindo a eliminação completa da carga bacteriana em larvas de peixes-zebra infectados, indicando seu potencial terapêutico (Assafiri *et al*., 2021).

Por fim, a fagoterapia pode ser integrada a estratégias combinadas com antibióticos, potencializando a eficácia dos tratamentos e reduzindo a recidiva das infecções causadas por *K. pneumoniae* resistentes. Estudos demonstram que a fagoterapia tem vantagens sobre a antibioticoterapia, como uma maior penetração nos biofilmes (Tian, *et al.,* 2021), possui um menor impacto na microbiota do paciente (Patangia *et al*., 2022) e principalmente, um processo fácil e rápido de descoberta, diferente dos antibióticos naturais, que demoram um longo tempo para serem desenvolvidas (Subramanian, 2024; Osman *et al*., 2023).

283 Dessa forma, a fagoterapia representa uma ferramenta essencial para enfrentar a crescente
284 ameaça das infecções por *Klebsiella* multirresistente, contribuindo para a mitigação da crise
285 global da resistência antimicrobiana.
286

3 OBJETIVOS

O presente estudo teve como objetivos gerais:

1 - O isolamento e caracterização de fagos de Klebsiella pneumoniae, utilizando amostras de esgoto e de efluentes poluídos, visando sua utilização em fagoterapia.

2 - Contribuir para o *corpus* de pesquisa envolvendo fagos, com a finalidade futura de implementar a fagoterapia regionalmente, tendo em vista a falta de pesquisas envolvendo o isolamento de fagos na Universidade Federal de Uberlândia.

3 - Estabelecer protocolos para o isolamento de fagos das amostras de esgoto e efluentes poluídos, além das ferramentas para caracterização genômica, morfológica, de atividade lítica e de atividade em biofilmes desses bacteriófagos.

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5 MANUSCRITO

Prospection of *Klebsiella pneumoniae* phages and their activity on multidrug-resistant strains of *Klebsiella* spp. of clinical importance.

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Prospection of *Klebsiella pneumoniae* phages and their activity on multidrug-resistant strains of *Klebsiella* spp. of clinical importance.

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Short title: *Klebsiella pneumoniae* phages that infect multidrug-resistant *Klebsiella pneumoniae*.

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Abstract:

Phages have gained attention as alternative therapies against multidrug-resistant (MDR) bacteria. *Klebsiella pneumoniae* is a major nosocomial pathogen associated with antibiotic resistance. This study reports the isolation and characterization of phages against *K. pneumoniae*.

Phages were isolated from samples from sewage and a contaminated river using *Klebsiella quasipneumoniae* and *Klebsiella pneumoniae*. Phage diversity was assessed by Random Amplification of Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR), and morphology was analyzed by transmission electron microscopy (TEM). Phage activity was tested during biofilm formation and on preformed biofilms. Host range was evaluated using eight MDR *Klebsiella* isolates.

Five phages were isolated from *K. quasipneumoniae* (KqP1 to KqP5) and five from *Klebsiella pneumoniae* (K2010P1 to K2010P5). RAPD-PCR revealed four *K. quasipneumoniae* phages with similar patterns (except for KqP2), suggesting relatedness, and all *Klebsiella pneumoniae* phages also appeared related within their group. TEM of KqP3 showed *Siphovirus*-like morphology. Among four *K. quasipneumoniae* phages tested, only KqP1 inhibited biofilm formation and disrupted the biofilm that was already formed. A cocktail containing these phages also disrupted the biofilm. All *K. pneumoniae* phages inhibited and disrupted biofilms effectively. Seven of the phages lysed three out of eight MDR *Klebsiella* strains tested, demonstrating promising potential against MDR *Klebsiella* infections.

Introduction:

Antimicrobial resistance (AMR) is one of the most significant threats to global healthcare systems today. Current estimates suggest that AMR may lead to 10 million deaths annually, alongside economic losses amounting to trillions of U.S. dollars[1]. Alarmingly, projections indicate that by 2050, none of the currently available antibiotics may remain effective unless novel therapeutic strategies are developed[2]. Among the most concerning pathogens are those belonging to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which are major causes of nosocomial infections due to their multiple resistance mechanisms[3]. *Klebsiella pneumoniae*, a frequent opportunistic pathogen, is closely associated with healthcare associated infections (HAIs) and resistance genes. A multicenter cohort study, which analyzed bloodstream infections across nine hospitals, identified *K. pneumoniae* as the second most prevalent gram-negative bacterium in both community and healthcare settings, surpassed only by *E. coli*[4,5]. Another study, conducted in Manaus, located in the state of Amazonas in northern Brazil, revealed that 80% of isolated bacterial strains harbored resistance genes[6]. In dumpsters near hospitals, a strain of *K. quasipneumoniae* harboring blaNDM-blaCTX-M15 genes, which confers resistance to carbapenems, was isolated from flies in Rio de Janeiro, revealing that multidrug resistant (MDR) bacteria are present outside health care units[7]. A review of 62 studies reported a 42.14% mortality rate of patients infected with resistant strains compared to 21.16% for susceptible ones[8].

Given the growing prevalence of MDR pathogens, alternative antimicrobials are being extensively researched. Bacteriophages, first discovered in the early 20th century, are now gaining renewed attention as therapeutic agents in the fight against AMR[9]. These viruses specifically infect and lyse bacteria, effectively killing them while sparing host eukaryotic cells[10]. This specificity makes bacteriophages a promising tool for controlling infections caused by MDR bacteria. As research progresses, they may play a pivotal role in addressing one of the most pressing challenges in modern healthcare.

In this study, we sought to isolate and characterize novel *K. pneumoniae* bacteriophages for the potential implementation of phage therapy in our region and to contribute valuable data to the growing body of research on this promising alternative treatment.

Materials and Methods

Bacterial strains and growth conditions

Two strains used to isolate the bacteriophages: clinical isolate *Klebsiella quasipneumoniae* ATCC 700603 (also described as *K. pneumoniae*, nevertheless in this research the *quasipneumoniae* subspecies will be used, as it is described this way in the ATCC[11]), which produces beta lactamase SHV-18 and is usually used as quality control in antimicrobial tests; a strain designated *Klebsiella* sp. (*Klebsiella* 2010) of unknown origin, whose partial sequence of 16S rRNA gene was obtained to ensure it was a *Klebsiella* sp. strain. Biochemical tests were also performed to ensure strain genus identification. The primers used for PCR and sequencing (515F and 806R) were described elsewhere[12,13]. Both were cultured in Luria-Bertani (LB) medium supplemented with 10 mM $MgSO_4$, (LB/$MgSO_4$) with 2% agar in case of culture medium in plates, according to protocols established by Townsend *et al.*[14], Sambrook & Russell[15]. Briefly, an isolated colony cultured overnight in LB/$MgSO_4$-agar was inoculated in 2 mL LB/$MgSO_4$ and incubated at 37 °C overnight in a shaker at 200 RPM. Then, 100 µL was pipetted into 5 mL of LB/$MgSO_4$ and incubated for 2 h at 37 °C to reach the exponential growth phase.

Phage isolation and purification

Three samples were used: sewage from Umuarama campus of Universidade Federal de Uberlândia (UFU), water from Uberabinha river in Uberlândia city, both in the state of Minas Gerais, and water from Aricanduva river, which is highly polluted, in São Paulo city, in the state of São Paulo, all located in southeastern Brazil. The treatment of the samples was based on a protocol described by Ceyssens[16]. A volume of 40 mL of each sample was treated with 300 µL of chloroform by incubating it at room temperature in a shaker at 200 RPM for 20 min. Then, the samples were centrifuged for 25 min at 5000 xg at 4 °C, after which the supernatants were filtered with 0.45 µm sterile filters and stored at -20 °C. To scan for the presence of lytic bacteriophages, 100 µL of the filtrate was added to 5 mL suspension of an exponential growth phase of each *Klebsiella* strain, as described above, and incubated overnight at 37 °C at 200 RPM. Then, the cultures were treated with 100 µL of chloroform and 10 µL of each supernatant was pipetted onto lawns of the respective *Klebsiella* strain and incubated overnight at 37 °C. After verifying the presence of lysis, we proceeded to the purifying step, which consisted of pipetting serially diluted samples of the lysate onto lawns of the bacteria, picking isolated lytic plaques with a sterile pipette tip, placing them individually in microtubes containing 200 µL LB/$MgSO_4$, treating the suspensions with chloroform and placing them onto bacterial lawns to pick isolated lytic plaques again. This step was repeated a total of three times to ensure pure clones, which were stored in SM buffer[17].

Lysis activity of the isolated phages against other *Klebsiella pneumoniae* strains

The ten isolated phages were tested against *Klebsiella pneumoniae* isolates with various antibiotic resistance profiles (Table 1) to verify whether they lyse MDR strains. The test was carried out by making lawns of the selected strain, then 10 µL of the stocks of each phage was pipetted onto different divisions of the plate. Any lysis observed was considered to have effect on the designated strain.

Ions and temperature test on KqP culture

Only *K. quasipneumoniae* phages (KqP) were tested, as the bacteria is a clinical strain (ATCC 700603). Two experimental groups were established, one incubated at 30 °C and the other at 37 °C, with four different medium conditions: (1) a control group with standard LB, (2) LB containing 10 mM $MgSO_4$, (3) LB containing 10 mM $CaCl_2$, and (4) LB containing 10 mM $MgSO_4$ and 10mM $CaCl_2$. First, 100 µL of an overnight suspension of *K. quasipneumoniae* was inoculated in 5 mL of LB, with 10 µL of phage stock and incubated overnight under agitation at 37 ºC. After which the cultures were treated with 300 µL of chloroform, agitated for 15 minutes and centrifuged. The supernatants, containing the bacteriophages, were collected and quantified by placing serially diluted suspensions onto bacterial lawns. The experiment was performed in triplicate.

Bacteriophage DNA extraction

DNA extraction was performed using a precipitation method, as previously described, with some modifications[15]. Firstly, 500 µL of phage suspensions were treated with 2 I.U. of DNase I (New England BioLabs) according to the manufacturer's instructions. The extraction process began with the addition of 1 µL of 0.5 M EDTA (pH 8.0), proteinase K (Ludwig Biotec) to a final concentration of 50 µg/mL, and SDS to 0.5% (w/vol). The mixture was agitated and incubated at 56°C for 1 h.

Subsequently, an equal volume of Tris-treated phenol/chloroform (1:1, v/v) was added to the mixture, followed by agitation and centrifugation at 3000 ×g for 5 min at room temperature. The aqueous phase was carefully separated, and 1/10 (vol/vol) of its volume of 3 M sodium acetate pH 5.2 and 7/10 (vol/vol) of isopropanol were added. The solution was mixed and then incubated at -20°C overnight.

Then, samples were centrifuged at 11,000 ×g for 15 min at 4 °C. The supernatants were discarded, the pellets were washed with 300 µL of 75% ethanol, followed by centrifugation under the same conditions. The pellets were air-dried for 10 min and dissolved with 30 µL of modified TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The samples were incubated at 56°C for 15 min, agitated, briefly centrifuged, and stored at -20°C. DNA concentration was quantified using a Qubit fluorometer 2.0 (Invitrogen).

Random Amplification of Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR)

The approach was performed to characterize genomically the isolated phages, following the method described by Johansson *et al*[18], and Gutiérrez *et al*[19] with some modifications. Four primers were used: RAPD5 (5'-AACGCGCAAC-3'), OPL5 (5'-ACGCAGGCAC-3'), P1 (5' -CCGCAGCCAA-3') and P2 (5'-AACGGGCAGA-3').

The RAPD-PCR was carried out using the mix GoTaq (Promega), supplemented with $MgCl_2$ to reach a final concentration of 3 mM, 8 µM primer, and 30 ng of phage DNA was used with a final volume of 25 µL, with the following cycling conditions: 5 cycles at 94 ºC for 45 s, 30 ºC for 2 min and 72 ºC for 1 min; 30 cycles at 94 ºC for 5 s, 36 ºC for 30 s, 72 ºC for 30 s with an addition of 1 s after each cycle; and the final step of 75 ºC for 10 min. Samples obtained after the amplification reaction were subjected to 0.6% agarose gel electrophoresis stained with DSview (Sinapse Biotecnologia Ltda). O´GeneRuler 1 kb Plus DNA Ladder (ThermoFisher) was used as DNA molecular weight marker. The similarity matrix was calculated based on the dice correlation coefficient, and its corresponding dendrograms (Figures 2 and 3) were deduced using the unweighted pair group method, using software GelJ V.2[20].

Transmission electron microscopy (TEM)

Transmission electron microscopy was performed following the protocol described by Ghaznavi-Rad et al[10], with modifications. KqP3 was selected due to its lytic activity against a MDR *Klebsiella* strain. Briefly, approximately 10 µL of 10^8 PFU/mL of phage stock was pipetted onto 300 mesh formvar carbon-coated grids (Sigma-Aldrich). The grids were left undisturbed for 5 min, after which they were negatively stained with 2% (w/v) uranyl acetate and air-dried. The grids were then examined using a transmission electron microscope (Hitachi HT7700), operating at an accelerating voltage of 80 kV with magnification of 60.000.

Biofilm inhibition and disruption assays

Two assays were carried out: inhibition assay, to assess the prevention of biofilm formation; and disruption assay, to assess the disruption of preformed biofilms. The experiments were conducted as described by Jeon & Yong (2019)[21], with modifications. Bacterial suspension was adjusted to an OD_{600} of 0.2 and 150 µL of the suspension was dispensed into each well of a sterile, flat-bottom 96-well polystyrene microtiter plate. Two controls were used: one group of wells consisting of 150 µL LB/$MgSO_4$ broth and another group consisting of bacterial suspension without the addition of phages. The amount of phages added to the wells were 10^7 PFU of KqP1, KqP3, and KqP4, or 10^5 PFU of KqP5; and 4.5 x 10^5 of K2010P1, K2010P2, K2010P3, K2010P4 and 4.5 x 10^4 of K2010P5; a phage cocktail containing the same amounts of four (KqP) or five (K2010P) phages combined was also tested. KqP2 was not used due to

734 the difficulty in obtaining its culture. In the inhibition assay, individual phages or the cocktail
735 were added simultaneously with the bacterial suspension to the wells and the plates were
736 incubated at 37 °C for 6 h. The disruption assay was performed by first allowing the biofilm to
737 form over 24 h, after which the medium was carefully removed, replaced with fresh LB broth
738 containing the individual phages or the cocktail and the plates were incubated for 6 h.
739 At the conclusion of each experiment, the medium of the wells was gently removed, the wells
740 were washed with sterile water and allowed to air-dry to ensure the biofilm stability. The
741 biofilms were fixed with 150 µL methanol for 10 min, after which the methanol was removed,
742 and the plates were air-dried for 5 min. Subsequently, 150 µL of 1% (w/v) crystal violet solution
743 was added to each well and left to stain for 30 min. Excess stain was removed by washing the
744 wells three times with PBS, and the bound crystal violet was dissolved with 150 µL of 33%
745 (v/v) acetic acid. The absorbance of each well was measured at 600 nm using a
746 spectrophotometer. All experiments were performed in triplicate.
747 **Lytic activity analysis against MDR *K. pneumoniae* isolates:**
748 Bacterial lawns were prepared as described above and a volume of 10 µL of each phage
749 preparation was spotted on the plates, which were incubated overnight at 37 °C. The bacterial
750 strains used in lytic activity tests are described (Table 1).
751
752 Table 1: Multidrug-resistant *Klebsiella* isolates and resistance associated-gene profile

Isolate	Subspecies	Origin	Carbapenem Resistance	EsβL[a]	Polymyxin	Other[b]
K35	*K. pneumoniae*	Rectum	+	+		++++++[c]
K150	*K. pneumoniae*	Rectum	+	+	+	+
K158	*K. pneumoniae*	Rectum	+	+	+	+
K142	*K. pneumoniae*	Urine	+	+		+
K155	*K. pneumoniae*	Lung	+	+	+	+++++
K57	*K. pneumoniae*	Urine	+	+	+	+++++
RB7	*K. quasipneumoniae*	Hospital food	+	ND[d]	ND	ND
HV55B	*K. michiganensis*	Hospital food	+	+	ND	+++++

753 [a] Extended spectrum β-lactamase producer.
754 [b] Other types of antibiotic resistance genes, such as mcr-1 (colistin), AadA1 (streptomycin and spectinomycin),
755 and others.
756 [c] Crosses used quantitatively according to the number of genes that confer other types of resistance.
757 [d] Not determined.
758

759 **Statistical analysis:**
760 For the test of addition of ions and of different temperatures on the culture of *K.*
761 *quasipneumoniae* phages, the results were analyzed using Two-way ANOVA with Geisser-

Greenhouse correction when applicable. For the biofilm assay, the results were analyzed using a non-parametric Student-t test with Welch's correction when applicable or Mann-Whitney test when data weren't parametric. Statistical significance was defined as $p < 0.05$.

Results

Isolation of phages

A total of 10 phage clones were isolated, five from *K. quasipneumoniae* and five from *K. pneumoniae* 2010. They were named based on the bacterial strain from which they were isolated: KqP stands for *Klebsiella quasipneumoniae* (Kq) phage, as K2010P stands for *K. pneumoniae* 2010 (K2010) phage, followed by their identification number: KqP1, KqP2, KqP3, KqP4, KqP5, K2010P1, K2010P2, K2010P3, K2010P4 and K2010P5. All phages showed lytic activity against the *Klebsiella* strain used for their isolation (Figure 1). Kq phages showed a clear lysis plaque and K2010 phages exhibited a small halo around the plaques. Notably, in many attempts the culture of KqP2 was not successful. Addition of Mg^{2+} and/or Ca^{2+} and incubation either at 30 °C or 37 °C did not improve their cultivation (results not shown).

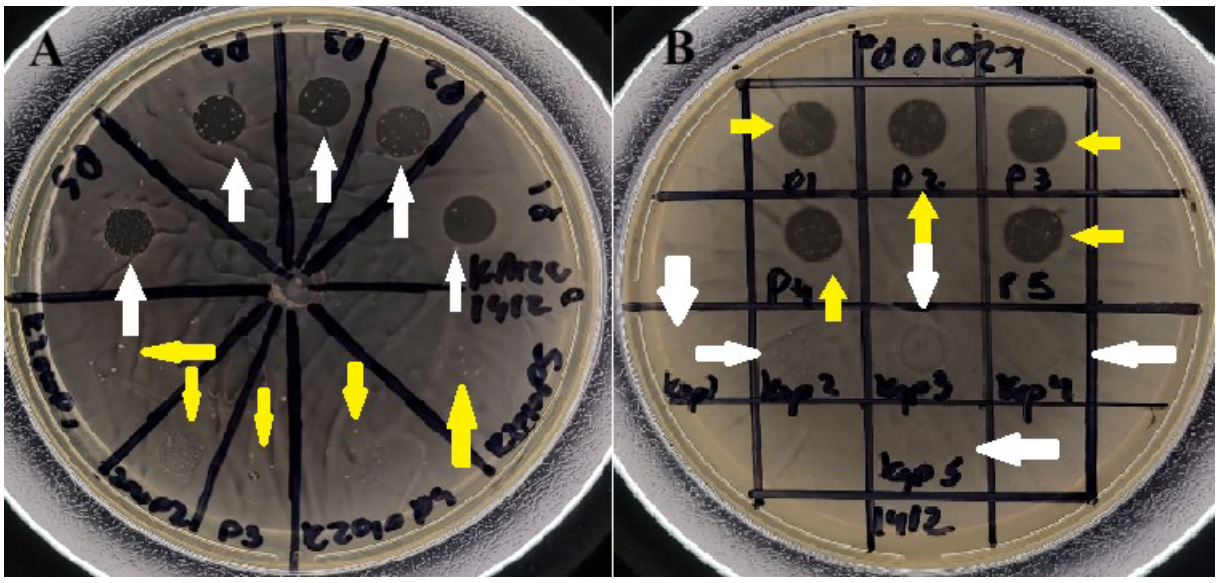


Figure 1: Lysis activity of isolated phages against the *Klebsiella* strains used for their isolation. Lawn of *K. quasipneumomiae* (A) and *K. pneumoniae* 2010 (B) spotted with phages KqP1 to KqP5 (white arrows) and K2010P1 to K2010P5 (yellow arrows).

Genomic analysis by RAPD-PCR

Genomic fingerprinting of the phages was only possible with primer OPL5. The results showed three patterns (Figure 2): (A) four *K. quasipneumoniae* phages (KqP1, KqP3, KqP4 and KqP5) shared similar band patterns, suggesting genetic relatedness, with KqP4 and KqP5 showing identical pattern, while (B) KqP2 showed a pattern distinct from the other phages, and (C) all

K. pneumoniae 2010 phages showed similarity in their band profiles, with K2010P1 and K2010P3 showing identical pattern.

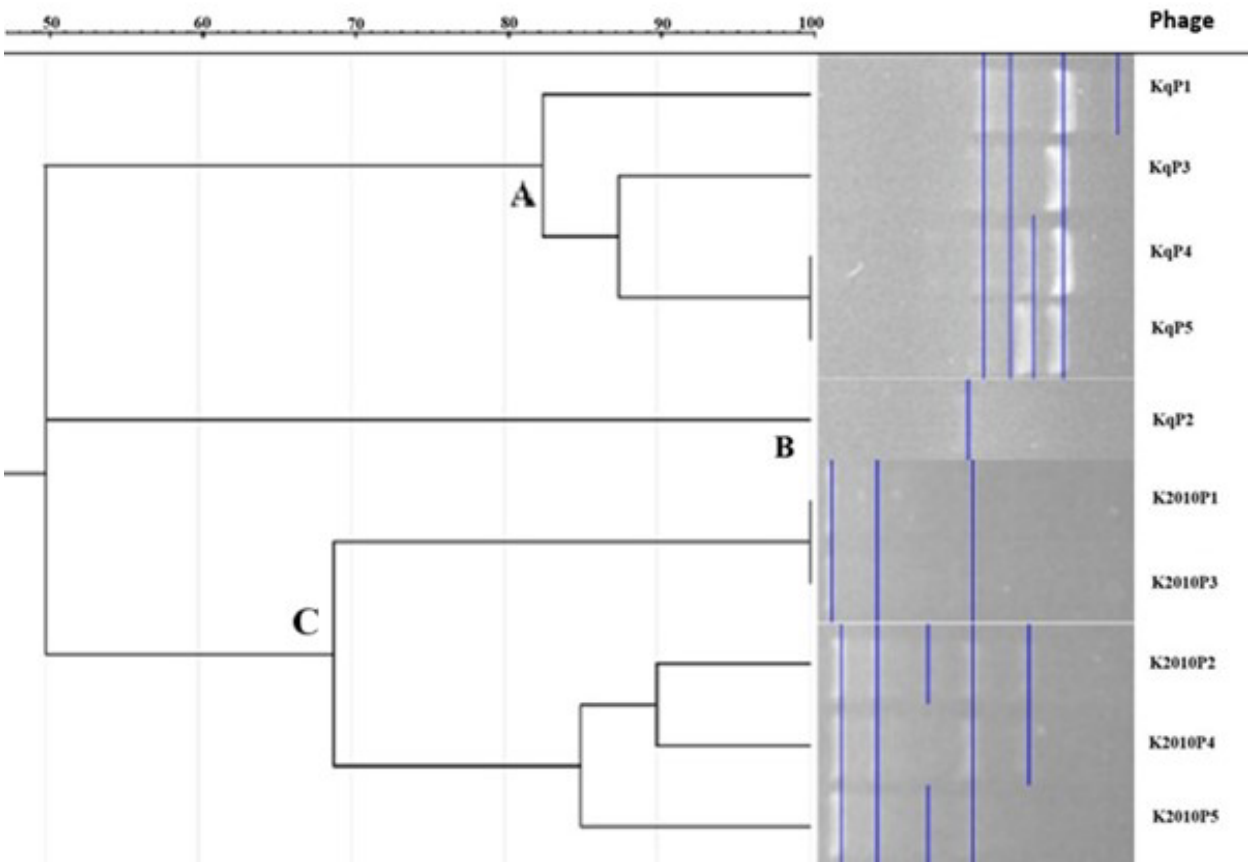


Figure 2: Dendrogram of the electrophoretic band patterns generated by RAPD-PCR with *K. quasipneumoniae* and *K. pneumoniae* phages using primer OPL5. Similarity matrix calculated based on the dice correlation coefficient, and its corresponding dendrogram was deduced using the unweighted pair group method. **A** through **C** represent the groups of identified patterns.

Transmission electron microscopy (TEM)

KqP3 was chosen for TEM due to its lytic activity against pathogenic *K. pneumoniae* K158 isolate (see in Lytic activity against MDR *K. pneumoniae* isolates), and because it rendered the highest viral titer. The head showed to be icosahedral (approximately 60 nm in length and 50 nm in width) and the tail appeared to be flexible (length over 100 nm and approximately 10 nm width) (Figure 3). Although it was not possible to visualize the terminal fibers, these morphologic characteristics suggest that KqP3 is a siphophage type virus, which has long and flexible tails[22].

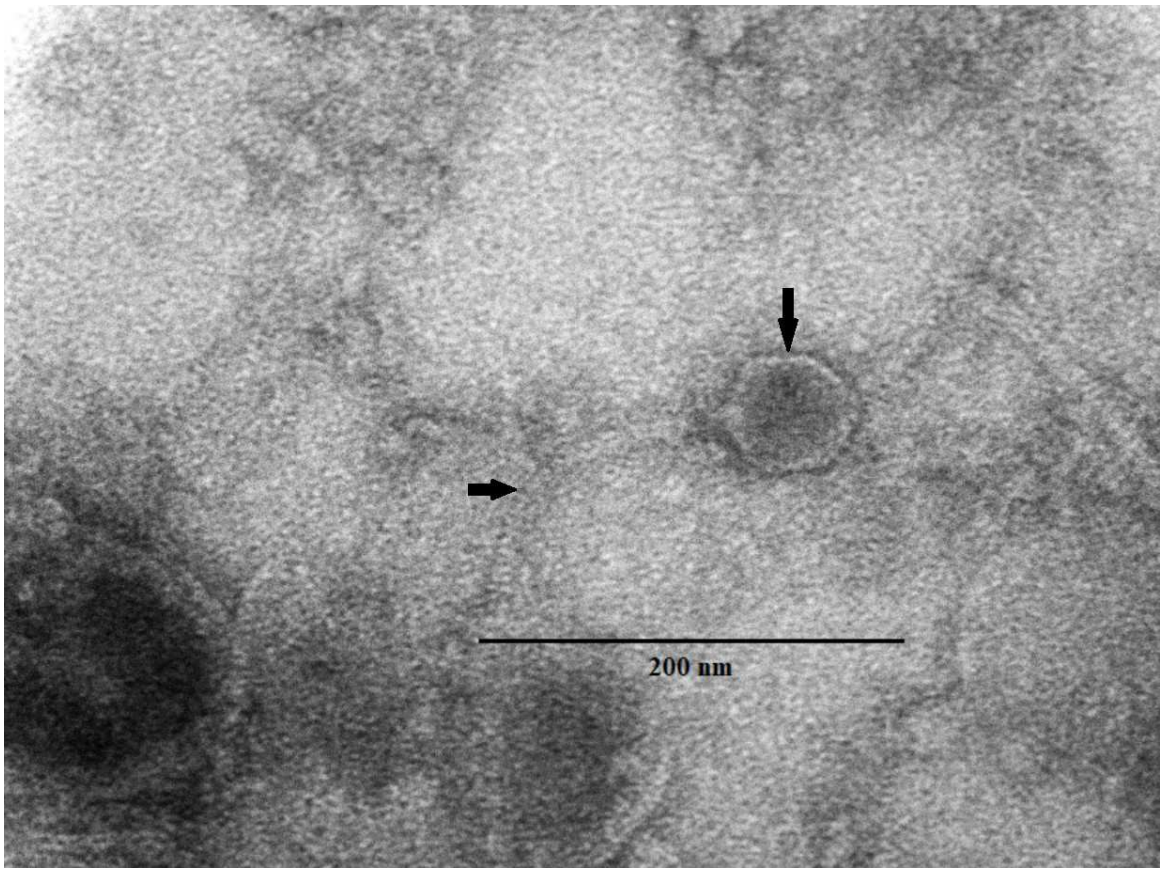
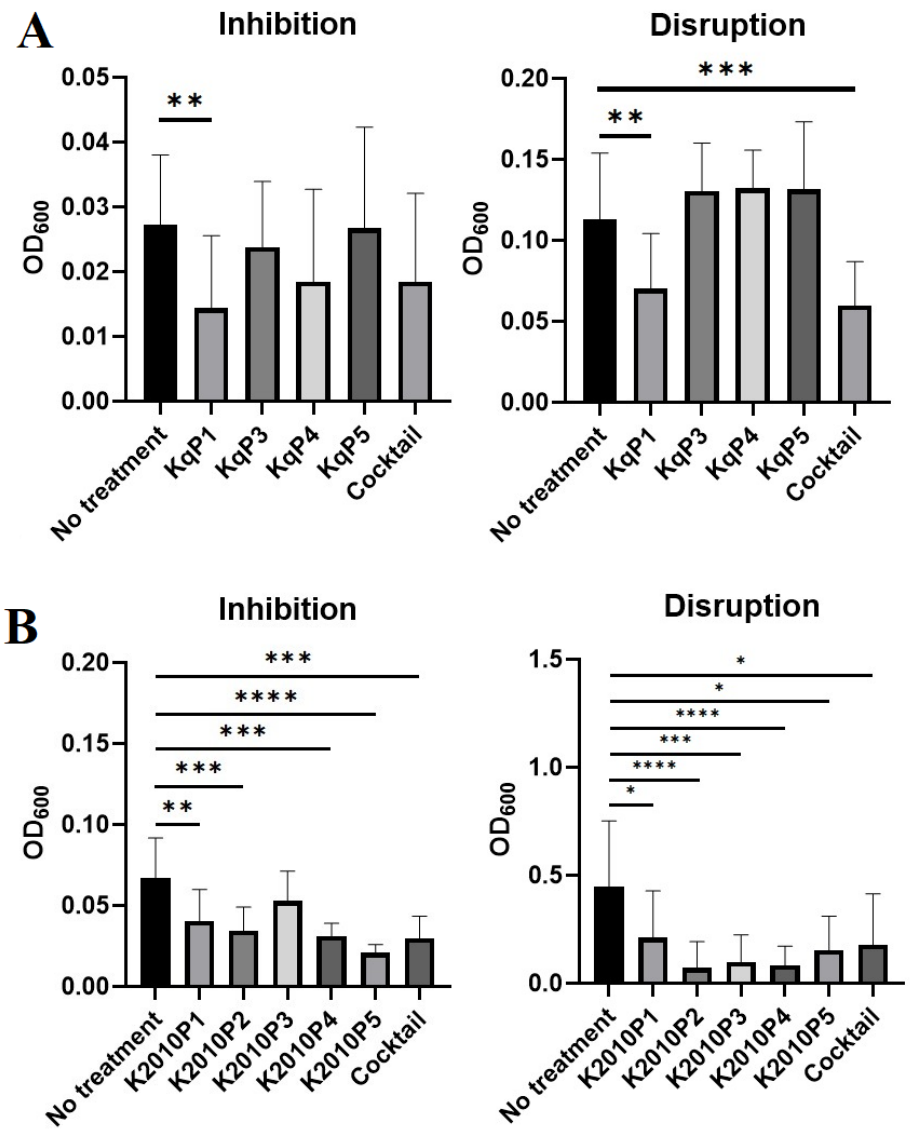


Figure 3: Transmission electron microscopy of KqP3 stained with 2% (w/v) uranyl acetate. The scale bar is shown. Vertical arrow points to the head and horizontal one to the tail.

Biofilm inhibition and disruption assays

To assess whether the phages could prevent biofilm formation or disrupt it, the assays were conducted with the *Klebsiella* strains used as hosts. The results demonstrated that KqP1 was the only *K. quasipneumoniae* phage that inhibited biofilm formation (Figure 4A). Although KqP1 was also present in the cocktail, there was no significant difference compared to the control wells that received no phage treatment ($p = 0.095$). In the disruption assay, the wells that received either KqP1 alone or with other phages in the cocktail showed a significant decrease in optical density compared to wells that received no phage.

Regarding the biofilm assays with *K. pneumoniae* 2010 strain, all phages, individually or in the cocktail, inhibited biofilm formation as well as disrupted biofilm that was already formed (Figure 4B). Individual K2010P5 showed the highest activity in the inhibition of biofilm formation, while K2010P2 and K2010P4 exhibited the highest levels of biofilm disruption.



825 **Figure 4:** *Klebsiella quasipneumoniae* (A) and *Klebsiella pneumoniae* (B) biofilm inhibition and disruption assays
826 by individual phages and phage cocktails. In the inhibition assay, bacterial and phage suspensions were added to
827 the wells at the same time, while in the disruption assay, biofilm was allowed to form in the wells 24 h before
828 phage suspension was added. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.
829

830 **Lytic activity against MDR *K. pneumoniae* isolates:**

831 KqP3 and KqP4 showed lytic activity against the *K. pneumoniae* K158, a multidrug-resistant
832 isolate (Figure 4A). Also, KqP1, KqP4, K2010P1, K2010P3, K2010P4 and K2010P5 lysed *K.*
833 *pneumoniae* K57 strain, with KqP3 presenting a weak lytic activity on it (Figure 4B), and all
834 KqP except KqP2 lysed *K. michiganensis* HV55B isolate (Figure 4C).

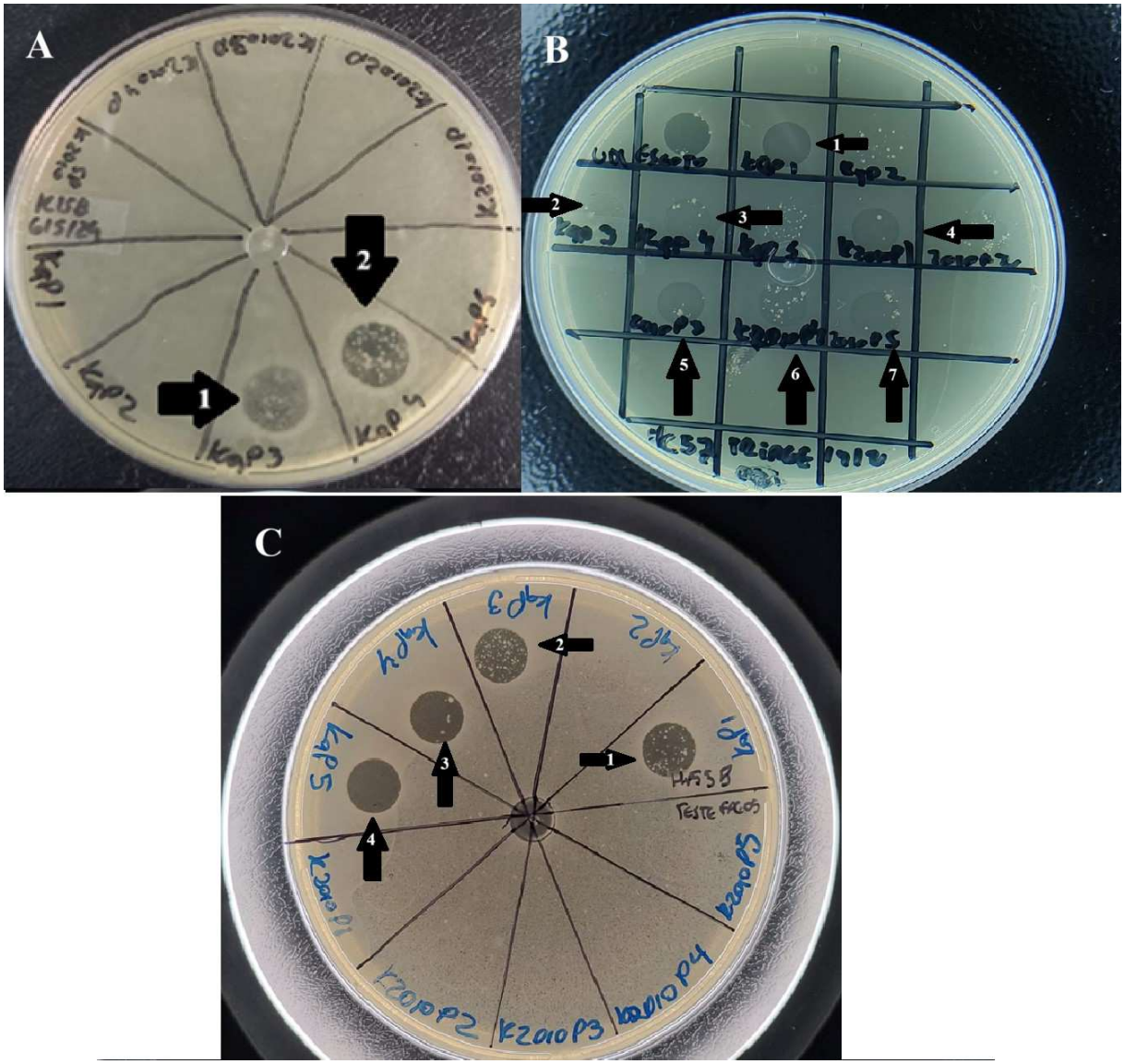


Figure 5: Lytic activity assays with the isolated phages against multi-drug-resistant *K. pneumoniae* strains. Lawns of *K. pneumoniae* K158 (A), *K. pneumoniae* K57 (B) and *K. K. michiganensis* HV55B (C) isolates. Arrows indicate lytic activity. (A): arrow 1 – KqP3 and arrow 2 - KqP4; (B): arrow 1 – KqP1, arrow 2 – KqP3, arrow 3 – KqP4, arrow 4 – K2010P1, arrow 5 – K2010P3, arrow 6 – K2010P4 and arrow 7 – K2010P5; (C): arrow 1 – KqP1, arrow 2 – KqP3, arrow 3 – KqP4 and arrow 4 – KqP5.

Discussion

Concerning the growing threat of AMR, phage therapy has emerged as a promising area of research. In this study, *Klebsiella pneumoniae*, a member of the ESKAPE pathogens known for its acquisition and dissemination of MDR genes such as those conferring carbapenem resistance[23], was targeted for bacteriophage isolation. It was reported that *K. quasipneumoniae*, which belongs to the same complex as *K. pneumoniae*, was less virulent than other strains of

Klebsiella because it harbors less virulent genes[24]. Nevertheless, it was shown that its infection spectrum is similar to the one caused by *K. pneumoniae* and it presents hypervirulent phenotypes in certain infection conditions. As the efficacy of first-line antibiotics continues to decline, reliance on second-line antibiotics increases; however, these treatments are often more costly, require prolonged administration, and result in extended hospital stays, thereby elevating the risk of hospital-acquired infections and further dissemination of resistance genes[25]. This underscores the urgent need for alternative therapeutic approaches such as bacteriophage therapy.

To address this, five phages infecting *K. quasipneumoniae* (ATCC 700603) and five infecting *K. pneumoniae* 2010 were isolated from sewage-contaminated water in a highly polluted section of São Paulo's Aricanduva River. These findings support previous reports that sewage-contaminated environments serve as reliable reservoirs for bacteriophages[26].

The adsorption and injection of genetic material by phages into the bacterial cells may require specific ions, such as magnesium (Mg^{2+}) and calcium (Ca^{2+}). However, an imbalance in the concentration of these ions —whether in excess or in shortage— can inhibit their life cycle[14]. Phages may also need metallic ions to maintain the structure of their proteins, as seen in the tail spike protein gpV of *Escherichia coli* P2 phages, which contains a metal-binding region with iron, calcium, and chloride ions[27]. While specific studies on *Klebsiella* phages and metallic ions are limited, it is reasonable to infer that similar principles may apply, given the conserved nature of phage biology across different bacterial hosts. In our study, however, no significant changes in phage titers were observed with addition of these ions. This observation aligns with Kuhn and Kellenberger's experiments[28], which showed that phages T3 and T7 were able to infect *E. coli* efficiently even under reduced intracellular levels of potassium and magnesium. However, a low concentration of magnesium reduced the burst size, suggesting that not all phages require precise ionic conditions for effective infection but highlighting the need to evaluate their importance for newly isolated phages[29].

The RAPD-PCR was used preliminarily to assess the diversity of the phages isolated in the present study. The results were obtained only with primer OPL5, which were similar to the results described by Gutiérrez *et al*[16], who reported that OPL5 along with primer P2 yielded the most reproducible results. The results in our study showed that four out of the five phages isolated from *K. quasipneumoniae* shared similar band patterns, suggesting that they belong to the same family and the remaining one probably belongs to a different family (Figure 2). Similarly, three phages isolated from *K. pneumoniae* 2010 strain also shared similar band patterns among themselves, suggesting that they belong to the same family. In addition, the

band patterns obtained with *K. quasipneumoniae* phages and the ones obtained with *K. pneumoniae* 2010 phages are not similar, therefore suggesting that the isolated phages belong to three different families. Also, although KqP4 and KqP5 showed identical band patterns, results obtained with the lytic activity, which showed that only KqP4 caused lysis against *K. pneumoniae* strain 158, indicated that they are distinct. Therefore, in combination, at least nine different phages were isolated in our study.

Regarding morphological features, electron microscopy of KqP3, a phage that showed lytic activity against the MDR *K. pneumoniae* strain 158, revealed characteristics consistent with siphophages, including viruses from the formerly named *Siphoviridae* family[30]. Specifically, KqP3 has approximately 60 nm icosahedral head, 55 nm diameter and a seemingly flexible tail over 100 nm in length (Figure 3). These traits are similar to those observed in phages LAPAZ[30] and IME268[31], both isolated from *K. pneumoniae*.

In the biofilm inhibition and disruption assays using *K. quasipneumoniae*, KqP1 was the only phage that showed activity in both tests (Figure 4A). Also, the phage cocktail exhibited a more significant reduction in biofilm biomass. This enhanced efficacy of the cocktail could be the results from the combined action of multiple phages targeting distinct bacterial receptors or biofilm components, a synergy that has already been described for *Klebsiella* phage cocktails and may broaden host range and biofilm degradation activity[32,33]. Although the other *K. quasipneumoniae* phages exhibited strong lytic activity in bacterial lawns, they showed limited or no efficacy against biofilms, suggesting that the biofilm matrix impedes their activity. This observation is consistent with previous studies demonstrating that phages often differ between infecting planktonic cells and cells in biofilms, due to their physical and biochemical barriers, which limit phage penetration and access to bacterial hosts through various mechanisms[34].

Comparable investigations using *K. pneumoniae* phage PG14 reported notable disruption of preformed biofilms, with the degree of disruption correlated to the presence of depolymerase enzymes, which degrade polysaccharides and may compromise bacterial cell integrity and biofilm structure[35,36]. This suggests that KqP3, KqP4, and KqP5 either lack depolymerase production or produce enzymes that are ineffective against the biofilm of the designated strain, this is supported by the fact that the phages didn't demonstrated depolymerase activity with the presence of halos around the plaques.

All K2010 phages inhibited biofilm formation, with K2010P5 showing the strongest activity and the phage cocktail also significantly reduced early biofilm establishment (Figure 4B). These results further reinforce the previously noted observation that phages generally show greater efficacy during the initial stages of biofilm development, when bacterial cells and matrix

components remain more accessible[37]. The enhanced antibiofilm activity of certain phages is attributable to depolymerases and other enzymes that degrade extracellular polymeric substances, thereby impairing formation of biofilm and bacterial adhesion[36].

Similarly, all K2010 phages tested demonstrated significant activity against mature biofilms, which could be attributed to the possible production of depolymerases[36] (Figure 4B). Also, the observation of halo in the plaques formed by K2010 phages is also an indicative of depolymerase activity. K2010P2 and K2010P4 exhibited the most pronounced disruption after 6 hours of treatment. However, the phage cocktail showed disruption activity that was comparable to the least effective individual phages, K2010P1 and K2010P5. This suggests possible antagonistic or competitive interactions among phages in the cocktail, which may reduce efficacy against complex biofilm structures, as previously seen in phage-biofilm dynamics where phages may interfere with each other, resulting in an impairment of overall biofilm clearance[38]. The amount of viruses used in the assays varied from 10^5 to 10^7 PFU/well for Kq phages and 4.5 x 10^4 to 4 x 10^5 PFU/well for K2010 K2010 phages. Our findings suggest that, rather than depending on high initial doses, the success of a treatment may rely on the intrinsic properties of the phage and its ability to propagate *in situ*. Therefore, low-dose strategies—if guided by accurate phage selection—could offer effective alternatives for biofilm-targeted applications, potentially minimizing the risk of resistance development and reducing production costs.

The isolated phages exhibited clear plaques (Figure 1), characteristic of lytic phages, with halo formation present in K2010 phages, indicating presence of depolymerase activity[39]. Although phages with depolymerase activity may target a broader range of hosts and degrade biofilms more effectively[40], phages that do not have it should be not disqualified for therapeutic use. In addition, they may be genetically engineered to include depolymerase activity, underscoring the value of isolating broad-host-range phages[41,42]. Of the 10 phages isolated in the present studies, KqP3 and KqP4 lysed *K. pneumoniae* K158, an MDR isolate; KqP1, KqP3, KqP4, K2010P1, K2010P3, K2010P4 and K2010P5 lysed *K. pneumoniae* K57; and, notably, all KqPs except KpP2 lysed *K. michiganensis* HV55B isolate. These results suggest that KqP could have a broader range of activity, even against other *Klebsiella* species. Also, our findings highlight the necessity of isolating more phages, to create a *K. pneumoniae* phage library, as the lytic activity was observed in only three of the eight *Klebsiella* strains used in the present study.

One limitation of the present study is the reproducibility issue regarding the RAPD-PCR method,[19,43]. Nevertheless, the qualitative aspect of RAPD-PCR and its power to discriminate even smallest changes in the genome, due to the small size of the primer used in the reaction

and the possibility of amplifying diverse genomic loci is what made the test a useful tool to approach the diversity of the phages isolated in our study[44,45].

Further steps are required to advance this work, including the genomic characterization of the isolated phages to ensure their safety and to identify any undesirable genes, such as those associated with lysogeny or antimicrobial resistance. In addition, it is essential to conduct more comprehensive in vitro and in vivo assays to assess the efficacy, stability, and therapeutic potential of these phages under diverse conditions. In particular, studies using appropriate animal models are necessary to evaluate the phages' pharmacodynamics, pharmacokinetics, immunogenicity, and effectiveness in treating infections in vivo, thereby providing critical preclinical data to support their potential clinical application.

Other studies highlight the synergistic role of antibiotics in aiding phages to combat biofilms, enhancing bacterial eradication beyond what phages can achieve alone, which demonstrates a need to test its anti-biofilm activity in combination with antibiotics[37].

Conclusions

In this study, 10 *Klebsiella pneumoniae* phages were successfully isolated from a highly polluted river using two *K. pneumoniae* strains, *K. quasipneumoniae* ATCC and *K. pneumoniae* 2010. RAPD-PCR, used to assess the diversity of the phages isolated in the present study, combined with lytic activity against antibiotic-resistant *Klebsiella* isolates revealed that at least nine different *Klebsiella pneumoniae* phages were isolated. Transmission electron microscopy revealed that KqP3 is a siphophage-like virus, which, along with other isolated phages, demonstrated lytic activity against three out of eight antibiotic-resistant bacterial strains, including a multidrug-resistant strain and a different species of *Klebsiella*. Only one phage isolated from *K. quasipneumoniae* showed biofilm inhibition and disruption activity, while all *K. pneumoniae* phages inhibited biofilm formation and disrupted preformed biofilm.

The findings of this study highlight the feasibility of isolating bacteriophages from regional environmental samples, enabling the identification of phages that lyse multidrug-resistant (MDR) bacterial strains that circulate locally. This work represents a significant step toward the development and implementation of phage therapy, particularly in the context of personalized or regionally adapted therapeutic strategies.

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Authors' Contributions

P.A.M.S.: Conceptualization, methodology, investigation, formal analysis, visualization and writing – original draft. **S.A.F, T.M.L.C.S., and L.S.R.**: Investigation and visualization. **J.Y.**: Conceptualization, methodology, resources, writing – revision & editing, supervision, project administration and funding acquisition. **S.R., D.W.F.B. and R.M.R.**: Methodology and resources

Conflict of Interest

The authors have no financial conflicts of interest to disclose.

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6 NORMAS DA REVISTA

A revista escolhida para publicação do manuscrito foi a ‘’Memórias do Instituto Oswaldo Cruz’’, que trabalha com formato neutro de submissão. Devido a isso a estrutura antiga do artigo, que foi escolhida para submissão na revista ‘’Phage’’ se manteve.

Normas:

The manuscript should be prepared using standard word processing software and should be printed (font size 12) double-spaced throughout the text, figure captions, and references, with margins of at least 3 cm. The figures should come in the extension .tiff, .jpg or .png, with a minimum resolution of 300 dpi. Tables and legends to figures must be submitted all together in a single file. Figures must be uploaded separately as supplementary file.

Title: with up to 250 characters

Author's names: without titles or graduations

Institutional affiliations: full address of the all authors

Abstracts: Provide an abstract up to 200 words. Abstracts of research articles should be structured into 5 sections as follows: BACKGROUND, OBJECTIVES, METHODS, FINDINGS and MAIN CONCLUSIONS, each section addressing respectively the problem, the aim of the study, the main methodological approach, the most important findings and the conclusions of the study.

Key words: 3-6 items must be provided. Terms from the Medical Subject Headings (Mesh) list of Index Medicus should be used.

Sponsorships: indicate the sources of financial support.

Introduction: should set the purpose of the study, give a brief summary (not a review) of previous relevant work, and state what new advance has been made in the investigation. It should not include data or conclusions from the work being reported.

Materials and Methods: should give full and clear information to permit the study to be repeated by others. Standard techniques need only be referenced. However if a modification has been done in a standard protocol, it must be clearly described.

Ethics: when reporting experiments on human subjects, indicate whether the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 1983. When reporting experiments on animals, indicate whether the institution's or a national research council's guide for, or any national law on the care and use of laboratory animals was followed. In case the research work is making use of natural resources (plant, microorganisms,

biodiversity samples) authors must provide a statement that the research work is compliant with the national regulations on this subject.

Results: should be a concise account of the new information discovered, with the least personal judgement. Do not repeat in the text data already described in tables and illustrations.

Discussion: should emphasize the relevance of the new information and relate the new findings to existing knowledge. Only unavoidable citations should be included.

Acknowledgements: should list and give full credit to all of those (apart from authors) who has helped the achievement of the research work (including the funding organizations).

Conflict of Interests: authors must disclose any conflict of interest related to their research work.

Author's contribution: state each author's contribution to the research work.

7 CONSIDERAÇÕES FINAIS

Foram isolados com sucesso 10 fagos capazes de infectar bactérias do gênero *Klebsiella*, incluindo as espécies pneumoniae e quasipneumoniae, a partir de um rio poluído. A RAPD-PCR permitiu distinguir ao menos oito perfis genômicos diferentes, com capacidade para lisar cepas multirresistentes, inibir biofilmes e atuar sobre biofilmes pré-formados, destacando os fagos K2010Ps, possivelmente codificadores de depolimerases, evidenciado pelo halo observado nas placas, além da visualização do fago KqP3 em microscopia eletrônica, que revelou sua cauda longa e flexível.

Para o avanço do estudo, é necessária a caracterização genômica detalhada dos fagos, visando identificar e excluir possíveis genes indesejáveis associados à lisogenia e resistência antimicrobiana. Adicionalmente, são fundamentais testes in vitro e in vivo, principalmente em modelos animais, para avaliar a eficácia, estabilidade e potencial terapêutico, gerando dados pré-clínicos essenciais para a futura aplicação clínica. Este trabalho demonstra a viabilidade do isolamento de fagos específicos para *Klebsiella* na Universidade Federal de Uberlândia, ampliando as perspectivas para terapias personalizadas e adaptadas regionalmente.