FULL ARTICLE

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Isolation of lytic bacteriophages of *Escherichia coli* from swine

Isolamento de bacteriófagos líticos de Escherichia coli de origem suína

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ABSTRACT

Bacteriophages (phages) are small viruses that infect bacteria and represent nature's most abundant biological entities. After infection, new phages are produced during their lytic reproductive cycle, causing disruption of the bacterial cell and releasing bacteriophages into the environment. Thus, phages have been used as a possible strategy for controlling bacterial infections in several areas, including pig farming, where multi-resistant pathogenic strains of *Escherichia coli* represent one of the leading agents associated with diseases in swine. In this sense, phagotherapy as an alternative treatment requires the selection of specific bacteriophages for the strains of interest. Therefore, this work aimed to isolate *E. coli* lytic bacteriophages from fecal swine samples, using standard *E. coli* strains as hosts for the infection. Four infective bacteriophages for *E. coli* ATCC 8739 and two for each of the *E. coli* K12 strains MG1655 and DH5α were isolated. The phage suspensions obtained had their virulence (titer) determined. Infection and cell lysis assays revealed that they were capable of breaking bacterial cells when added to liquid cultures, reducing cell growth by approximately 75% after 2 h, evidencing the possibility of being used as an alternative tool to antibiotics in the treatment of bacterial infections, in addition to the traditional antibiotics. Further studies using *in vivo* models should be considered.

Keywords: Bacteriophages. Isolation. Swine production.

RESUMO

Bacteriófagos (fagos) são pequenos vírus que infectam bactérias e representam as entidades biológicas mais abundantes na natureza. Após infecção, durante o ciclo reprodutivo lítico, novos fagos são produzidos, causando ruptura da célula bacteriana e liberação de novos bacteriófagos no ambiente. Os fagos têm sido utilizados como estratégia de controle de infecções bacterianas em diversas áreas, inclusive na suinocultura, onde cepas patogênicas multi-resistentes de *Escherichia coli* representam um dos principais agentes associados a doenças em suínos. Nesse sentido, a fagoterapia como tratamento alternativo requer a seleção de bacteriófagos específicos para as cepas de interesse. Assim, o objetivo deste trabalho foi isolar bacteriófagos líticos de *E. coli* de amostras fecais de suínos, utilizando cepas padrão de *E. coli* como hospedeiras da infecção. Para isso, quatro bacteriófagos infectantes para *E. coli* ATCC 8739 e dois para cada uma das cepas de *E. coli* K12 MG1655 e DH5α foram isolados. As suspensões de fagos obtidas tiveram sua virulência (título) determinada. Ensaios de infecção e lise celular revelaram que foram capazes de lisar as células bacterianas quando adicionados às culturas líquidas, reduzindo o crescimento celular em aproximadamente 75% após 2 horas, evidenciando a possibilidade de serem usados como uma ferramenta alternativa aos antibióticos no tratamento de infecções bacterianas, além dos antibióticos tradicionais. Novos estudos usando modelos *in vivo* devem ser considerados.

Palavras-chave: Bacteriófagos. Isolamento. Produção de suínos.

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Introduction

Pig farming is one of the most essential activities in agribusiness worldwide. The European Union, the United States, China, Brazil, and Canada are among the largest producers. Brazil is the fourth largest worldwide producer and exports approximately 1 million tons of pork meat to over 90 countries (Brazilian Pork, 2021; Wu et al., 2020). The intense progress of world pork production in the last three decades is due, among other factors, to the advance in pig nutrition. However, among the limitations of the productive potential of this activity, the urgent need to find effective alternatives for antibiotics stands out. Among the diseases that affect pigs, Escherichia coli is one of the main pathogenic agents, characterized by a considerable index of antimicrobial resistance. This situation is aggravated by the ability of this microorganism to disseminate genes associated with resistance through horizontal transfer to animal or human pathogens, representing a risk to public health (Costa et al., 2009; Wu et al., 2020).

The indiscriminate use of antimicrobials and the consequent increase in the number of events related to resistance to conventional antibiotics, characterized by the evolution of new resistance mechanisms with global dissemination, has driven therapies using bacteriophages with some advantages since they are devoid of any metabolic apparatus, being an obligate intracellular parasite that has no affinity for eukaryotic cells (Davies & Davies, 2010; Harada et al., 2018; World Health Organization, 2020).

Bacteriophages, called phages, can infect only bacterial cells, probably representing the planet's oldest and most abundant biological entity. Estimates indicate that there are over 10^{31} phages in the biosphere. Through infection and replication within the host cell, new virions known as infectious viral particles are produced, which contribute to bacterial population control and gene transfer between bacterial species, being primarily responsible for the adaptive evolution of bacterial genomes (Harada et al., 2018; Hatfull & Hendrix, 2011). They are able to interact with membrane receptors on the surface of bacterial cells. Studies suggest the existence of approximately 10 bacteriophages for each type of bacterial cell, some of which are highly specific for a particular species and others capable of recognizing different types of receptors (Leiman & Shneider, 2012).

Potentially successful applications of lytic bacteriophages have been described for the prevention and treatment of *Salmonella* spp and *E. coli* in poultry, calves, and swine, as well as *E. coli* O157:H7 in cattle. Moreover, the use of phages against diseases caused by *Pseudomonas aeruginosa* and *Acinetobacter baumanii* in pigs has been reported, in addition to the reduction of food contamination by *Salmonella* spp (Jamal et al., 2019; Johnson et al., 2008; Sriprasong et al., 2022).

Several routes may be used to administer phages, including topical, oral, and intravenous. They destroy the host pathogen and, in some cases, increase the sensitivity of the host bacteria to antibiotics. Several studies have shown that combining phage therapy and antibiotic treatment can be effective against bacterial infections. Clinical trials of phage therapy have shown promising results for several human diseases and conditions (Kiani et al., 2021; Vera-Mansilla et al., 2022). In addition to human health, phages have been used as a strategy to control bacterial infections in agriculture, food safety, and veterinary sciences, especially in animals affected by diseases caused by pathogens *E. coli*, *Salmonella* spp, *Pseudomonas* spp, among others (Jamal et al., 2019; Li et al., 2024).

In veterinary medicine, especially in pig farming, reports indicate the emergence of several multi-resistant strains of *E. coli* since it represents one of the primary pathogens associated with swine diseases, with emphasis on multi-resistant enterotoxigenic *E. coli* (ETEC) strains, causing diarrhea in piglets (Costa et al., 2009; Fairbrother et al., 2005). Post-weaning diarrhea in piglets and impacting animal health represent a significant economic loss in pig farming. The emergence of resistant enterotoxigenic strains (ETEC) limits therapeutic options, increases the cost of treatment, and increases mortality rates. In this sense, there is a tendency to seek solutions that include, among other alternatives, bacteriophage therapy (Laird et al., 2021).

Thus, phages from swine feces showed potential for prophylactic use and phagotherapy against *E. coli* ETEC O149, a strain associated with post-weaning diarrhea in piglets (Jamalludeen et al., 2007), in addition to being

capable of infecting resistant avian pathogenic *E. coli* (APEC) (Zhou et al., 2015). Furthermore, an *in vivo* therapeutic study using mice as models showed that a phage isolated from swine feces could act as an antibacterial agent against the pathogenic strain of *E. coli* (Sui et al., 2021).

The isolation of phages from healthy or diarrheal swine fecal samples revealed the presence of polyvalent and specific phages for enterotoxigenic (ETEC) and enteropathogenic (EPEC) *E. coli*. Although the presence of these phages in swine intestines is common, since they contribute to the regulation of bacterial communities, they were more abundant in the post-weaning stage compared to the pre-weaning stage and with a predominance of specific strains in each rural property (Lin et al., 2021). Other therapeutic applications include phage-encoded lysines, which effectively control infections and destroy bacteria in the tested area 2-5 h after application. Phages have also been used to control *Staphylococcus aureus*, *E. coli,* and other pathogenic bacteria in sewage treatment systems (Jamal et al., 2019).

Although regulatory agencies have approved some phage-based products, there is concern about issues related to the safety and efficacy of these applications, considering the possibility of virulence transmission, the difficulty of high-quality and clinically safe phage preparations, and possible development of host immune responses after administration (Jamal et al., 2019; Kiani et al., 2021; Luong et al., 2020;).

In this context, considering the emergence of multidrug-resistant strains of microorganisms associated with pig farming and their potential impact on animal and human health, as well as the environment, the objective of this work was to isolate lytic bacteriophages of *E. coli* as an alternative to chemical antimicrobials commonly used in pig farming.

Materials and Methods

Bacterial strains

The standard strains of *E. coli* DH5α (Hanahan, 1983), ATCC 8739 (Gunsalus & Hand, 1941) and K12 MG1655 (Jensen, 1993) were used in this work. The bacterial strains were grown in Luria-Bertani (LB) culture medium (Miller, 1972) at 37 °C, under agitation at 120 rpm, and stored in glycerol 50% (v/v) at -20 °C. Solid and semi-solid LB medium were prepared by adding 15 g/L and 7.5 g/L of agar, respectively.

Sampling and recovery of bacteriophages

Eight swine fecal samples were collected from a Swine Teaching and Learning Unit (400 m^2) that involves a complete cycle of intensive production system composed of Landrace x Large White females. They were collected immediately after defecation from eight animals in different rearing stages: piglets (four), sow (one), and finishing (three). All animal procedures were performed with the approval of the Ethics Committee on Animal Use (CEUA) of the Catarinense Federal Institute (under protocol Number 435/2023/CEUA-IFC).

Sample processing was performed using three methodologies: one adapted from Lin et al. (2021) and two modified from Atterbury et al. (2005). In the methodology based on Lin et al. (2021), referred to as Method 1, fecal samples (1 g) were separately suspended in SM buffer (Sambrook & Russell, 2001) in a 1:20 ratio (g/mL). Next, the suspension was homogenized and centrifuged at 13,000 ×g for 15 min. The supernatant was filtered through a cellulose acetate filter with pores of 0.45 μm, followed by storage at 4 °C. The double agar technique tested the presence of lytic phages in the filtered supernatant.

For the modified methodologies of Atterbury et al. (2005), the first 1 g of each stool sample was suspended separately in 15 mL of Luria Bertani (LB) (Miller, 1972), followed by the addition of the mixture of 100 μL of each culture (OD_{600nm} = 0.6 to 0.9) of *E. coli* strains DH5α, ATCC 8739 and K12 MG1655. The suspensions were incubated at 37 °C under agitation at 120 rpm for approximately 4 h. Next, a volume of 2 mL was centrifuged at 13,000 ×g for 30 min, and the supernatant was tested for lytic phages using the double agar technique (Method 2).

Another similar methodology was tested (method 3), proceeding with a 1:10 dilution (g/mL) of the stool samples in NaCl 0.9% (m/v), followed by the addition of a mixture of 100 μL of the culture of each strain of *E. coli* (OD_{600nm} = 0.6 to 0.9) and incubated at 37 °C for approximately 4 hours. After this time, a 2 mL aliquot of each sample was centrifuged at 13,000 ×g for 30 min, and the supernatant was tested for the presence of lytic phages using the double agar technique.

Prospection, isolation, and titration of bacteriophages

Sample processing was carried out using the double agar technique to verify the presence of lytic phages while observing the formation of lysis plaques. One drop (10 μL) of the bacteriophage suspensions was poured onto the semisolid LB medium containing the tested *E. coli* strain, already solidified on the solid LB medium in a Petri dish, followed by incubation under the same conditions described above.

Isolation and propagation were performed as described by Sambrook & Russell (2001) after a few modifications. Briefly, for the isolation of bacteriophages,

after the appearance of lysis plaques, SM buffer (5 mL) was added to the plates, followed by manual orbital agitation for virus detachment for approximately 5 min. Afterward, the buffer was collected using a micropipette and transferred to microtubes (2 mL). The tubes were centrifuged at 13,000 ×g for 30 min, and the supernatant was transferred to another sterile microtube. This procedure was performed twice.

Then, the infection procedure was repeated for the third passage using the isolated bacteriophage strain. After the appearance of lysis plaques, SM buffer (5 mL) was added to the surface of the medium, followed by shaking the Petri dishes manually for 5 min at room temperature. The SM buffer was collected and transferred to a sterile microtube, where chloroform (100 µL) was added. The tube was shaken, and cell debris was removed by centrifugation at 13,000 ×g for 30 min. The supernatant was filtered through a cellulose acetate membrane filter with pores of 0.45 µm to obtain purified viral particles stored at 4 °C. This process was performed to isolate one single lineage of bacteriophages.

To obtain the title of the isolated phages, serial dilutions of the final sample containing the viruses previously collected from each lysis plaque after the propagation process was performed. Thus, 100 µL of this sample was transferred to a sterile microtube containing 900 µL of NaCl 0.9% (w/v) as a diluent solution. Another 100 µL was taken and transferred from this dilution to a new microtube, also containing 900 µL of diluent solution. Seven identical dilutions were performed and tested for standardization of the best dilution. Lysis plaques were counted visually, and the number of viral infectious units (phage titer) was expressed as plaque-forming units per mL (PFU/mL) (Adams, 1949; Kęsik-Szeloch et al., 2013).

Infection assays

For the infection tests, *E. coli* strains ($OD_{600\ nm}$ = 0.1) were cultivated in 100 mL Erlenmeyer flasks containing 10 mL of liquid LB medium. The flasks were incubated in a shaking incubator (37 °C and 120 rpm), and the OD 600 nm was determined every 30 min or 1 h to evaluate growth along all phases of the bacterial growth curve.

After 1 h 30 min of cultivation (OD 600 nm approximately 0.7), when the cultures were in the log phase of cell growth, the isolated bacteriophage suspensions (100 µL) were added. In controls, the same volume of SM buffer was added.

Results and Discussion

Selection of lytic bacteriophages

All fecal samples used in this work came from animals in the post-weaning phase. A study of isolation of pathogenic *E. coli* bacteriophages from the intestine of piglets resulted in a higher number of bacteriophages in post-weaning fecal samples than in pre-weaning samples. Moreover, in this same study, two feces samples from pre-weaning animals showed a complete absence of bacteriophages for *E. coli* (Lin et al., 2021).

The formation of lysis plaques was not observed when tested with the three strains of *E. coli* used in this work (Table 1). According to Pelzek et al. (2008), for bacteriophage propagation techniques, an important parameter to be considered is the multiplicity of infection, represented by the ratio between the number of detectable infecting bacteriophages and the number of vulnerable bacterial cells. This ratio varies by type of bacteriophage, with most requiring a value ≥ 3 to ensure that at least 95% of the bacteria are infected.

The number of specific bacteriophages isolated directly from the sample using Method 1 was insufficient to

Table 1 – The lysing capacity of suspensions of bacteriophages from swine fecal samples collected at different stages of life

Sample	Origin	*E. coli*			Method*
		ATCC 8739	K12 MG1655	DH5α	
C	piglets	Negative	Negative	Negative	A
T1	finishing	Negative	Negative	Negative	A
T2	finishing	Negative	Negative	Negative	A
T3	finishing	Negative	Negative	Negative	A
C1AAG	piglet	Positive	Negative	Negative	B
C1BAG	piglet	Positive	Positive	Positive	B
C2AG	piglet	Positive	Positive	Negative	B
MAG	sow	Positive	Positive	Positive	B
C1A	piglet	Negative	Negative	Negative	C
C1B	piglet	Negative	Negative	Negative	C
C2	piglet	Positive	Negative	Negative	C
M	sow	Positive	Positive	Positive	C

*A, a methodology adapted from Lin et al. (2021); B and C, adapted from Atterbury et al. (2005). Negative – without PFU and Positive – PFU present and characteristic.

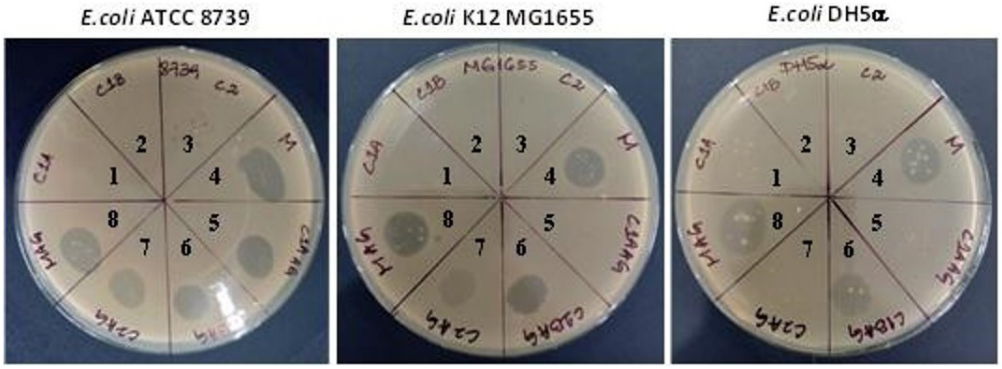

Figure 1 – Lysis plaques in cultures of *E. coli* DH5α, ATCC 8739, and K12 MG1655 using bacteriophages isolated from different samples and different methodologies adapted from Atterbury et al. (2005). Fecal sample 1 (C1A), 2 (C1B), and 3 (C2) from piglets; sample 4 (M) from sows; Samples 1 to 4 were prepared by diluting (1:10) in NaCl 0.9% (m/v), and samples 5 (C1AAG), 6 (C1BAG), 7 (C2AG), and 8 (MAG) were prepared by diluting 1 g of feces into 15 mL of LB.

guarantee infection and allow the visualization of lysis plaques. According to Gill & Hyman (2010) and Pelzek et al. (2008), environmental samples may have a low titer, and no bacteriophage may be found initially. For these situations, it is necessary to enrich this sample for a specific subset of bacteriophages using successive rounds of amplification with one or more hosts to increase the number of bacteriophages in the source sample.

In this sense, two methodologies adapted from Atterbury et al. (2005), referred to as Methods 2 and 3, were used, in which the samples were previously enriched before the isolation process (Figure 1; Table 1). Studies show that most phage isolation protocols, particularly those designed for phage therapy, employ slight variations of the classic enrichment protocol to enhance detection sensitivity. Some use a mixture of hosts to obtain a broader host range of phages, while in other studies, this feature is determined immediately after the isolation (Hyman, 2019).

According to the formed lysis plaques, it was possible to observe that the 1:10 dilution (m/v) of the feces samples (Method 3) was efficient only for the sample from adult female swine (Figure 2, Sample 4 - M) for all *E. coli* strains. This same sample also presented lysis plaques when isolated using the other methodology.

Using Method 2, lytic phages of *E. coli* ATCC 8739 were isolated from the three samples from the daycare center and adult females (Figure 2, Samples 5 - C1AAG to 8 - MAG). On the other hand, when the strains of *E. coli* K12 MG1655 and DH5α were used, the lysis profile was similar, highlighting a sample from piglets and sow (Figure 2, Samples 6 - C1BAG and 8 - MAG, respectively), while another sample from piglets (Figure 2, Sample 7 – C2AG) showed no apparent plaque lysis for DH5α, and plaque lysis that had a thin surface layer of bacteria when tested with K12 MG1655. Notably, the C1AAG and C1BAG

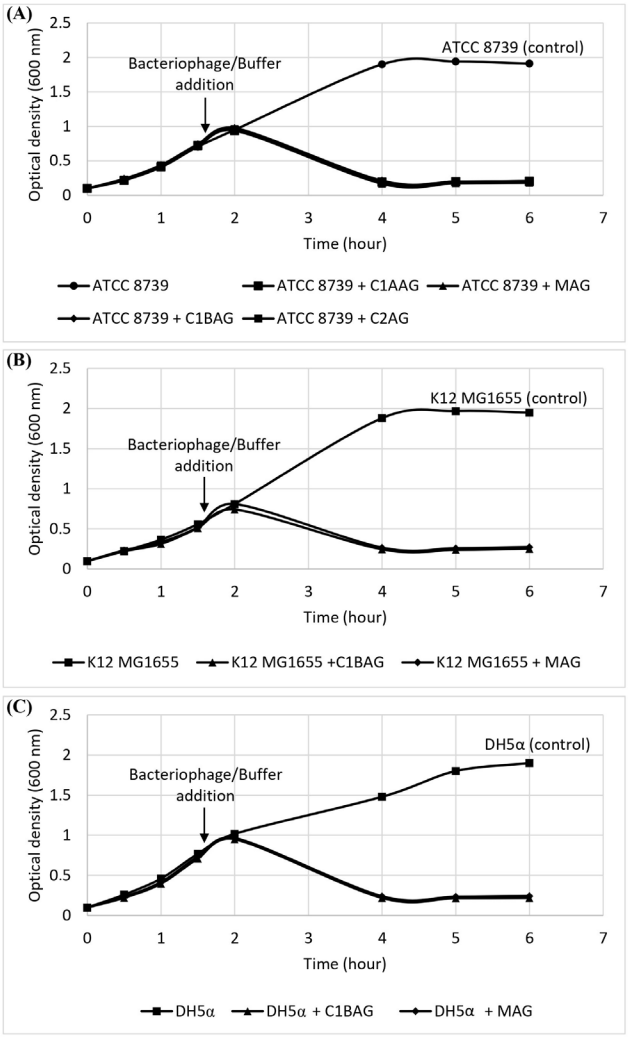

Figure 2 – Infection assays of isolated bacteriophages. (A) *E. coli* ATCC 8739; (B) *E. coli* K12 MG1655; and (C) *E. coli* DH5α. Values represent three experiments carried out independently. The standard deviation was less than 5% among replicates.

samples were obtained from fecal samples of animals of the same pen. Despite this, they showed different behavior in

forming lysis plaques in contact with the K12 MG1655 and DH5α strains. The results also showed that the 5 – C1AAG sample had specific bacteriophages for *E. coli* ATCC 8739 without showing apparent lysis plaques when tested with K12 MG1566 and DH5α.

Based on these results, the following samples were selected for isolation and bacteriophage propagation: *E. coli* ATCC 8739 (Samples 5 - C1AAG, 6 - C1BAG, 7 - C2AG, and 8 - MAG), *E. coli* K12 MG1655 (Samples 6 – C1BAG and 8 - MAG) and *E. coli* DH5α (Samples 6 – C1BAG and 8 - MAG).

Isolation, purification, and titration of selected bacteriophages

The selected samples were tested individually, and the results showed the appearance of lysis plaques. Some samples showed almost complete lysis of the cell mass. The purified bacteriophages from the third passage of the isolation and propagation step gave rise to stocks of phage suspensions. The titers (virulence) of these stocks were determined through serial dilutions in NaCl 0.9% (w/v) (Table 2).

Clinical tests using phage therapy to treat infections caused by different pathogenic bacteria used doses of bacteriophages ranging from 3.5×105 to 5.0×109 PFU, applied topically or intravenously (Kortright et al., 2019). Thus, the estimated titers (PFU/mL) obtained in the isolates allow the use of these phage suspensions to test their lytic potential.

Infection assays of isolated bacteriophages

Figure 2 shows the results of the infection assays of isolated bacteriophages. Cells maintained a growth profile for approximately 20 min after the addition of bacteriophages, followed by a drop in OD_{600nm} of 80%, 67%, and 76% for ATCC 8739, K12 MG1655, and DH5α, respectively, after 2 h of cultivation, remaining constant until the end of the experiment (Figures 2A, B, and C).

Table 2 – Estimated titer of bacteriophage suspensions isolated after third passage

Sample	Origin	*E. coli* host	Titer* (PFU/mL)
C1AAG	Piglet	ATCC 8739	4.30×10^{12}
C1BAG	Piglet	ATCC 8739	3.80×10^{9}
C2AG	Piglet	ATCC 8739	4.40×10^{10}
MAG	Sow	ATCC 8739	1.10×10^{11}
C1BAG	Piglet	K12 MG1655	1.00×10^{9}
MAG	Sow	K12 MG1655	2.00×10^{8}
C1BAG	Piglet	DH5α	4.00×10^{10}
MAG	Sow	DH5α	6.60×10^{9}

*Each value represents the mean number of lysis plaques observed at different dilutions in experiments performed independently, expressed as PFU/mL.

The results suggest that bacteriophages infected and lysed the cells after being added to the bacterial culture, evidenced by a reduction in the OD600nm and an apparent clarification of the culture medium. On the other hand, the controls continued increasing cell density until they reached the stationary phase of growth once they received the addition of SM buffer.

The amount of infective viral particles present in each culture (PFU/mL of culture) was approximately 4.30×10^{10} of C1AAG, 3.80×10^{7} of C1BAG, 4.40×10^{8} of C2AG, 1.10×10^{9} of MAG, for *E. coli* ATCC 8739 as a host. For K12 MG1655 as host, the number of viral particles was 1.00×107 from C1BAG and 2.00×106 from MAG, and for DH5α, 4.00×10^{8} from C1BAG and 6.60×10^{7} from MAG.

This work used two methods to detect isolated bacteriophages: lysis plaques and culture lysis. According to Hyman (2019), in the lysis plaque method, the size of the plaques may suggest phage size since larger phages diffuse more slowly, resulting in smaller plaques. Although, some phages do not form plaques due to limited diffusion in agar or low productivity. On the other hand, the culture lysis method displays the advantage of detecting cell lysis by turbidity. However, cell debris from early infections may bind and inactivate the phages, disrupting subsequent infections.

Despite the limitations of the methods used to detect isolated phages, both proved effective for this work, as they are commonly used for isolating and characterizing bacteriophages for phage therapy.

Phage therapy has many advantages, especially with the emergence of antibiotic-resistant bacteria, which can contribute to reducing dependency on antimicrobial agents, resulting in better economic and animal welfare outcomes. However, they have a narrow host range, limiting a broad-spectrum application. Furthermore, studies have shown that phages are unstable in the stomach and upper small intestine. There is a possibility that their administration may cause an immune response in the treated animal, in addition to the possibility of the evolution of bacteria resistant to virulent phages (Harada et al., 2018; Laird et al., 2021; Zhang et al., 2015).

The therapeutic application of bacteriophages may not completely replace the administration of antibiotics because they are inappropriate in some clinical conditions. However, synergistic approaches between phages and antimicrobial agents can combine the strengths of both treatments. In this sense, as with any therapeutic drug, the ideal circumstances for phage therapy should reconcile the reduction of side

effects and minimize the damage caused to the beneficial microbial community (Kortright et al., 2019).

Conclusion

To isolate *E. coli* lytic bacteriophages from swine feces, four isolates were obtained using *E. coli* ATCC 8739 as a host, and two isolates for each of the K12 strains MG1655 and DH5α, all isolates from fecal samples of piglets and sows. The isolation of lytic bacteriophages with different strains of *E. coli* as hosts brings the prospect of using these isolates after *in vitro* assays to evaluate the ability to infect and lyse multidrug-resistant strains of pathogenic *E. coli* associated with swine diseases. The lytic capacity of the isolates against multidrug-resistant strains may represent an advance in the biosafety of *in vivo* assays as a proposal for phagotherapy since these bacteriophages can be replicated in the non-pathogenic strains used in this work (ATCC 8739, K12 MG1655, and DH5α).

From the initial results obtained in this work, new studies can be developed to test strains of *E. coli* originating from the production chain of pigs known to be multi-resistant, in addition to *in vivo* assays to treat diarrhea in piglets in the post-weaning phase, among other applications. In this sense, studies of alternatives to conventional antibiotics represent essential strategies for effective treatment in the face of the worrying scenario of the evolution of multidrug-resistant strains associated with the main bacterial diseases.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethics Statement

All the experimental procedures using animals were previously approved by the Ethics Committee on Animal Use of the Catarinense Federal Institute, under protocol Number 435/2023/CEUA-IFC.

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