Plaque morphology of lytic bacteriophages infecting *Escherichia coli* isolated from sewage and river samples of Curitiba, Brazil

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Morfologia de placa de bacteriófagos líticos infectando Escherichia coli *isolada de amostras de esgoto e rios de Curitiba, Brasil*

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Abstract

Bacteriophages, natural viruses targeting bacteria, present a promising solution to the persistent challenge of antimicrobial-resistant bacteria in human and veterinary medicine. Despite a century of efforts, this issue remains unresolved. Regardless, this paper focused on illustrating the plaque morphologies that were disclosed by bacteriophages isolated from rivers and urban sewage from Curitiba, Paraná, southern Brazil. Laboratory analysis unveiled distinctive features in the macromorphology of *Escherichia coli* phage plaques, representing various morphotypes. Obtaining this data is a relatively quick and inexpensive process that highlights the significance of primary screening for the successful recovery of phages to be further used in combating *E. coli* through phage technology.

Keywords: Antimicrobials. Bacteriophage. *Escherichia coli*. River. Sewage.

Resumo

Os bacteriófagos, vírus naturais que atacam bactérias, apresentam uma solução promissora para o desafio persistente das bactérias resistentes aos antimicrobianos na medicina humana e veterinária. Apesar de um século de esforços, esta questão permanece sem solução. Todavia, este artigo se concentrou em ilustrar as morfologias de placas reveladas por bacteriófagos isolados de rios e esgotos urbanos em Curitiba, Paraná, sul do Brasil. A análise laboratorial revelou características distintivas na macromorfologia das placas de fagos de Escherichia coli*, representando vários morfotipos. A obtenção destes dados é um processo relativamente rápido e barato, que destaca a importância da triagem primária para a recuperação bem-sucedida de fagos para serem posteriormente utilizados no combate à* E. coli *através da tecnologia de fagos.*

Palavras-chave: *Antimicrobiano. Bacteriófago.* Escherichia coli. *Rio. Esgoto.*

Introduction

Bacteriophages, commonly referred to as phages, are viruses that exclusively infect and replicate within bacterial cells. Widely acknowledged as the most prevalent biological entities on Earth (Guo et al., 2021; Kasman and Porter, 2022), these viruses co-exist with their bacterial hosts, adapting to diverse environments such as soil, freshwater, oceans, sewage water, and hospital settings (Huang et al., 2018). Remarkably, bacteriophages also inhabit the gastrointestinal tracts of humans and animals (Zalewska-Piątek and Piątek, 2020), representing natural components of the mammalian ecosystem (Mills et al., 2013; Duerkop, 2018).

Escherichia coli, a well-known pathogen, poses a significant threat by causing diseases in animals and displaying resistance to multiple antibiotics (Brennan et al., 2016; Harada et al., 2016). It contributes to bovine mastitis (Sun et al., 2021) and calf diarrhea (Feuerstein et al., 2021), resulting in substantial losses for the dairy industry (Halasa et al., 2007; Olde et al., 2008; Blum et al., 2017; Lippolis et al., 2017; Sharifi et al., 2018). Furthermore, antibiotic-resistant strains of *E. coli* are pervasive in livestock manure, posing risks to human, animal, and environmental health (Gros et al., 2012; Manyi-Loh et al., 2018).

Conventional manure treatment processes inadequately remove antibiotic resistance genes, leading to their dissemination in soil and water (Resende et al., 2020). Once exposed to resistant bacteria, humans struggle to combat subsequent clinical infections unresponsive to standard antibiotics (Haulisah et al., 2021; Ma et al., 2021; Zalewska et al., 2021). Given the ongoing challenge of mitigating bacterial resistance in antimicrobial therapy, there is a growing interest in exploring the antimicrobial properties of bacteriophages (Górski et al., 2018; Zalewska, 2023). The specificity for targeted bacterial strains and non-toxic characteristics (Sillankorva et al., 2012) has reignited enthusiasm for bacteriophage therapy, showcasing its potential in controlling various bacterial infections (Hibstu, 2022). Utilizing the pathogenic-specific antimicrobial potential of the bacteriophage therapy holds promise for reducing the prevalence of antibiotic-resistant bacteria, a critical goal in both medical and veterinary fields (Mahony et al., 2011; Saini et al., 2013; Tsonos et al., 2014; Anyaegbunam et al., 2022; Hitchcock et al., 2023). In this scenario, lytic phages emerge as an attractive alternative to combat antimicrobial-resistant pathogens (Montso et al., 2019).

For at least four decades, sewage water has been recognized as a valuable source of bacteriophages, extensively studied and harvested (Alharbi and Ziadi, 2021). This study aims to elucidate various plaque morphologies exhibited by *E. coli* bacteriophages isolated from sewage. The results contribute to a descriptive model, building upon previous investigations (Shende et al., 2017; Ngu et al., 2020). Ultimately, the findings of this research aim to support the exploration of bacteriophage isolation for biocontrol and therapeutic purposes in addressing bacterial infections.

Material and methods

Bacterial strain/host bacteria

To isolate bacteriophages, the commercial strain *E. coli* ATCC®25922™, sourced from the Xenobiotics Research Unit of the Pontifical Catholic University of Paraná (Brazil), was utilized. The strain was maintained in Tryptic Soy Broth (TSB) supplemented with 20% glycerol at -20 °C for storage and served as an inoculum when required.

Collection and preparation of samples

The phage isolation method was adapted from studies conducted by Smith et al. (1982) and Berchieri Jr et al. (1991), who successfully employed raw sewage water. This modified technique has been widely adopted for phage isolation (Paisano et al., 2004; Fortier and Moineau, 2009; Bonilla et al., 2016).

Water samples were obtained from the Belém River (25°27'00.4"S 49°14'59.8"W – Site I and II), characterized as the most polluted river in Curitiba, Paraná, Brazil, and classified as sewage. Additional samplings were conducted at Vila Formosa River (25°31'04.4"S 49°19'18.2"W) and Barigui River (25°25'53.1"S 49°18'47.9"W), both also located in Curitiba. Post-collection, the samples were immediately placed in an ice bath and transported to the laboratory.

Subsequently, 300 mL of river water was combined with 100 mL each of Luria-Bertani broth and TSB broth. The mixture was then incubated in an incubator at 37 °C for one hour. Following this, 0.5 mL of a suspension of the *E. coli* ATCC®25922™ strain, prepared in TSB, was added. After 16 hours of incubation at 37 °C, 500 μL of chloroform were added for every 10 mL of the culture. The mixtures were shaken to put cells and solvent in contact. This mixture underwent centrifugation for 10 minutes at 7,000 rpm. To the resulting supernatants, 10 μL of chloroform were added, and the crude lysate, containing the phages, was stored at 4 °C. Plaque formation assays were carried out spreading 100 μL of 108 cfu mL^{-1} of 24h-old bacterial cells onto TSB agar agar in standard disposable Petri dishes (90 × 15 mm). After 24 to 48h of phage/bacterium contact, macroscopically visible lytic plaques had their diameters measured (N®S, E®W) using a digital caliper.

Results and discussion

Six distinct bacteriophage plaque morphotypes against *E. coli* were identified, as depicted in Figure 1. Type #1 displayed large plaques with halos; type #2 exhibited large plaques without halos; type #3 presented large plaques with clear centers and halos; type #4 showcased diffused plaques with clear centers and turbid edges; type #5 manifested uncountable plaques resembling rainfall drops; and type #6 featured clear small-sized plaques with pin-headed shapes. Samples from the Belém River (Site #I) showed similar plaque morphology, characterized by large and nitid lytic zones with and without halos, consistent with type #1 and #2 morphology. Conversely, samples from the Belém River (Site #II) displayed diffused large plaques with clear centers and turbid edges (type #3 and #4). Plaques from the Vila Formosa and Barigui Rivers exhibited two dis-tinct morphologies: uncountable phage lysis (type #5) and clear, small-sized, pin-headed plaque shapes (type #6).

Previous studies have successfully isolated phages from sewage and urban rivers (Aghaee et al., 2021; Menon et al., 2021; Ballesté et al., 2022). This study focused on the preliminary assessment of plaque morphological characterization of bacteriophages isolated from urban rivers which receive in natura sewage. Such an environment was chosen as the primary source for obtaining phages due to its richness, availability, and ease of depicting plaque characterization (Shukla and Hirpurkar, 2011). Plaque morphological characterization is integral to the initial screening process in bacteriophage studies, providing visual evidence of their existence and lytic capability against targeted bacteria. This serves as a key process for further studies involving phage collection, offering a fast and cost-effective method to illustrate the biodiversity of bacteriophages isolated from a single sample.

Moreover, potential biotechnological and medical applications of similar studies' results have emerged, allowing the use of detected bacteriophages in constructing new tools for genetic engineering and bacteriophage therapy (Jurczak-Kurek et al., 2016). The observed variations in plaque features for *E. coli* bacteriophages align with previous reports (Shende et al., 2017; Ngu et al., 2020), potentially indicating different phages (Tiwari et al., 2010; Ghasemian et al., 2017). Factors such as varying growth medium compositions can alter bacterial cell wall structure and phage receptor availability.

Despite differences in bacteriophage halo and morphology sizes, it has been reported that there is no direct relationship between the size of the phage lysis plaque and its effectiveness (Ghasemian et al., 2017). Therefore, the lytic effect of bacteriophages with larger plaque lysis cannot be conclusively deemed superior to phages with smaller sizes.

When analyzing bacteriophages in terms of plaque morphology and characterization, they can exhibit vast diversity in size, morphology, and genomic organization (Hatfull and Hendrix, 2011; Doore and Fane, 2016; Simmonds and Aiewsakun, 2018). Specific tests such as polymerase chain reaction and transmission electron microscopy should be performed for a deeper understanding of their nature, identifying and classifying them according to the guidelines of the International Committee on Taxonomy of Viruses (Fauquet et al., 2005).

The information and characteristics obtained during the initial phase of research are valuable for further studies, emphasizing the importance of primary screening for the successful recovery of selected phages for use in medical practice. Additionally, exploring relationships with other literature can lead to the application of phages for the control and elimination of animal and human diseases.

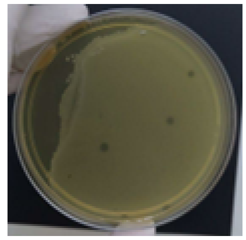
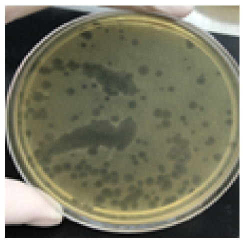
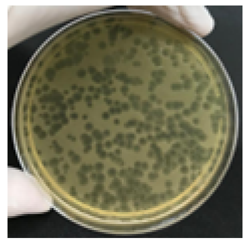
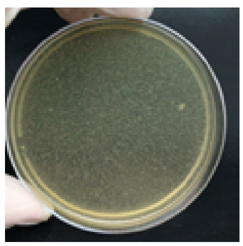
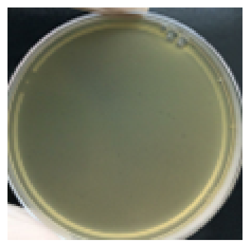
Origin - Source of sample	Lowest dilution A	Highest dilution B	Observed macromorphology (A)	Observed macromorphology (B)
Belém River (Site #I)			Large plaques (type #1) with the presence of halos[1,2]	Large plaques (type #2) without the presence of halos[1,2]
Belém River (Site #II)			Large plaques (type #3), with clear centers	Diffused large plaques (type #4), with clear centers and turbid edges[1]
Barigui River + Vila Formosa River			Uncountable plaques (type #5), resembling rainfall drops	Clear small-sized, pin--headed shape[1,2] (type #6)

Figure 1 - Source, image and macroscopic description of *Escherichia coli* bacteriophages collected from sewage and river samples in Curitiba, Brasil. Following descriptions from [1]Shende et al., 2017, and [2]Ngu et al., 2020, with modifications.

Conclusion

The morphological plaque analysis conducted on a diverse collection of bacteriophages sourced from sewage and river environments has not only provided an array of phage samples but has done so through a cost-effective prospective methodology. The observed six distinct morphotypes underscore the rich diversity within the collected phages, rendering them particularly attractive for potential biotechnological applications.

The variability in plaque formation exhibited by these investigated phages holds significant promise, suggesting adaptability and versatility in targeting various bacterial strains. This adaptability, coupled with the ability to propagate on wild strains of bacteria, underscores the potential efficacy of these bacteriophages in phage therapy. The implications of this research extend beyond mere characterization, presenting a compelling case for the practical utilization of these phages in addressing challenges within biotechnological and therapeutic realms. This study thus not only enriches our understanding of bacteriophage diversity but also substantiates their tangible potential for transformative applications in the fields of medicine and biotechnology.

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