Original Research Article

Isolation and Phenotypic Characterization of Lytic Bacteriophage with Activity Against *Pseudomonas aeruginosa*

ABSTRACT

Aims: To perform the isolation and phenotypic characterization of bacteriophage with lytic activity against *Pseudomonas aeruginosa* from untreated water. To demonstrate that this type of viral agent can be isolated from the environment and used for the biocontrol of resistant bacterial types, such as *Pseudomonas aeruginosa*.

Study design: This study was an experimental study.

Place and Duration of Study:~~The study was carried out at,~~ The study was conducted at, Bacteriology and Mycology Laboratory in the Veterinary Hospital at the School of Agricultural Sciences,Innovation and Business of the University of Passo Fundo (ESAN/UPF) and Center for Diagnosis and Research in Animal Health of the University of Passo Fundo (CDSA/UPF), between April 2022 and June 2022.

Methodology: Samples of untreated water were inoculated with the host bacterium strain *Pseudomonas aeruginosa* ATCC 27853 in an enriched media~~.~~ After the incubation period in ~~an enriched medium~~, ~~we obtained~~ a phage filtrate was obtained by ~~centrifuge~~ centrifugation followed by~~and~~ filtration ~~steps~~. We verified the presence of bacteriophages using spot test and we ~~carry~~ carried out its purification by the method of sterile toothpick plate transfer on bacterial overlay semi-solid agar ~~semi solid~~. Amplification was performed using an SM buffer elution procedure to produce a stock of viral material. We performed titration and phenotypic characterization regarding the lysis spectrum and efficiency of phage infection in the host, ~~through~~ using an assays ~~in Petri dishes~~ with bacterial overlay semi solid agar in Petri dishes.

Results: We managed to isolate a morphologically characterized virulent ~~cycle~~ lytic bacteriophage with approximately 1 mm of diameter, high clarity in the inhibition area, the presence of halo and well-demarcated edges. The bacteriophage, named as Phage UPF_PaBP1, demonstrated the infection capacity of the target bacteria in all tested dilutions~~,~~ and a stock preparation with a ~~titration titre~~measured at~~of~~ 6.5 x 10□ PFU/ml was obtained for future use.

Conclusion: The isolated phage showed strong lytic activity against the bacterial host, a finding that nourishes our expectations regarding the use of this phage as a biocontrol agent and phage therapy.

Keywords:Bacteriophages; Pseudomonas aeruginosa; biocontrol;antimicrobials, phage therapy; lytic phage.

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1. INTRODUCTION

The bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*) makes ~~up~~ a group of ~~pathogens~~ multidrug resistant bacteria named under the acronym “ESKAPE” along with five other bacteria: *Enterococcus faecium,Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter spp.* The pathogens of the ESKAPE group are ~~closely related to~~associated with an increase in mortality rates and ~~an increase in~~treatment costs related to health (1,2,3).

The World Health Organization (WHO) classifies this bacterium as a pathogen of critical priority, which is the highest priority among the three urgency categories for development of new antibiotics (critical, high and medium). High resistance rates are reported worldwide (2, 4, 5). The ability of *P. aeruginosa* to induce a broad spectrum of infections, which are usually difficult to treat, is directly related to the fact that this pathogen has several resistance mechanisms. In addition, inappropriate use of antimicrobials is also an aggravating factor for the developm~~e~~ent of antimicrobial resistance among pathogens, further limiting the arsenal of viable therapeutic options (6,7,8).

Faced with this worrying scenario, great efforts have been made to ~~re~~search new therapeutic agents that can replace conventional antimicrobials or, at least, reduce the development of antimicrobial resistance (AMR) to them (9, 10, 11). ~~Because~~ Since, bacteriophages (viruses that infect bacteria)~~they~~ are species-specific, they ~~bacteriophages (viruses that infect bacteria)~~ represent an interesting alternative for biocontrol. Also called phages, bacteriophages are recognized as the most abundant biological entity on the planet (12, 13, 14). Phages depend on the presence of the bacterial host for their survival and replication, being able to replicate through two distinct cycles: the lysogenic cycle and the lytic cycle. In the lytic cycle, the phage infects and leads to host cell lysis (cell disruption), rapidly, showing higher replication rates (15, 16).

The administration of phage to lyse a bacterial pathogen that is causing an infection is called phage therapy. The phages listed for therapy are those that have the characteristic of lytic cycle replication (17, 18, 19). The utilization of lytic phages provides not only the eradication of target pathogens due to their bactericidal action, but is also effective in preventing the development of resistance (20, 21). In addition, high phage specificity avoids side effects by not interfering with populations of bacteria that make up the natural microbiota. They also feature multiplicity in place infections, that is, they multiply naturally in the presence of the host (22, 23).

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Bacteriophages can be used in a variety of ~~eradication~~ bacterial eradication strategies. As they are ubiquitous, phages comprise a large repertoire of therapeutic possibilities, with numerous viral entities and phage combinations still unexplored (24, 25, 26). In this context, we propose to isolate bacteriophages from environmental matrices (non-treated effluent and raw lake water) with activity against the bacterium *Pseudomonas aeruginosa*~~, as .~~ T~~t~~he use of phages as a therapeutic tool~~s~~ in the treatment of infections represents an alternative approach to the use of conventional antimicrobials, especially ~~concerning~~ with multidrug-resistant bacteria (17, 18, 19).

2. MATERIAL AND METHODS

We used the methodology of Azeredo (2014) for the isolation and amplification of bacteriophages (27).

2.1 Host Bacteria

For the isolation and characterization of bacteriophages, we used as host bacterium the American Type Culture Collection (ATCC) standard strain 27853 of ~~the~~ *P. aeruginosa*~~bacterium~~, obtained from the bacteriology library of the Laboratory of Bacteriology and Veterinary Mycology of the Veterinary Hospital (HV) of the School of Agricultural Sciences, Innovation, and Business of University of Passo Fundo (ESAN - UPF).

2.1.1 Reactivation, Purity Confirmation and Enrichment

The bacteria were reactivated ~~(~~by inoculating10 µl of the stock containing *P. aeruginosa* ATCC 27853 to 4 ml of ~~broth) in~~ Brain Heart Infusion (BHI), a non-selective enrichment broth~~,~~ and incubated at 37 ± 1 °C for 24 h. Afterward, we performed seeding on cetrimide agar (selective medium), incubated for 24 h at 37 ± 1 ºC and performed the gram staining and biochemical tests to confirm the purity of the isolate.~~.~~ After reactivation and confirmation of purity, we inoculated *P. aeruginosa* ATCC 27853 in 3 ml of Tryptic Soy Broth (TSB) broth and incubated for 37 ± 1 °C overnight for use ~~in~~ iat the later stages. After reactivation of the host bacterium we observed, Gram negative bacilli ; growth and pigment production on selective agar); and biochemical tests according to the characteristics of the bacteria (fermentation of glucose, lactose and negative sucrose; positive catalase; positive oxidase; positive motility). So, we confirmed the purity of the isolate.

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2.1.2 Search for Prophages

To avoid false positive results ~~for~~ due to the presence of lysogenic bacteriophages, the host bacterium (*P. aeruginosa* ATCC 27853) was tested for the presence of prophage in its DNA. We prepared Petri dishes with a thin layer of Trypticase Soy Agar (TSA). The plates with TSA agar were dispensed with 5 ml of Molten Top-Agar (MTA), a~~n~~semi-solid agar ~~semi-solid~~ composed of TSB and agar (15 g of TSB and 3 g of agar in 500 ml of water distilled), previously inoculated with 100 µl of *P. aeruginosa* ATCC 27853. After solidification of the overlay, we pipetted 10 aliquots of 10 µl of the inoculum with the same bacteria ~~as~~ fromthe overnight growth in the TSB~~.overnight and dispensed them onto the plate.~~ We wait for the complete drying of ~~aliquots~~aliquots and the plates were incubated for 24h at 37 ± 1 ºC. After the incubation period plates were read and no areas of lysis were observed, confirming the absence of prophage in the bacteria and suitability for use as a host in the isolation of bacteriophages.

2.2 Source of Bacteriophage Isolation

As a source for the isolation of bacteriophages, we collected samples from two collection points. A sample (200 ml) was collected from the inlet tank of the Effluent Treatment Station (ETE) of the UPF sewage network and the other sample (200 ml) was collected from raw water from a lake, also located in the UPF.

2.2.1 Enrichment and purification of the bacteriophage isolation source

To remove the solids in coarse suspension, we centrifuged 140 ml of each collected sample ~~of isolation sources~~, separately, in 4 vials (35 ml each) in a refrigerated centrifuge at 4ºC ~~programmed with~~at 5000 revolutions per minute (rpm) for 20 minutes. For each of the

samples, we performed a pre-filtration of the supernatant ~~in~~ using a membrane filters of polyethersulfone (PES) of 0.45 µm porosity, for sample clarification, removing ~~solids smaller~~ suspended solids ~~cells~~ and most prokaryotic cells. We collected the respective filtrates in sterile flasks and ~~later we added~~transferred 100 ml of the filtrate in flasks containing 100 ml of double strength TSB~~in double concentration~~. We immediately added 50 µl of the ~~bacteria~~ host bacteria *P. aeruginosa* ATCC 27853 to the flask, from ~~of~~ the TSB overnight ~~and~~ incubated under agitation at 120 rpm for 24h at 37 ± 1 ºC ~~for removal of aliquots,~~ and ~~we re~~incubated the flasks for up to 48h. After th~~ese~~ incubation period~~s~~, ~~we~~ performed ~~a new~~ centrifugation was done at 4 ºC ~~with a rotation of~~at 5000 rpm for 20 minutes ~~and~~ followed by two steps of filtration. T~~:~~ the first, with PES membrane filters with porosity of 0.45 µm; and the second ~~in~~ using PES membrane filters with porosity of 0.22 µm, for removal of remnants bacteria ~~removal~~from the sample~~sample remnants~~.

2.3 Isolation of bacteriophages

2.3.1 Verification of the presence of bacteriophages in samples

To confirm the presence of bacteriophages from the samples, we performed a spot test, ~~*that is*~~*i.e.*~~,~~, a plate assay ~~forming~~ using a~~n~~semi-solid agar overlaid with~~y of agar semi-solid containing~~*P. aeruginosa* ATCC 27853. For this, Petri dishes containing TSA agar were prepared with a bacterial overlay (5 ml of MTA inoculated with 100 µl of the bacteria. The plate was inoculated with ~~and inoculation of~~ the 10 µl of the filtratewith the aid of a micropipette.We deposited 10 drops (spot) of 10 of the filtrate on one plate.~~,~~T~~t~~o verify the formation of lytic areas in the bacterial overla.y~~. For this, Petri dishes containing TSA agar were prepared with a bacterial overlay (5 ml of MTA inoculated with 100 µl of the bacteria) and, with the aid of a micropipette, we deposited 10 drops (spot) of 10 µl of the filtrate on them. We~~ the plate was incubated ~~the plate~~ at 37 ± 1 ºC after ~~total~~ complete drying of the drops~~., and performed T~~the visual inspection was performed ~~within~~ after 6, 18 and 24 hours, to check the presence or absence of clear areas (bacterial lysis), which indicate the presence of bacteriophages.

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2.3.2 Plaque purification

After confirming the presence of lysis zones, procedures were initiated to isolate and purify bacteriophages from the samples. For that, we use two different methods. In one of the methods, from the previous step (spot test), we collected the center of the lysis zones with the aid of a sterile toothpick and inoculated by stinging in semi-solid agar with a bacterial overlay of *P. aeruginosa* ATCC 27853. We spread the inoculum on the Petri dish with the aid of a sterile strips of paper~~,~~ with changing the strip at each line, and playing the previous track. The second methodology was performed using directly the filtrate of the sample, soaked in a strip of sterile paper, striated over the entire area of the semi-solid agar with a bacterial overlay of *P. aeruginosa* ATCC 27853, changing the strip every line and playing the previous track. This method is useful when it is not possible to proceed immediately using the spot test board.

The plates of both techniques were incubated (37 ± 1 ºC for 24 hours). P~~On the boards Petri~~etri plates with lysis areas~~, we~~ were selected ~~phage plates~~ considering the phenotypic characteristics of the clear area (plaque) ~~visible~~,in terms of ~~such as~~ size (in mm), halo (presence or absence) and appearance (clear or cloudy). With the aid of a sterile wooden toothpick, we transferred the selected phages again individually to~~for~~ a new TSA plate with bacterial overlay, with the aid of a toothpick and sterile paper, as previously described, and incubated at 37 ± 1 ºC for 24 hours. That procedure was repeated until all bacteriophage plaques were uniform, to guarantee the purity of the isolated phage.

2.4 Amplification of bacteriophages

We performed amplification to create a stock solution of the isolated bacteriophage, producing a viable amount for use in future trials. The production technique used was stock preparation from a single lysis plate and elution. We selected three phage plaques from the isolation step, which were collected with a sterile toothpick stick and inoculated by sting, individually, in ten plates of TSA, previously prepared with a bacterial overlay of *P. aeruginosa* ATCC 27853. Sterile paper strips were used to spread the phages throughout the petri dish, this time without changing the strip of paper to each line, to guarantee maximum replication of bacteriophages. We incubated at 37± 1 °C for 18-24 h and, after the incubation period, 5 ml of SM buffer was added to each plate (5.8 g NaCl; 2 g MgSO4; 50 ml of Tris/HCl ph 8.8; 1 l of distilled water) a solution buffer used in bacteriophage storage. We incubated the plates containing SM buffer under stirring at 50 rpm at 4°C for 5 h. We collected the SM Buffer for sterile vials, centrifuged at 4 °C with 5000 rpm for 20 minutes, filtered the supernatant and stored it in sterile bottles.

2.5 Titration of bacteriophages in UFP

For the titration of bacteriophages in Plaque Forming Units (PFU) we performed serial dilution from the stock solution. For the dilution, we prepared homogenization of 100 µl of phage stock in 900 µl of SM buffer (10^{-1} dilution) in sterile Eppendorf, and so on successively, up to 10^{-10} dilutions. On TSA plates we added 5 ml of MTA and 100 µl of *P. aeruginosa* ATCC 27853 grown overnight in TSB. In addition to these, we added 100 µl of each dilution of bacteriophages, individually. After drying, the plates were incubated at 37 ± 1 ºC for 24 h, and we performed the counting of lysis plates for titration in Forming Units of Plates per milliliter (PFU/ml), according to the equation:

$$Phage\ titre\ (PFU/ml) = \frac{Number\ of\ lysis\ plates \times Dilution\ factor}{Phage\ sample\ volume\ (ml).}$$

2.6 Phenotypic characterization

2.6.1 Lytic Spectrum

The characterization of the lytic spectrum of the isolated bacteriophage was carried out from the visual analysis ~~visual~~ of the morphology of the lytic plaques obtained in the phage isolation step. The areas of lysis were described regarding the size of the lysis plaques (in mm), halo (presence or absence), appearance (clear or cloudy) and edge demarcation characteristic (well-demarcated or poorly demarcated).

2.6.2 Infection Efficiency

The infection efficiency assay is intended to verify the amount of phage capable of infecting (lyse) the host bacterium (*P. aeruginosa* ATCC 27853), using different dilutions performed from the stock solution. Petri dishes with TSA agar with overlay bacteria were divided into eight equal parts. Each quadrant received an aliquot of 10 µl of each dilution (10^{-1} ~~at~~ to 10^{-8}), incubate for 24 h at 37 ± 1 ºC and check for the presence or absence of lysis in each inoculated dilution. The infection efficiency rating is instituted according to the magnitude of the lysis verified on visual inspection, in the scores: are high when areas of total lysis were verified, moderate when verified the presence of plaques individual, low when there are few individual plates and null when there are no areas whitening (43)

3. RESULTS

3.1 Verification of the presence of bacteriophages in the sample, isolation of bacteriophages and purification of phage plaques

The assay to verify the presence of bacteriophages was positive only for the sample collected from the effluent and was negative for the sample collected from lake, after incubations of 24 h and 48 h. The readings taken after 6 hours of incubation did not show lytic areas ~~lithic~~. With 18 h of incubation, we verified the appearance of lysis areas in all inoculum points, only from the effluent filtrate. 24-hour reading did not show a change from the one performed at 18 hours. After thirteen purification steps, performing the selection of the phage plate, transferring with a toothpick and spreading with the use of paper strips, it was possible to obtain lysis plates with a single morphological pattern. The isolated bacteriophage was named *Pseudomonas aeruginosa* Phage UPF_PaBP1.

3.2 Amplification and titration in PFU/ml of the isolated bacteriophage

We produced approximately 50 ml of bacteriophage stock solution in the SM buffer in the amplification step, stored in sterile flasks at -80 ºC so that it can be used in trials and future work. Aliquots were kept at 4 °C to continue the tests of this experiment. The stock solution had a titration of 6.5 x 10□ PFU/ml.

3.3 Phenotypic characterization

3.3.1 ~~Lithic~~ Lytic Spectrum

Phage UPF_PaBP1 phage lysis plates showed the following characteristics morphological features: approximate size of 1mm; the presence of a halo (ranging from 0.5 – 1.5 mm, depending on incubation time and phage titer); clear appearance, demonstrating 100% of transparency; and regular edges of well-demarcated feature. Plates incubated by a time equal to or greater than 24 hours and a lower phage titer in the dilution, showed larger halos than those incubated for up to 18 hours and with a higher titer of phage on the dilution. The characteristics of the bacteriophage lytic plaque can be seen in figure 1 and halo size variation can be seen in figure 2.

Fig. 1. Morphological characteristics of the lysis plaque formed by the UPF_PaBP1.

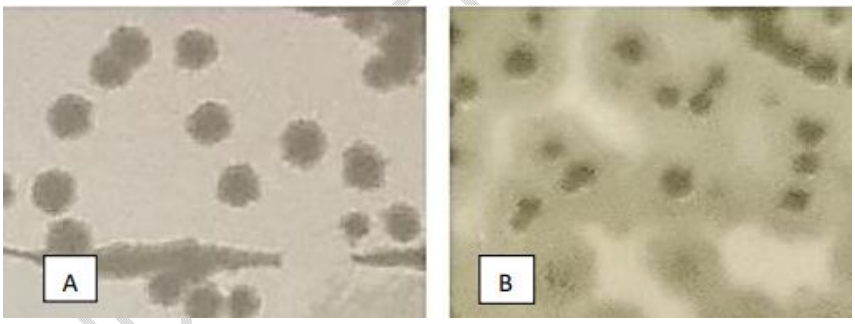

Fig. 2. Halo size variation. 2A. Halo ≤ 0.5 mm. 2B. Halo ≥ 1.5 mm.

3.3.2 Infection efficiency

In the plate assay to evaluate the infection efficiency of Phage UPF_PaBP1, we observed plaque formation at all dilutions (figure 3). From 10^{-1} dilution to 10^{-3}, we classified the phage's ability to infect as high, as it presents 100% lysis in the area where the bacteriophage solution was deposited, also advancing with 100% lysis in regions beyond the inoculation area. The 10^{-4} dilution was classified as moderately high, as we visualized an area with 100% lysis and an area that advanced beyond the inoculation with the formation of isolated phage plaques. We classify the 10^{-5} dilution as moderate-low, as we only visualized the formation of isolated phage plaques in the area and beyond the aliquot

deposit area. Dilutions 10^{-6}, 10^{-7} and 10^{-8} were classified as low-null, by the verification of a few phage plaques in the aliquot deposit area.

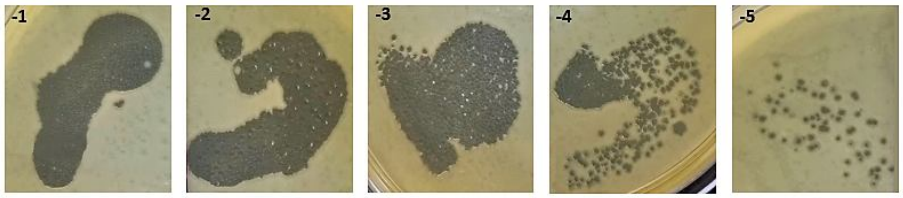

Fig 3. Bacteriophage infection efficiency at different dilutions.

4. DISCUSSION

Bacteriophages are the most abundant biological entities on the planet, being more numerous than the bacteria themselves. It is estimated that there are about 10^{32} bacteriophages on the Earth, of varied morphological types. Phages co-exist and co-evolve with their host bacteria for about 3 to 4 billion years. We propose to isolate bacteriophages with lytic potential against *P. aeruginosa* due to the ubiquitous character and pathogenicity of this bacterium (28, 29, 30).

P. aeruginosa causes a wide range of infections in humans, animals and plants. Immunocompromised individuals are among the most susceptible to *P. aeruginosa* infection. Among these, are those affected by burns, those with cystic fibrosis (CF), cancer and diabetes mellitus. Infections caused by *P. aeruginosa* include endocarditis, pneumonia, meningitis, osteomyelitis, urinary tract infections and sepsis, being an important cause of high morbidity and mortality in a hospital environment. Immunocompetent individuals also can be affected by infections of *P. aeruginosa*, one of the main causes of folliculitis, otitis and keratitis acquired from exposure to recreational waters (6, 31). The presence of *P. aeruginosa* in production animals and the food industry represents a risk to consumers of animal products (such as meat, eggs, milk and dairy products), compromising food quality and biosecurity (32,33). In addition to animal production, companion animals are also affected by *P. aeruginosa* infections, usually from difficult treatment (given the ineffectiveness of conventional antimicrobials against this pathogen) that upset animal welfare (34).

For the isolation of bacteriophages, we selected two collection sources: effluent water untreated and raw lake water, as bacteriophages are often isolated from aquatic environments. Phages can also be isolated from sites in which their hosts, which varies depending on the bacterial species (29, 30, 31). *P. aeruginosa* is a ubiquitous free-living bacterium, often found in natural waters (such as rivers and lakes). Its presence occurs both in waters rich in nutrients, such as sewage, as well as in nutrient-poor waters. The presence of *P. aeruginosa* in drinking water is less recurrent and is generally associated with the formation of biofilms in plumbing, for example. Despite being considered ubiquitous, *P. aeruginosa* is much more abundant in environments with intense human contact and less in untouched environments, being thus, it can be said that *P. aeruginosa* prevails in places correlated to human activity (31, 35).

In our work, we were able to isolate a bacteriophage from the sample collected from effluent. We ~~did~~ could not ~~verify~~ demonstrate the presence of bacteriophages ~~in~~ in the sample collected ~~in~~ from the lake. As both samples were ~~manipulated~~ processed concomitantly, using the same conditions of processing and storage, we ruled out the possibility of technical failure in the procedure. We believe that this finding is due to the fact already described in the literature, that lytic phages dominate waters rich in nutrients (effluent, as an example) and waters poor in nutrients are dominated by lysogenic phages (36). The verification of the presence of lysogenic phages in a sample is not always possible from the plaque assay, as the formation of lysis plaques occurs much less markedly than for lytic phages or may not occur at all. For confirmation of the presence of plate-tempered phages, it is indicated that the host cells are exposed to stressors, such as radiation and antibiotics, to induce the release of virions (37).

The bacteriophage isolated from the effluent sample showed a characteristic of lytic replication, due to the formation of clear areas on the agar, called “plaques” of bacteriophage. Plaques are described as clear circular areas of reduced turbidity, present in bacterial overlays, which form as a result of bacterial lysis induced by virion propagation (38). Lytic replication cycle bacteriophages have plates with typically clear morphology. In contrast, cloudy plaques may indicate the presence of lysogenic (tempered) bacteriophages in the sample. In addition, the high turbidity on plaques can also be the result of old bacterial cultures (39). The bacteriophage isolated in this study, however, showed 100% clarity in the bacterial overlay, indicating an occurrence of lytic bacteriophage.

We obtained bacteriophage plaques approximately 1 mm in diameter. Plates phages of different sizes and morphologies are frequently described in the literature. The plaque evaluation is important because they represent an indicator of the viability of the phage. Plates of 1 mm in diameter are recurrently classified as small plates. Bacteriophage plaques ≥ 4 mm in diameter are considered large plaques. It is important to emphasize that the size of the bacteriophage plaque is not related to greater or lesser capacity for infection, but to the structural characteristics of the bacteriophages. Bacteriophages with a large capsid (head) tend to form lytic plaques smaller, while bacteriophages with smaller heads tend to form larger lytic plaques. We believe that the bacteriophage isolated in this work belongs to the first group.(39).

The isolated bacteriophage showed high clarity in the area of phage plaque formation and managed to infect its host in all dilutions, with effective infection high, visually, up to 10^{-4} dilution. In addition, the presence of a halo was also observed. Halo is the description for the region that forms around a lithic slab that is less turbid than the bacterial overlay and more turbid than the lytic plaque area (38). The halos are formed by the action of depolymerases, enzymes that degrade important components of the biofilm matrix and capsule constituents. Depolymerase is a phage by-product that has a bactericidal effect and can be used in antibacterial strategies. The size of the halo formed by the action of depolymerase tends to increase with incubation time (40, 41, 42). We noticed this tendency of halo formation and increase in plates incubated for a long time equal to or greater than 24 hours.

We believe that the relative titer phage:bacterium also exerted an influence on the size of the halo, because in plates with a lower titer of phage in relation to the host, we noticed the formation of larger halos. It is worth noting that depolymerases degrade bacterial

exopolysaccharides when there is no further propagation of the bacteriophage (42). Therefore, we hypothesized that the higher the titer of bacteria, the sooner the propagation of phages ceases and the action of depolymerases begins, which would explain the higher halo the lower the phage titer, since in these plaques the depolymerases would act, consequently, for a longer time against bacterial cells. This hypothesis, however, needs confirmation through testing and further investigation. In any case, verifying the presence of the depolymerase enzyme is a finding that further reinforces our expectations about the use of this bacteriophage as an antimicrobial agent.

5. CONCLUSION

A bacteriophage with action against the bacterium *P. aeruginosa*, which is named *Pseudomonas aeruginosa* Phage UPF_PaBP1, was successfully isolated in this study. Through the trials performed in vitro, we found that Phage UPF_PaBP1 is a virulent bacteriophage and showed strong lytic activity against the bacterial host used in the isolation (*P. aeruginosa* ATCC 27853). These findings nourish our expectations regarding the use of this phage as a biocontrol agent in vivo infections, environment and in food. With the phenotypic and biological characteristics of the phage described, we can explore other properties of this bacteriophage and use it in countless assay futures.

REFERENCES

1. Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, Paterson DL, Walker MJ. Antimicrobial Resistance in ESKAPE Pathogens. Clinical Microbiology Reviews May 2020, 33 (3) e00181-19; DOI: 10.1128/CMR.00181-19.
2. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. Front Microbiol. 2019 Apr 1;10:539. doi: 10.3389/fmicb.2019.00539.
3. Founou RC, Founou LL, Essack SY. (2017). Clinical and economic impact of antibiotic resistance in developing countries: a systematic review and meta-analysis. PLoS ONE 12:e0189621. 10.1371/journal.pone.0189621.
4. Ponce de Leon A, Merchant S, Raman G, Avendano E, Chan J, Tepichin Hernandez G, Sarpong E. Pseudomonas infections among hospitalized adults in Latin America: a systematic review and meta-analysis. BMC Infect Dis. 2020 Mar 27;20(1):250. doi: 10.1186/s12879-020-04973-0.
5. Sader HS, Flamm RK, Jones RN. Frequency of occurrence and antimicrobial susceptibility of Gram-negative bacteremia isolates in patients with urinary tract infection: results from United States and European hospitals (2009-2011). J Chemother. 2014 Jun;26(3):133-8. doi: 10.1179/1973947813Y.0000000121. Epub 2013 Dec 6..
6. Wilson MG, Pandey S. Pseudomonas Aeruginosa. 2020 Sep 10. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan–. PMID: 32491763.
7. Morel CM, Lindahl O, Harbarth S. Industry incentives and antibiotic resistance: an introduction to the antibiotic susceptibility bonus. J Antibiot. 2020. https://doi.org/10.1038/s41429-020-0300-y.
8. Botelho J, Grosso F, Peixe L. Antibiotic resistance in Pseudomonas aeruginosa - Mechanisms, epidemiology and evolution. Drug Resist Updat. 2019 May;44:100640. doi: 10.1016/j.drup.2019.07.002. Epub 2019 Jul 19.

9. Raman G, Avendano E, Chan J, et al. Risk factors for hospitalized patients with resistant or multidrug-resistant Pseudomonas aeruginosa infections: a systematic review and metaanalysis. Antimicrob Resist Infect Control 7, 79 (2018).
10. Ma YX, Wang CY, Li YY, Li J, Wan QQ, Chen JH, Tay FR, Niu LN. Considerations and Caveats in Combating ESKAPE Pathogens against Nosocomial Infections. Adv Sci (Weinh). 2019 Dec 5;7(1):1901872. doi: 10.1002/advs.201901872. Erratum in: Adv Sci (Weinh). 2020 Apr 22;7(8)
11. Santaniello A, Sansone M, Fioretti A, Menna LF. Systematic Review and MetaAnalysis of the Occurrence of ESKAPE Bacteria Group in Dogs, and the Related Zoonotic Risk in Animal-Assisted Therapy, and in Animal-Assisted Activity in the Health Context. Int J Environ Res Public Health. 2020 May 8;17(9):3278. doi: 10.3390/ijerph17093278.
12. Ferriol-González C, Domingo-Calap P. Phages for Biofilm Removal. Antibiotics 2020, 9, 268.
13. Al-Ishaq RK, Skariah S, Büsselberg D. Bacteriophage Treatment: Critical Evaluation of Its Application on World Health Organization Priority Pathogens. Viruses. 2020 Dec 30;13(1):51. doi: 10.3390/v13010051. 44
14. Furfaro LL, Chang BJ, Payne MS. Applications for bacteriophage therapy during pregnancy and the perinatal periodFrontiers in Microbiology, 2018.
15. Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage 21 2011 , 1 , 31–45.
16. Tortora GJ. Microbiologia [recurso eletrônico] / Gerard J. Tortora, Berdell R. Funke, Christine L. Case, 10. ed. – Dados eletrônicos. – Porto Alegre : Artmed, 2012.
17. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon ST. Phage therapy in clinical practice: treatment of human infections. Curr Pharm Biotechnol. 2010 Jan;11(1):69-86. doi: 10.2174/138920110790725401.
18. De Kraker ME, Stewardson AJ, Harbarth S. Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? PLoS Med. 2016 , 13 , e1002184.
19. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011;1:66–85
20. Tinoco JM, Buttaro B, Zhang H, Liss N, Sassone L, Stevens R. Effect of a genetically engineered bacteriophage on Enterococcus faecalis biofilms. Arch. Oral Biol. 2016;71:80– 86. doi: 10.1016/j.archoralbio.2016.07.001.
21. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. Evol. Med. Public Health.2018,60– 66.
22. Penadés JR, Chen J, Quiles-Puchalt N, Carpena N, Novick RP. Bacteriophagemediated spread of bacterial virulence genes. Curr. Opin. Microbiol. 2015;23:171–178. doi: 10.1016/j.mib.2014.11.019.
23. Lin DM, Koskella B, Lin HC. Phage therapy: An alternative to antibiotics in the age of multidrug resistance. World J Gastrointest Pharmacol Ther. 2017 Aug 6;8(3):162- 173. doi: 10.4292/wjgpt.v8.i3.162.
24. Motlagh AM, Bhattacharjee AS, Goel R. Biofilm control with natural and geneticallymodified phages. World J. Microbiol. Biotechnol. 2016, 32, 67.
25. Torres-Barceló C, Hochberg ME. Evolutionary rationale for phages as complements of antibiotics. Trends Microbiol. 2016, 24, 249–256.
26. Tagliaferri TL, Jansen M, Horz HP. Fighting Pathogenic Bacteria on Two Fronts: Phages and Antibiotics as Combined Strategy. Front Cell Infect Microbiol. 2019;9:22. Published 2019 Feb 18. doi:10.3389/fcimb.2019.00022 22.
27. Azeredo J. Pseudomonas Bacteriophage Isolation and Production. In: Alain Filloux and Juan-Luis Ramos (eds.), Pseudomonas Methods and Protocols: Methods in Molecular Biology, vol. 1149, DOI 10.1007/978-1-4939-0473-0_4, © Springer Science+Business Media New York 2014. p. (23) - (32).

28. Huang C, Shi J, Ma W, Li Z, Wang J, Li J, et al. Isolamento, caracterização e aplicação de um novo bacteriófago específico de Salmonella em diferentes matrizes alimentares. Food Res Int. (2018) 111:631-41. doi: 10.1016/j.foodres.2018.05.071.
29. Brussow H, Kutter E. Phage ecology. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: biology and applications. Boca Raton, FL: CRC Press; 2005. p. 129–63.
30. Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. International Journal of Antimicrobial Agents, v. 30, p. 118-128, 2007.
31. Mena KD, Gerba CP. Risk assessment of Pseudomonas aeruginosa in water. Rev Environ Contam Toxicol. 2009;201:71-115. doi: 10.1007/978-1-4419-0032-6_3.
32. Maia AA, et al. Resistência antimicrobiana de Pseudomonas aeruginosa isolados de pescado e de cortes e de miúdos de frango. Ciênc. Tecnol. Aliment., Campinas , v. 29, n. 1, p. 114- 119, Mar. 2009.
33. Fernandes MC, Ribeiro MG, Siqueira AK, Salerno T, Lara GHB, Listoni FJP. Outbreak of bovine mastitis caused by multiple drug resistant Pseudomonas aeruginosa strains. Arq. Bras. Med. Vet. Zootec., v.61, n.3, p.745-748, 2009. 20
34. Sharma D, Pakravan N, Pritchard JC, Hartmann FA, Young KM. Mucoid Pseudomonas aeruginosa infection in a cat with severe chronic rhinosinusitis. Vet Clin Pathol. 2019 Jun;48(2):300-304. doi: 10.1111/vcp.12749. Epub 2019 Jun 18.
35. Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, MartínezGarcía E, Rojas-Acosta C, Catalina Gomez-Puerto M, Calum H, Whiteley M, Kolter R, Bjarnsholt T. 2020. The environmental occurrence of Pseudomonas aeruginosa. APMIS 128:220–231.
36. Jassim SAA, Limoges RG. (2013) The impact of changing environmental forces on cyanophage-host interactions in aquatic ecosystems. World J Microbiol Biotechnol 29 (10):1751–1762. doi:10.1007/s11274-013-1358-5.
37. Runa V, Wenk J, Bengtsson S, Jones BV, Lanham AB. Bacteriophages in Biological Wastewater Treatment Systems: Occurrence, Characterization, and Function. Frontiers in Microbiology. Vol 12. 2021. https://www.frontiersin.org/articles/10.3389/fmicb.2021.730071. DOI 10.3389/fmicb.2021.730071.
38. Abedon ST. (2018). Detection of Bacteriophages: Phage Plaques. In: Harper D, Abedon S, Burrowes B, McConville M. (eds) Bacteriophages. Springer, Cham. https://doi.org/10.1007/978-3-319-40598-8_16-1
39. Jurczak-Kurek A, Gąsior T, Nejman-Faleńczyk B, et al. Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. Sci Rep 6, 34338 (2016). https://doi.org/10.1038/srep34338
40. Vázquez R, García E, García P. Phage Lysins for fighting bacterial respiratory infections: A new generation of antimicrobials. Front. Immunol. 2018, 9, 2252.
41. Pires DP, Oliveira H, Melo LDR, Sillankorva S, Azeredo J. Bacteriophageencoded depolymerases: Their diversity and biotechnological applications. Appl. Microbiol. Biotechnol. 2016, 100, 2141–2151.
42. Knecht LE, Veljkovic M, Fieseler L. 2020. Diversity and Function of Phage Encoded Depolymerases. Frontiers in Microbiology 10:ARTN 2949.
43. Kutter E. Phage Host Range and Efficiency of Plating. In: Clokie M, Kropinski A. Bacteriophages: Methods and Protocols. Vol 501. Totowa, NJ. Humana Press. 2009.

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