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Characterization of a bacteriophage from urban sewage obtained with the bacterium *Staphylococcus aureus*

Anderson Luiz Pena da Costa[1,3*], Antonio Carlos Freitas Souza[2] and Rafael Lima Resque[3]

[1] Faculty of Pharmacy and Nutrition of The Macapa Institute of High Education – IMMES, Jovino Dinoa Street, 2085, Central - 68900-075 -Macapa - AP, Brazil

[2] Post-Graduation Program in Animal Sciences (PPGCA), Maranhao State University– UEMA, Avenue. Lourenço Vieira da Silva, 1000 - Jardim São Cristovão - 65055-310 – Sao Luís/MA, Brazil

[3] Post-Graduation Program in Pharmaceutical Sciences (PPGCF), Federal University of Amapa - UNIFAP, Juscelino Kubitschek Highway, KM-02 Jardim Marco Zero - 68.903-419 – Macapa, AP, Brazil

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Bacteriophages are viruses of bacteria that have received significant attention in the last decades due to their potential as an alternative to the antibiotics, as well as their applicability in the selective control of bacterial species harmful to food. In this context, this work reports the partial results of a viral filtrate named P4CSa that was obtained with the bacterium *Staphylococcus aureus* and characterized by the viral host range and the restriction fragment length polymorphism technique. The results indicate that the phage P4CSa probably belongs to the order *Caudovirales*, it presents a polyvalent host range, and it can be preserved for the long term in the form of filtrated lysates stored at 4°C, suggesting that the phage P4CSa may have the potential for the development of a pharmaceutical product indicated for the biocontrol of pathogenic bacteria.

Keywords: Bacteriophage, Anti-infective agents, Antibacterial.

INTRODUCTION

The bacterial resistance to the antibiotics is a biological phenomenon that is reported as a clinical problem since the year 1950 (1), representing a public health threat because it reduces the effective therapeutic options against bacterial infections and increases the duration, cost, and clinical complications in the course of the treatment (2).

Highlighting that the bacterial resistance generates longer periods of disease and transmission that consequently contribute to the occurrence of epidemic events of emergency and reemergence of infectious diseases (3-5). Therefore, in this context, the bacteriophages represent a viable strategy against bacterial resistance due to their capability of infecting and killing bacteria (6).

Thus, illustrating the applicability of the bacteriophages as anti-bacterial agents, Imkin and Nasanit (7) reported 2 bacteriophages that are stable in association with souring pads and dishwashing sponges that may be useful to prevent the contamination and growth of *Salmonella* spp. in residues of food in dishes. Yin et al. (8) reported the isolation of 3 bacteriophages with the capability of infecting and lyse the foodborne pathogen *Vibrio haemolyticus* with potential employability for the biocontrol of this bacterium in the aquaculture.

Regarding the use of bacteriophages in the treatment of bacterial infections in human beings, Wright et al. (9) reported the complete recovery of 24 patients with chronic otitis caused by *Pseudomonas aeruginosa* resistant to multiple antibiotics. According to several previous studies, cases of bacterial infections were successfully treated or partially improved after the administration of bacteriophages (10, 11).

Demonstrating these pieces of evidence presented in the recent scientific literature that many scientists are seeking for bacteriophages as a tool for the biocontrol of bacterial pathogens, as well as an alternative to the antibiotics in the therapeutics. Therefore, this work aimed to prospect bacteriophages in samples of a water body linked to the Amazon River in an urban perimeter where the biological and chemical contamination is considered high, for then isolate and characterize bacteriophages with potential biotechnological employability in the control of pathogenic bacteria and possibly to treat bacterial infections.

MATERIALS AND METHODS

Sample collection. The samples were collected from urban sewage discharged into a water body related to the Amazon river (coordinates 0° 0.112'N 51° 4.219'W) in an area called *Pedrinhas*. The collection procedure consisted of the transfer of the sewage to sterile cone tubes with the aid of a sterile Pasteur pipette carefully manipulated to avoid cross-contamination, then the samples were identified, packaged in a box of polystyrene filled with ice, and transported to the microbiology facility of the toxicology and pharmaceutical chemistry of the Faculty of Pharmacy from the Federal University of Amapá. The sample reported in this work was named P4C, standing for Pedrinhas 4th collection.

Viral enrichment and isolation. The viral enrichment is a procedure necessary to amplify the number of viruses in the sample and facilitate their isolation. For this purpose, this study used Müeller Hinton (MH) broth, solid (1.5% agar), and soft agar (0.6% agar) purchase from Kasvi ® (São José do Pinhais, PR, Brazil), and the bacterial host *Staphylococcus aureus* ATCC 6338 for isolation of bacteriophages as they are obligatory parasites of bacteria, that was purchased from the American Type Cell Culture

***Corresponding Author**: Mailing address. MPham. Anderson Luiz Pena da Costa, Faculty of Pharmacy, Macapa Institute of High Education – IMMES, Jovino Dinoa Street, 2085, Central - 68900-075 -Macapa - AP, Brazil. E-mail: pena.pharmacist91@gmail.com.*

(Manassas, VA, USA).

The enrichment, prospection and isolation procedures were performed according to protocol mentioned elsewhere (12). For the enrichment, 10 ml of sewage was mixed with 9 ml of MH broth and 1 mL of an overnight inoculum of the bacterial host in an Erlenmeyer flask that was gently mixed and incubated at 37°C for 24 h. The enrichment culture was centrifuged with chloroform (1:1 volume/volume) at 3000 rpm for 30 min and the aqueous phase was collected, filtrated through syringe filters with a pore size of 0.45 µm, serially diluted to 10^{-6} and cultivated by the double agar layer technique.

The double agar layer technique consisted in the addition of 100 µl of the filtrated lysate serially diluted and 100 µl of an overnight bacterial host to 3 ml of MH soft agar in a tube assay that was gently mixed and poured onto MH agar plates. After the solidification of the soft agar layer plates were incubated at 37°C for 24 h, and the lysate plaques formed were collected, enriched and cultured for three consecutive times to assure the isolation of a bacteriophage.

After the completion of these procedures, the bacteriophage obtained was named P4CSa, standing for Pedrinhas 4th collection obtained with *Staphylococcus aureus*.

Host range analysis. The host range of the phage P4CSa was evaluated to identify the capability of the phage to infect different bacteria species. The method employed was the one described by Gregoracci (13) and Costa *et al*. (14) that consists in the preparation of serial dilutions of the viral filtrate from which 100 µl was transferred to a tube with MH soft agar followed by the addition of 150 µl of an overnight culture of bacterial hosts. The tube was gently mixed and poured onto plates of MH agar, after the solidification of the up layer of agar, the plates were incubated at 37° C for 24 h, then the lysate plaques formed were counted and the viral titers were determined according to the equation below:

$$PFU = \frac{Number\ of\ plaques}{Dilution\ volume\ (ml) \times Dilution factor} \times \frac{1000\ \mu l}{ml}$$

The bacterial hosts used in this assay were *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae ATCC 4352, Proteus mirabilis* ATCC 15290, and *Pseudomonas aeruginosa* ATCC 2583 (purchased from the American Type Cell Culture ®, Manassas, VA, USA). The assay was performed in triplicate with each bacterial host, and the results are expressed as the mean of the lysate plaques with its standard deviation, and the viral titer in PFU x ml^{-1} (Plaque forming units per milliliter).

Stability analysis for the long term storage. The filtrate of the phage P4CSa lysate was stored at 4°C in a refrigerator according to Golec et al. (15) and the viability of the phage was evaluated by its capability of infecting and form lyse plaques in the bacterium host. Therefore, to get access to the virus viability under this condition of storage, the double agar layer technique as described above, according to Mirzaei and Nilsson (12) was adopted. The cultures to test the phage P4CSa stability were performed with a periodicity from one week to six months.

Molecular characterization by RFLP. Initially, the viral precipitation method described by Gregorraci (13) was employed for the obtained of a viral super concentrated solution whose nucleic acids were extracted with commercial kits (Quick-DNA/Viral RNA kit, Zymo Research ®, Irvine, CA, USA) following the instructions of the manufacturer. Then the genome obtained was digested with the enzymes Eco RI and Hind III for 1 hour at 37°C according to the manufacturer (Thermo Fisher ®, Waltham, MA, USA), and the fragments were resolved in agarose gel (1%, w/v), stained with ethidium bromide and visualized in UV transilluminator.

RESULTS

The enrichment assay for the bacteriophages showed a viral titer of 5.3×10^5 PFU $\times$ ml^{-1}. Indicating a considerable abundance of phages with the capability of infecting and lysing the host *Staphylococcus aureus* ATCC 6338 in the sewage sample analyzed.

The phage P4CSa presented plaques with a circular shape and 0.64 (± 0.15) mm in diameters as shown in Figure 1.

Regarding the ability to infect different bacteria, beside infect and lyse *Staphylococcus aureus* ATCC 6338, the phage P4CSa was also effective against *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 2583 presenting viral titers that ranged from 10^4 to 10^7 PFU $\times$ ml^{-1}. The viral titers of the phage P4CSa filtrate per host tested are presented in Table 1. During six months of study, aliquots of the filtrate of the phage P4CSa lysate were stored under 4°C at the laboratory refrigerator and cultured with the bacterial host *Staphylococcus aureus* ATCC 6338*,* resulting in the formation of lysates at all the time these were assayed.

This result demonstrates that the filtrate of the phage P4CSa lysate can resist the conditions of storage adopted in this study. This preservation condition can be suggested for long term storage of formulations contenting this phage.

Figure 1. The lysates formed by the P4CSa phage over the bacterial growth.

The results of the restriction enzymes digestion can be seen in Figure 2, it is possible to observe the restriction fragments of the DNA of the phage λ with Eco RI and with Hind III, followed by the digested P4CSa genome with Eco RI, the P4CSa genome digested with Hind III, and the genomic P4CSa, while the wells with the identification STD is from a different phage obtained with *Escherichia coli* ATCC 8739 whose chemical composition of the genome was confirmed to be ds DNA. This other phage was used as standard for comparison of the results of the molecular biology assays conducted with the phage P4CSa.

DISCUSSION

The viral titer obtained in this work is a little lower than those reported by Elbreki et al. (16) which informs that the concentrations of phage in sewage samples often presents values in the order of 10^8 phages $\times$ ml^{-1} to 10^{10} phages $\times$ ml^{-1}.

But two facts may explain this difference. The first is related to the method employed, which used chloroform, a solvent that is harmful to phages having lipid rich structures such as, envelops and lipoproteins (17). And the second concern is host specificity of some phages (18). Therefore, the results obtained cannot address the abundance and diversity of the bacteriophages in the sewage sample analyzed, but it demonstrates that sewage might be a good source of bacteriophages able to infect and lyse the bacterium *Staphylococcus aureus*, as also demonstrated by Synnott et al. (19), Wang et al. (20), and Nasser et al. (21) that successfully isolated phages

with high biotechnological potential from sewage samples. Highlighting that the presence of bacteriophages depends on the availability of bacterial hosts in the environment (22).

Regarding the morphological properties of the plaques, according to Saad et al. (23), the small size of the plaques observed suggests that the phage P4CSa might present a large viral particle, but a transmission electron microscopy analysis is necessary to confirm this prediction.

With respect to the host range, it represents the number of strains a bacteriophage is capable of infecting, and it can be classified as broad when the virus infects several strains of the same genus or different species, narrow when the virus can infect just specific bacterial strains, usually of the same species, monovalent when the virus infects a single bacterial species, or polyvalent when the phage can infect more than one bacterial species (24).

Since the phage P4CSa could infect the species, *Staphylococcus aureus* ATCC 6338, *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 2583, its host range is considered polyvalent. However, more tests with different strains of these bacterial species are necessary to evaluate if the phage P4CSa has a broad or a narrow host range. Because the interaction between the bacteriophage and the bacterial host is determined by genetic factors related to the expression of surface molecules used by the phages for the recognition and initiation of the infection process (25), attributing selectivity of the virus by the host, which can limit the number of strains these viruses may infect according to the genetic variability among the bacteria regarding the molecules used by the phages as receptors (26, 27).

In this context, regarding the P4CSa potential applicability, according to Shende et al. (28), the bacteriophages with the host range of the polyvalent type are strong candidates for the therapeutic application against common bacterial pathogens as an alternative to the antibiotics. Especially if they have a broad host range that allows the recognition of different receptors in the bacterial cell, increasing the likelihood of success in the treatments where there is little information about the identity of the etiologic agent, also prevents the development of resistance in the bacteria against the phages when used therapeutically in combination with different bacteriophages in the form of phage cocktails (26, 29, 30).

Concerning the sensitivity and resistance of the phages to environmental stresses, this is a very important characteristic that must be considered for the therapeutic and biotechnological applications (26, 31), since the resistance/sensitivity to environmental stress is an intrinsic characteristic of each virus and there is no universal method for their storage (15). Therefore, it must be determined experimentally for each bacteriophage isolate.

In this study, it was demonstrated that the lysate

Table 1. Host range of the phage P4CSa.

Bacterial Host	**Dilution used in the plaque count**	**PFU count** ±SD	**PFU × ml^{-1}**
S. aureus ATCC 6338	10^{-3}	201.66 (±3.51)	$2.0x10^{6}$
E. coli ATCC 8739	10^{-4}	167 (±7.57)	$1.7x10^{7}$
P. aeruginosa ATCC 2583	10^{-2}	41.33 (±5.51)	$1x10^{4}$
E. faecalis ATCC 29212	10^{-1}-10^{-6}	0	0
K. pneumoniae ATCC 4352	10^{-1}-10^{-6}	0	0
P. mirabilis ATCC 15290	10^{-1}-10^{-6}	0	0

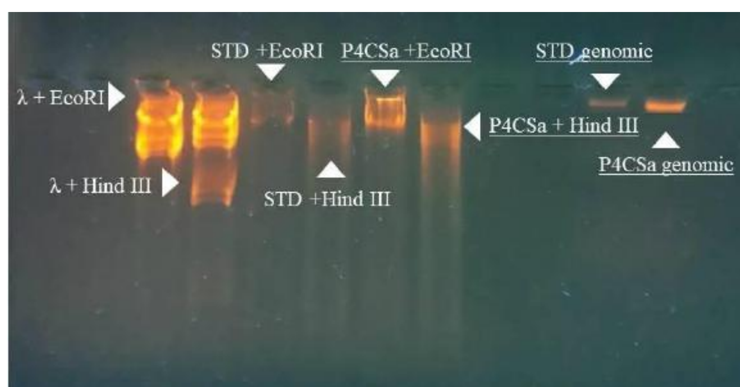

Figure 2. Electrophoresis of the restriction digestion.

In this study, it was demonstrated that the lysate filtrate of phage P4CSa can be kept viable at 4°C for more than six months. This finding also suggests the storage temperature be considered in the preparation and storage of pharmaceutical formulations containing the phage P4CSa.

The method adopted for obtaining of nucleic acids demonstrates to be not effective because it didn't provide an adequate amount of genetic material for the observation of restriction fragments; however, it was enough to conclude that the genetic material of the phage P4CSa is composed of double-strand DNA.

According to Ackermann (32) and Ackermann & Prangishvili (33), the classification of the bacteriophages is based on the presence of common features that are hierarchically organized into order and families, considering the morphology of the viral particle as the main criteria for the classification of these viruses, followed by the composition of the genetic material, genomic architecture, and structural elements.

Therefore, even though the molecular characterization by restriction fragment length polymorphism did not provide observable of fragments, due to the stereospecificity of the enzymes Eco RI and Hind III by ds DNA in the regions with GAATTC / CTTAAG and AAGCTT / TTCGA, respectively (34, 35, 36), it is possible to affirm that the genetic material of the phage P4CSa is composed of double-strand DNA, and the comparison with the STD sample results reinforces this conclusion since its genome is composed of ds DNA, since this sample belongs to the family *Myoviridae*, order *Caudovirales*. Therefore, following the reasoning adopted in the classification of the bacteriophages, and the methodological approach (site of collection, prospection, isolation, and host range), it is possible to infer the phage P4CSa belongs to the order *Caudovirales*.

Because the phages from the order *Caudovirales* have genetic material composed of ds DNA, it is possible the exclusion of phages possessing their genome made of ss DNA as the viruses of the families *Microviridae* and *Inoviridae*, and those whose genetic material is made of RNA, as the viruses of the family *Leviviridae* (32, 33, 37-39).

Being also possible to exclude the phages from group Salterprovirus and the families *Guttaviridae* and *Cystoviridae*, because these viruses have their genomes made of segmented ds DNA, and in the electrophoresis of the genomic material of the phage P4CSa it was observed just one band, not several bands as seen in the viruses with segmented genomes (32, 33, 37).

Considering the use of chloroform in the obtaining and isolation processes, the viral families contenting structures that are rich in lipids can be excluded, such as the families *Corticoviridae, Cystoviridae*, *Rudiviridae*, *Lipothrixviridae*, *Plamaviridae*, *Fuseloviridae*, *Tectviridae*, and *Globulaviridae* (32, 33, 37-39).

Also, being possible to exclude the family *Ampulaviridae* due to the ability to infect a single host of the species *Acidum convivator,* the family *Clavaviridae* which infects hyperthermophilic archaea, and the family *Bicaudoviridae* that is obtained in aquatic environments with temperatures in the order of 75-90°C (33, 37, 38, 39).

CONCLUSION

The results demonstrate that the phage P4CSa can infect different bacteria species and that the filtrate of culture lysates is a suitable way to preserve the phage for the long term. However, these results are preliminary and wider characterization studies are needed, such as the classification of the isolate by transmission electron microscopy to determine the viral family to which the phage belongs as established by the International Committee for Virus Taxonomy (40).

Molecular assays to assure the absence of island of pathogenicity and resistance genes to antibiotics in the viral genome must be conducted to ensure safety in the therapeutic application, as well as a wider analysis of the host range involving a greater diversity of bacteria, mainly clinical isolates (24). And stability analyzes of the phage P4CSa considering different environmental conditions such as pH, temperature and ionic strength need to be conducted for determining the ideal conditions for the production and storage of future formulations containing this phage as an antibacterial agent (26).

The Applications of bacteriophages is still an open field, involving the therapeutic use, hospital sanitization, the promotion of food safety by preventing foodborne pathogens, tool for the bacterial biocontrol in any industrial sectors in which the contamination by bacteria represents a problem in production (41). Therefore, the research on bacteriophage biology and their biotechnological application is promising and still is a field little explored in Brazil.

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