Anti-bacterial analysis of a filtrate containing five large amazonic bacteriophages

Análise antibacteriana de um filtrado contendo cinco bacteriófagos amazônicos grandes

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Keywords

Phages
Myoviridae
Electron transmission microscopy
Anti-infective agents

Bacterial resistance to antibiotics is a public health issue with notable implications for the treatment of bacterial infections, as well as the epidemiological profile of these infections in hospital settings and the community, given the transmissibility of bacterial Infectious agents; therefore, alternatives to antibiotics are urgent to mitigate this public health problem. Thus, a search for phages from sewage with potential for bacterial biocontrol was conducted using classical microbiological approaches to evaluate their resilience to storage conditions and their lytic activity over pathogenic bacteria, followed by characterization using transmission electron microscopy. As a result, a filtrate containing five large myoviruses capable of lysing three clinically significant bacterial species and remaining viable in storage at 4°C for lengthy periods was obtained, demonstrating the potential for biotechnological applications in bacterial control.

Palavras-chave

Fagos
Myoviridae
Microscopia eletrônica de transmissão
Agentes anti-infecciosos

A resistência bacteriana aos antibióticos é um problema de saúde pública com implicações notáveis no tratamento das infecções bacterianas, bem como no perfil epidemiológico dessas infecções em ambientes hospitalares e na comunidade, dada a transmissibilidade de agentes infecciosos bacterianos; portanto, alternativas aos antibióticos são urgentes para mitigar esse problema de saúde pública. Assim, uma busca de fagos de esgoto com potencial para biocontrole bacteriano foi realizada usando abordagens microbiológicas clássicas para avaliar sua resiliência às condições de armazenamento e sua atividade lítica sobre bactérias patogênicas, seguida de caracterização por microscopia eletrônica de transmissão. Como resultado, obteve-se um filtrado contendo cinco miovírus grandes capazes de lisar três espécies bacterianas clinicamente significativas e permanecer viáveis em armazenamento a 4°C por longos períodos, demonstrando o potencial para aplicações biotecnológicas no controle bacteriano.

INTRODUCTION

The bacterial resistance to antibiotics is a natural phenomenon that has been accelerated and intensified since the discovery of these drugs, and it has been causing worldwide public health concerns due to a decrease in effective treatment options for bacterial infections caused by resistant pathogens, increases in treatment and prevention costs, as well lengthening the hospitalization time and thus increasing the risks of epidemiological events. As a result, research for new antibacterial medications and prophylactic strategies is critical in combating this public health concern (LEVY; MARSHALL, 2004; BLAIR et al., 2015; ASLAM et al., 2018; KUMAR et al., 2020).

In this context, phages are viruses that selectively infect and kill bacteria, are abundant and diverse in nature (HATFULL; HENDRIX, 2011), have the ability to track bacterial evolution (GOLKAR et al., 2014), and have unique pharmacological proprieties (unlike chemical antibiotics) (ABEDON; THOMAS-ABEDON., 2010) making them a promising strategy against bacterial resistance to the antibiotics, which motivated the conception and subsequent execution of this research.

Moreover, the biotechnological applications of bacteriophages are not limited to their use in nanomedicine for the treatment of bacterial infections and diagnosis by phagotyping (REHMAN et al., 2019), but also include food contamination prevention, bioremediation of polluted areas, water treatment, and the development of biosensors for the detection of pathogens in environmental, clinical, or food samples (SHARMA et al., 2017) by combining the biological component of phages with materials capable of generating signals detectable through immunological, enzymatic, molecular or physical methods (AHOVAN et al., 2020).

In addition, bacteriophages are important technologically as tools in synthetic biology for the construction of circuits that enable the planning and development of vaccines by

expressing epitopes of interest in viral particles, as well as the production of nanomaterials with unique morphology and properties by modifying viral proteins so that they interact with specific materials that assume conformations similar to those of phages after they have been engineered (LEMIRE et al.., 2018).

Emphasizing that bacteriophages became possible the phage display technology, in which protein fragments of interest are expressed in the capsid proteins of phages, allowing researchers to investigate the interaction of such fragments with proteins from a complex biological system (ARAP., 2005), which led to Gregory Winter and George Smith receiving the 2018 Nobel Prize in Chemistry for the development of humanized antibodies for the treatment of neoplasms and inflammatory disorders (SERVICE, 2018).

Thus, using a biotechnological approach, this study describes the morphological characterization of five large bacteriophages as well as their lytic activity against bacterial species linked to common infections around the world, which can become severe and difficult to treat when these pathogens are antibiotic-resistant. Noting that the findings of this study also contribute to the recognition of viruses in the Brazilian Amazon, as well as the field of virology in general, because the majority of current knowledge about these biological entities comes from phages isolated from North America and Europe, there is still a scarcity of data on phages from South American and African ecosystems (ACKERMANN, 2007; ACKERMANN, 2011).

MATERIAL AND METHODS

SAMPLE COLLECTION

Four sewage samples were obtained from a polluted branch of the Amazon River called Pedrinhas (coordinates 0 ° 0.112'N 51 ° 4.219'W), where earlier investigations on bacteriophage had been undertaken. The samples were then labelled P1C, P2C, P3C, and P4C, where the P represents Pedrinhas, the number represents the collection number, and the letter C represents collection. This study exclusively presents the results of P4C enriched with *Escherichia coli*, which was called P4CEc after being processed in the laboratory.

Each sample was collected using sterile Pasteur pipets to transfer sewage to sterile cone tubes and then transported to the Microbiology facility of the Toxicology and Pharmaceutical Chemistry laboratory of the Pharmacy course at the Federal University of Amapa, where microbiological experiments were performed.

VIRAL ENRICHMENT AND ISOLATION

The phage enrichment was performed using the bacterium *Escherichia coli* ATCC 8789, which was centrifuged at 4000 rpm for 30 min with chloroform after a period of culture in broth with the sample at 37° C for 24 h, with the aqueous phase being collected, filtered through syringe filters with a pore size of 0.45 µm, serially diluted and cultivated by the double-agar-layer technique for the initial prospection, followed by the isolation procedure which was performed through plaque collection followed by subsequent cultures of purification as described by Mirzaei and Nilsson, (2015).

For the cultures of prospection, isolation, and purification the culture medium used was the müeller hinton in broth, solid (1.5% agar) and soft (0.6% agar) consistencies, and the incubation conditions were 37 ° C for 24 hours. The viral titer of the P4CEc filtrate was obtained according to Costa et al. (2019) applying the counts of plaques formed using the formula:

$$\text{PFU} = \left(\frac{\text{Number of plaques}}{\text{Dilution volume in mL} \times \text{Dilution factor}}\right)\left(\frac{1000\mu\text{L}}{mL}\right)$$

The results are expressed with the mean of the plaque forming units (PFU) counts, followed by the standard deviation, and the viral titer in PFU x mL^{-1}.

HOST RANGE

The evaluation of how many different hosts the filtrate P4CEc is capable of infecting and lyse was accessed according to the method described above in 2.2, but instead of just the bacterium *Escherichia coli* ATCC 8789, the viral titers of the filtrate was also determined using the bacteria *Pseudomonas aeruginosa* ATCC 4352, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6338*, Klebsiella pneumoniae* ATCC 4352, and *Proteus mirabilis* ATCC 1529. In addition, the results of the viral titer obtained using each host are expressed with the mean of the plaque-forming units (PFU) counts, followed by their standard deviation, and the viral titer in PFU x mL^{-1}.

EFFICIENCY OF PLATING

The efficiency of plating (EOP) is a continuation of the host range analysis, and aims to mathematically define the efficiency of the P4CEc filtrate against different hosts considering the viral titers obtained with a test host and the titer achieved with the host of obtaining, according to (Imklin and Nasanit, (2020). By using the following formula:

$$EOP = \frac{\text{Viral titer in the test host}}{\text{Viral titer in the obtaining host}}$$

Where the efficacy is defined with bases in the following nominal values: EOP> 0.5 Highly effective, EOP> 0.2 Moderately effective, EOP> 0.001 to 0.2 Low effectiveness, and EOP = 0 or <0.001 Not effective.

MORPHOLOGICAL CHARACTERIZATION BY ELECTRON TRANSMISSION MICROSCOPY

The viral particles were observed using negative stain with phosphotungstic acid at pH 7.2 and transmission electron microscopy. The acid was added to the dried lysates on the

surface of formvar-coated 300 mesh copper grids, which were then dried completely before being sent to the transmission electron microscope, which operated at 80 kV with a nominal magnitude range of 80.000 to 150.000.

The constituents of viral particles were measured and recorded using Icy license GPLv3 image processing software, and the classification was done according to the International Committee on Taxonomy of Viruses as published by Maniloff and Ackermann (1998).

STORAGE VIABILITY FOR LONG TERM

The ability of the P4CEc filtrate to remain viable when stored at 4° was qualitatively assessed according to Golec et al. (2011), using the spot assay, which consisted of adding 10 µL of the filtrate over a pour plate culture of *Escherichia coli* ATCC 8789 on soft agar, followed by incubation at 37 ° C for 24 h, where the formation of lysate in bacterial growth is interpreted as a signal that the filtrate could maintain its infectivity and ability to lyse the host, suggesting resilience to the storage condition for the long-term preservation (SYNNOTT et al., 2009).

The spot assay was conducted weekly for the first month, then monthly for the next five months.

STATISTICAL ANALYSIS

The host range was analysed in triplicates after obtaining the P4CEc filtrate via multiple cultures with the host *Escherichia coli*, and measurements of the viral particle components included five measurements of the capsid diameter, capsid length, tail length, and tail diameter from random phage particles using the image processing software Icy license GLv3, and the mean and standard deviation from these experiments was calculated using descriptive statistics from the Microsoft Excel ®2010 data analysis package.

The host range assessment was expressed as the mean count of plaque-forming units, followed by the standard deviation value and the results obtained in each triplicate, and the results referring to the measurements of the components of the viral particles found in the filtrate are expressed in terms of mean and standard deviation accompanied by the values of the 5 random measurements performed.

RESULTS AND DISCUSSION

After viral enrichment and the use of the isolation technique by plaque collection followed by multiple cultures using the double-agar-layer method, a viral filtrate was obtained, which was subjected to microbiological tests with different bacteria, evaluation of viability for long term storage, and viral characterization. As a result, a filtrate presenting a titer of 1.6×10^6 UFP x mL^{-1} was obtained, which formed plaques with a mean diameter of 0.44 mm and a standard deviation of ±0.30 mm, with no change in plaque morphology. However, transmission electron microscopy investigation indicated that the filtrate did not contain one isolated phage, but rather five viruses. What highlights the importance of performing electron microscopy analysis not only during the characterization phase but also for ensuring microbiological isolation.

In this sense, plaque morphology is considered one of the primary criteria for identifying distinct bacteriophages, and their diameter features can provide predictive information regarding viral particle size, with large bacteriophages forming small plaques, and small bacteriophages forming large plaques (SAAD et al., 2019; SHENDE et al., 2017). Emphasizing that in the results obtained, the morphology of the plaque did not vary in terms of shape and appearance, but it did vary in terms of diameter (standard variation of ±0.30 mm) in the observed data.

Regarding the correlation between plaque diameter size and viral particle size, transmission electron microscopy analysis produced results that corroborate with SAAD et al., (2019) and agree with the results from COSTA et al. (2019 and 2020) that report lytic activity of bacteriophages from the same collection site (coordinates 0 ° 0.112'N 51 ° 4.219'W), which also produced small plaques of lyses, demonstrating conformity with Yuan et al. (2017), regarding the difficulty in isolating large phages under conventional conditions (0.5-0.7% soft agar, in the upper layer), because large viral particles present limited diffusion in the medium, explaining the formation of small plaques. Highlighting that this work did not aim to isolate large phages, suggesting that large phages might be abundant in the area the samples were collected.

Thus, these findings show that plaque morphology is not very reliable for ensuring the isolation of a single phage and that the isolation of bacteriophages by plaque collection followed by multiple passes by the double-layer agar method failed, indicating that three passes are insufficient to ensure the isolation of a single bacteriophage, also reinforcing the importance of electron microscopy transmission not only in the characterization process but also in the isolating process (CASEY et al., 2018).

All the bacteriophages detected by transmission electron microscopy in the filtrate belong to the order *Caudovirales*, which consists of phages with a protein capsid filled with DNA, to which is attached a tail whose function is to inject the viral material into the host cell, and structures of recognition, adsorption, and infection initiation at the distal ends (BEBEACUA et al., 2013). Moreover, this order is divided into three families that differ in their tail characteristics: the *Myoviridae* family has a long and contractile tail, the *Siphoviridae* family has a non-contractile long tail, and the *Podoviridae* family has a short and non-contractile tail (NELSON, 2004).

Furthermore, all five phages detected belonged to the *Myoviridae* family and have viral particles larger than those commonly reported in the literature, implying that the viruses detected also belong to the Jumbo category due to their large viral particles. The morphology of the phages in the filtrate P4CEc can be seen in figure 1 and the measurements of their components can be found in table 1.

Figure 1. Electromicrography of the bacteriophages detected in the filtrate P4CEc, all present morphology typical of viruses belonging to the order *Caudovirales* and family *Myoviridae*.

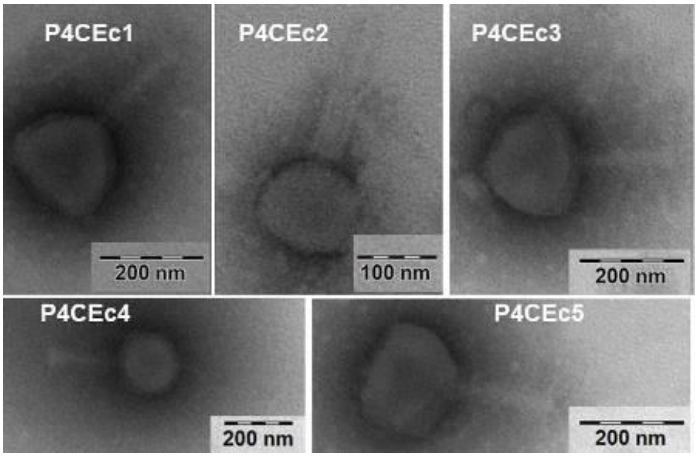

Table 1. Measurements of the diameter and length of the capsids and tails of the 5 phages detected in the filtrate obtained using *E. coli* as host; mean (standard deviation) [measures collected]

Component of the viral particle	P4CEc1	P4CEc2	P4CEc3	P4CEc4	P4CEc5
Capsid diameter	202.57 (±1.89) nm [203.41 nm, 200.17 nm, 200.95 nm, 204.45 nm, 203.87 nm]	132.93 (±7.05) nm [135.49 nm, 139. 02 nm, 123.88 nm, 127.09 nm, 139.93 nm]	206.15 (±8.52) nm [196.30 nm, 207.23 nm, 212. 82 nm, 215.77 nm, 198.67 nm]	193.12 (±2.01) nm [190.02 nm, 193.50 nm, 195.29 nm, 192.53 nm, 194.29 nm]	209.86 (±3.96) nm [207.82 nm, 215.25 nm, 204.65 nm, 210.20 nm, 211.41 nm]
Capsid length	195.03 (±4.37) nm [199.47 nm, 194.07 nm, 198.55 nm, 194.61 nm, 188.45 nm]	113.44 (±1.51) nm [111.60 nm, 113.56 nm, 115.32 nm, 112.31 nm, 114.45 nm]	194.31 (±3.41) nm [196.45 nm, 195.39 nm, 189.30 nm, 197.87 nm, 192.55 nm]	196.05 (±2.63) nm [193.,59 nm, 195.21 nm, 197.00 nm, 194.29 nm, 200.18 nm]	174.04 (±2.10) nm [171.39 nm, 174.79 nm, 176.19 nm, 175.59 nm, 172.27 nm]
Tail length	191.00 (±7.86) nm [197.85 nm, 188.02 nm, 197.98 nm, 192.07 nm, 179.10 nm]	179.12 (±5.57) nm [177.12 nm, 184.26 nm, 170.75 nm, 179.65 nm, 183.93 nm]	222.20 (±5.13) nm [218.42 nm, 217.50 nm, 226.68 nm, 228.73 nm, 219.70 nm]	198.37 (±6.51) nm [204,46 nm, 191.81 nm, 202.52 nm, 202.25 nm, 190.79 nm]	193.62 (±3.87) nm [191.91 nm, 190.55 nm, 195.39 nm, 190.68 nm, 190.60 nm]
Tail diameter	32.07 (±2.35) nm [32.46 nm, 30.00 nm, 35.99 nm, 30.97 nm, 30.97 nm]	46.57 (±1.94) nm [43.53 nm, 47.70 nm, 48.68 nm, 46.17 nm, 46.76 nm]	42.38 (±5.64) nm [40.41 nm, 33.61 nm, 47.77 nm, 43.82 nm, 46.34 nm]	41.87 (±1.58) nm [40.48 nm, 41,40 nm, 43.97 nm, 43,09 nm, 40.44 nm]	37.69 (±2.87) nm [34.65 nm, 37.05 nm, 42.34 nm, 38.07 nm, 36.36 nm]

Phages from the family *Myoviridae* are frequently isolated from diverse environmental and biological materials because they are resistant to various environmental challenges, including the use of organic solvents in the process of acquiring and isolating them in the laboratory (JOŃCZYK et al., 2011). However, phages with the dimensions reported in this paper are uncommon, ranging from 132.93 - 209.86 nm in capsid diameter by 113.44 - 196.05 nm in length, with tails

ranging from 179.12 - 222.20 nm in length by 32.07 - 46.57 nm in diameter. These dimensions indicate that the bacteriophages reported in this study belong to the jumbo category.

Jumbo bacteriophages are viruses with a large capsid and a genome of at least 200 kbp. They are typically isolated from gram-negative bacteria and differ from small bacteriophages in evolutionary origin and the synthesis of enzymes such as RNA and DNA polymerases, lysines, and hydrolases (YUAN; GAO, 2017; SAAD et al., 2019; BUTTIMER et al., 2016).

In this regard, the comparison of the dimensions reported in this work with the viral particles of jumbo bacteriophages published by other authors supports the jumbo classification for the five viruses found in the P4CEc filtrate. Drulis-Kawa et al., (2014) isolated a jumbo bacteriophage from the *Myoviridae* family with a genome of 375 kbp and a viral particle with a capsid of 131 nm, a tail of 136 nm in length, and a diameter of 19 nm. Attai et al., (2018) described the isolation of another myovirus with a 440 kbp genome, a capsid measuring 152 nm by 146 nm, and a tail measuring 136 nm. While Arens et al., (2018) reports two myoviruses with a genome of 235 kbp, a capsid of 143.6 nm, and a tail of 206 nm in length by 23.2 nm diameter.

In terms of antibacterial activity, the filtrate demonstrated lytic activity against three bacterial species other than the host used to obtain the filtrate (*Escherichia coli* ATCC 8789), being particularly effective against *Pseudomonas aeruginosa* ATCC 4352, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 6338. Table 2 shows the plaque-forming unit counts in each triplicate, the mean and standard deviation, the viral titer in PFU x mL-1, and the efficiency of plating.

Table 2. Host range and efficiency of plating of the filtrate obtained with *E. coli* and its viral titers against different bacterial hosts; average, (standard deviation), [plaque counts in the triplicates]

Bacterial Host	Dilution fator	PFU	PFU x mL^{-1}	EOP
S. aureus	10^{-3}	200 (±32.00) [200, 197, 203]	$2.0x10^6$	1,2
*E. coli**	10^{-3}	155 (±28.05) [157, 143, 167]	$1.6x10^6$	*
P. aeruginosa	10^{-4}	72,66 (±10.96) [64, 85, 69]	$7.2x10^6$	4,5
E. faecalis	10^{-3}	223 (±11.50) [229, 218, 223]	$2.2x10^6$	1,4
K. pneumoniae	10^{-1}-10^{-6}	-	-	-
P. mirabilis	10^{-1}-10^{-6}	-	-	-

*Host employed in the processes of prospecting, isolation and purification; PFU = Plaque-forming unit; EOP = Efficiency of plating (test host/host of obtaining)

The P4CEc filtrate's host range showed efficacy against four bacterial species that typically cause infections in humans and animals, as well as species linked to food-related disorders (KAPER et al., 2004; TONG et al., 2015; STRATEVA et al., 2016; MATOS et al., 2016). These findings indicated the therapeutic potential of phage treatment for treating human and animal infections (ELBREKI et al., 2015) or as a biotechnological tool for controlling pathogenic bacteria in food to increase food safety (HSIEH et al., 2011; ENDERSON et al., 2014).

In this context, the results obtained with *Pseudomonas aeruginosa* ATCC 4352, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 6338 classify the filtrate as highly efficient against these bacterial species, indicating that the phages in the filtrate present a profile of highly productive infection, which occurs when the phages produce viral titers superior to those of the primary host (MIRZAEI; NILSSON, 2015).

However, bacteria have a high level of genetic variability in terms of structural and functional elements, which can result in differentiated expression of surface molecules required for the phage to recognize the host (LEÓN; BASTÍAS, 2015), or even different expression of protective factors against phage infection by the bacteria, such as biofilms (DEVEAU et al., 2002), with the caveat that these differences can result in variations in viral titers even among bacteria of the same species. As a result, more research is needed to determine the biotechnological and therapeutic potential of the P4CEc filtrate, particularly studies incorporating clinical isolates when prospecting a future pharmaceutical product for treating human infections (ROSS et al., 2016).

Concerning the viability of the bacteriophages in the storage conditions tested, the filtrate remained viable during the six months of analysis, demonstrating that this filtrate and their phages can be stored for the long term for laboratory manipulation, as well as suggesting the potential for developing an antibacterial pharmaceutical product that can be stored for a long term under refrigeration at 4°C.

In this light, it is critical to emphasize that one of the most significant problems for developing pharmaceutical products and phage therapy is the resilience of phages to the environmental conditions of formulation preparation,

storage, and administration (VANDENHEUVEL et al., 2015; MALIK et al., 2017; ZHANG et al., 2018; BROWN et al., 2018; DANIS-WLODARCZYK et al., 2021).

According to Jończyk et al. (2011), phages can be affected in various ways by factors such as temperature, pH, ionic strength, and divalent ion concentration, which can cause damage to the structural apparatus of these viruses, and in general, those belonging to the *Myoviridae* family are the most resistant to these factors, as demonstrated by the results of Jurczak-Kurek et al. (2019), which isolated 83 bacteriophages and evaluated their susceptibility to different temperatures, pH values, ionic strength, and organic solvent, finding various profiles of viability among the isolates, correlating with Golec et al. (2011) regarding the lack of a universal strategy for preseving bacteriophages in the laboratory, since their viability to environmental stresses is an intrinsic aspect of each one of these viruses.

In this sense, this study shows that the phages in the filtrate P4CEc can survive at 4°C for up to six months, and it suggests that the physical-chemical properties of the filtrate can be addressed for developing future pharmaceutical formulations containing these phages as antibacterial agents; however, more research is needed to achieve this goal. Furthermore, these findings constitute a record of large bacteriophages in an Amazonian ecosystem, helping to catalog phage diversity in this massive biological hotspot.

CONCLUSION

The P4CEc filtrate contains five large bacteriophages from the *Myoviridae* family that were obtained from sewage samples discharged into a geographical location of the Brazilian Amazon, which is one of the world's greatest biological hotspots, but where little is known about the viruses that live in the most diverse ecosystems from the area.

In this sense, this study makes an unprecedented contribution to the recognition of bacteriophages in the Brazilian Amazon using transmission electron microscopy, as well as demonstrating through microbiological experiments that the viruses present in the P4CEc filtrate remain viable for more than six months and exhibit a satisfactory lytic activity against bacteria of significant clinical importance.

Thus, these results indicate a potential of the P4CEc filtrate for pharmaceutical development aiming the treatment of bacterial infections, as well as for the biocontrol of foodborne bacterial diseases.

However, further studies are needed to determine the optimal physicochemical conditions to maintain the phages viability in formulations, as well as deeper evaluations of the antibacterial activity of the filtrate against different strains of the tested species, including strains of epidemiological relevance with profiles of circulation in the community and the hospital sets, along with strains to susceptible to the drugs commonly used in the medical practice, to recognize the potential and limitations of the therapeutic use of P4CEc filtrate and its derivatives to rationalize its pharmacological use as an alternative to antibiotics.

ACKNOWLEDGMENTS

The authors acknowledge the Federal University of Amapa and the Evandro Chagas Institute for providing the support in the experimental conduction of this work.

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