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# Genomics and Phenotypical Characterization of Two New Lytic Bacteriophages for Biocontrol of *Salmonella enterica*

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author ESP designed the study, performed the statistical analysis, managed the analyses of the study, wrote the protocol and wrote the first draft of the manuscript. Authors BW, SC, VG and ASPN managed the analyses of the study, wrote the second draft of the manuscript and managed the literature searches. Authors RZ, VPDN, LRDS and LBR worked on the analysis, writing and the critical review of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** To perform the isolation, characterization and sequencing of the bacteriophages. To demonstrate that the bacteriophages can be used for biocontrol of different *Salmonella enterica* serovars.

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**Study Design:** This study was an experimental study.
**Place and Duration of Study:** Bacteriology and Mycology Laboratory in the Veterinary Hospital at the Faculty of Agronomy and Veterinary Medicine of the University of Passo Fundo (FAMV/UPF), Biotechnology Center (CBiotec) of the Federal University of Paraíba (UFPB), Center for Microscopy and Microanalysis at the Faculty of Veterinary of the Federal University of Rio Grande do Sul (UFRGS), between January – September 2016.
**Methodology:** Twelve *Salmonella enterica* serovars (*S.* Anatum, *S.* Agona, *S.* Brandenburg, *S.* Bredeney, *S.* Infantis, *S.* Lexington, *S.* Panama, *S.* Rissen, *S.* Schwarzengrund, *S.* Tennessee, *S.* Enteritidis ATCC 13076 and *S.* Typhimurium ATCC 14028) were selected to be the hosts. We isolate, purify, produce and determine the bacteriophage titers to verify the potential for lysis of these phages against the hosts. Having determined the action of the phages against the hosts, we performed the sequencing of the bacteriophages on the Illumina Mi-Seq platform and the morphology was performed by transmission electron microscopy (TEM).
**Results:** We isolated, characterized and sequenced the genome of two new bacteriophages, *Salmonella* phage UPF_BP1, belonging to the family *Podoviridae* and *Salmonella* phage UPF_BP2, family *Myoviridae*. UPF_BP1 has lytic action against seven tested *Salmonella enterica* serovars, while UPF_BP2 has action against the twelve tested serovars.
**Conclusion:** The two new bacteriophages have a lytic action against different *Salmonella enterica* serovars, feeding our expectations for the development of alternatives for the use of antimicrobials, being possible candidates for use as a biocontrol of *Salmonella enterica* in food, animals and the environment.

*Keywords: Bacteriophages; genome; Salmonella phage UPF_BP1; Salmonella phage UPF_BP2; Salmonella enterica; biocontrol.*

## 1. INTRODUCTION

Brazil began its intensive poultry production in the 1960s and is currently the third largest poultry producer and exporter in the world [1]. Given the prominence of production in the national and international scenario, the control of poultry health and diseases is of great importance, as it directly impacts public health and the economy. Food safety is characterized by concerns about the dangers that may be associated with products that are part of the human food chain, so any risk of physical, chemical or biological origin is unacceptable [2]. In view of this, it is necessary to maintain hygiene and quality standards as priorities in the poultry industry. Among the bacteria listed by the World Health Organization (WHO) that can be found in farms, we highlight *Salmonella* spp. According to the WHO list based on the urgency of new antibiotics, these bacteria fall into the high priority category [3]. The control of *Salmonella* spp. in slaughterhouses is rigorous, in accordance with MAPA Ordinance 210 (Ministry of Agriculture, Livestock and Food Supply), because in addition to being relevant as a cause of diseases transmitted by food, it has an economic impact, causing losses in the domestic market and in exports.

*Salmonella* spp. are the main bacteria related to foodborne diseases in several countries [4]. Salmonellosis in humans is often related to the ingestion of contaminated animal products [5]. The chicken meat products are the most common source of human infection [6,7]. *Salmonella* Enteritidis is the most common serovar in poultry, together with *S.* Pullorum and *S. gallinarum*, are involved with avian paratyphoid's, and also responsible for food-borne infections in humans [8,9,10]. Other serovars also include the list of the 15 most serotyped in environmental, food, human and animal samples, such as *S. anatum, S. agona, S. brandenburg, S. bredeney, S. infantis, S. lexington, S. panama, S. rissen, S. schwarzengrund* and *S. tennessee* [11].

In order to minimize the incidence of *Salmonella* spp. in poultry and throughout their production chain, as well as in the food industry, antimicrobials and chemical sanitizers are used. However, there are concerns about the selection of resistant bacteria. This fact limits the treatment options in cases of salmonellosis, since antibiotic residues in poultry products can generate infection that is difficult to control due to ineffective antimicrobial therapy [9,12]. Thus, there is a need for new methods for the control and prevention of *Salmonella* spp., with emphasis on the use of bacteriophages (phages) as an important alternative.

Phages are viruses that can infect and eliminate bacteria, and lytic phages can be used to treat bacterial infections in humans, as well as to improve food security [13,14]. Phages are considered one of the safest antibacterial, as they are highly specific to each species and cannot infect eukaryotic cells [15,17]. Before the appearance of antibiotics, it was suggested that bacterial infections could be prevented or treated with phage administration [13,18]. Phages are identified as potentially attractive therapeutic agents, since they are not harmful to humans and animals, they are rapidly changeable to contain the emergence of new pathogens. In addition to these characteristics, phages replicate only in specific bacterial cells, not destroying the adjacent microbiota [17,19].

Biocontrol using bacteriophages has been successfully tested in the treatment against *S.* Enteritidis, *S.* Heidelberg and *S.* Typhimurium serovars in the turkey breast, demonstrating a reduction of 4 logs when compared to untreated controls [20]. Other researchers used bacteriophage P22 as a biocontrol in liquid eggs, energy drinks, skimmed milk, apple juice and chicken breast inoculated with *S.* Typhimurium, there was a reduction of 2 logs when compared to untreated controls [21]. The results indicated that phages may be useful in controlling foodborne pathogens [21]. Thus, these studies corroborate the purpose of this experimental study, where the objective was to perform the isolation, sequencing and characterization of lytic bacteriophages to be used in the biological control of different *Salmonella enterica* serovars.

## 2. MATERIALS AND METHODS

The assays for isolation, purification, production, titration and phenotypical characterization of bacteriophages were performed in Bacteriology and Mycology Laboratory in the Veterinary Hospital at the Faculty of Agronomy and Veterinary Medicine of the University of Passo Fundo (FAMV/UPF).

### 2.1 *Salmonella enterica* Samples

For the isolation and evaluation of the phage lytic spectrum, a sample of each of the following *Salmonella enterica* serovars were used as a host: *Salmonella anatum, S. agona, S. brandenburg, S. bredeney, S. infantis, S. lexington, S. panama, S. rissen, S. schwarzengrund, S. tennessee, S. enteritidis* ATCC 13076 and *S. typhimurium* ATCC 14028. All bacteria were previously isolated and their purity was confirmed by culture in selective medium and biochemical tests. In Table 1, it can be seen the characteristics of host *Salmonella enterica* samples used in this study.

### 2.2 Verification of Host Bacteria for the Presence of Prophage

*Salmonella enterica* serovars were tested for the absence of prophage in their DNA to avoid a false positive result for the presence of bacteriophages. For this test, 100 µL of each host bacterium were cultured in Petri plates with a thin layer of tryptone soy agar (TSA) and a 5 mL overlay of a semi-solid medium composed of 94% tryptone soy broth (TSB) and 0.6% agar. Further, the host bacteria were individually inoculated in BHI broth (brain heart infusion) and incubated at 37 ± 1ºC for 24 h. After that time, 50 aliquots of 10 µl of the inoculum were used and compared with the hosts themselves in order to verify the presence or absence of phages. The plates were incubated for 24 h at 37 ± 1ºC to evaluate lysis areas [26].

### 2.3 Enrichment of Isolation Sources and Host Bacteria

After the incubation period the inoculum was transferred to the TSB broth in double concentration and incubated for an additional 24 h at 37 ± 1ºC. Two sources were used for isolation: samples from poultry feces and wastewater from poultry slaughter. To enrich the isolation sources, we used Erlenmeyer flasks with 100 mL of 0.9% saline, 10g of the stool sample and 10 mL of the effluent sample. 50 µL of each host bacteria prepared individually was also add to the Erlenmeyer flasks incubated at 37 ± 1°C for 24 h with shaking (120-180 rpm). Afterwards, we centrifuged at 9,000xg, 4°C for 10 min, filtered through a 0.22 µm membrane and collected the supernatant in sterile flasks [26].

The plate assay method was used as an initial test for the presence of phage, a procedure based on the semi-solid overlay technique with modifications [27]. An individual inoculum of each host bacterium was added to tubes with 5 mL of semi-solid medium, which was used to form an overlay on Petri plates already prepared with TSA agar. We pipette 10 aliquots of 10 µL supernatant (filtrate) into the plates and incubated at 37 ± 1ºC for 24 h and checked for phage lysis plates.

**Table 1. Characteristics of host *Salmonella enterica* samples**

| *Salmonella enterica* serovar | Biofilm formation capacity at 36ºC [23,25] | Virulence genes [22, 23] | | | | | | | | Antimicrobial Resistence [23,25] | Isolation area | Year |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cell invasion | | Fimbriae | | Effector proteins | | | Biofilm | | | |
| | | invA | hilA | ipfA | agfA | avrA | sopE | sivH | spiA | | | |
| 1- Brandenburg [24] | No | + | + | - | + | - | + | + | + | SOX | Carcass after cleaning | 2012 |
| 2- Anatum [24] | Strongly | + | + | - | + | + | - | + | + | SOX, SUL | Cloacal swab | 2012 |
| 3- Tennessee [24] | Weak | + | + | + | + | + | - | + | + | SPT, SOX, TET, SUT, GEN, CAZ, ATM, AMC, CTX | Frozen carcass 24 h | 2012 |
| 4- Agona [24] | Weak | + | + | + | + | + | - | + | + | SOX, SUL, ENRO | Coop | 2012 |
| 5- Bredney [24] | Moderate | + | + | - | + | + | - | + | + | SPT, SOX, SUT, CHL, SUL | Cloacal swab | 2012 |
| 6-Schwarzemgrund [24] | Weak | + | + | - | + | + | - | + | + | SPT, SOX, SUT, CHL, SUL | Cloacal swab | 2012 |
| 7- Infantis [24] | Weak | + | + | + | + | + | - | + | + | SPT, SOX, SUT, CHL, SUL, GENENRO, CAZ, ATM, AMC, CTX | Coop | 2012 |
| 8- Rissen [22] | No | + | + | + | + | + | - | + | + | SUL | Cloacal swab | 2013 |
| 9- Lexington [22] | No | + | + | + | + | + | - | + | + | SUL | Sponges before washing | 2013 |
| 10- Panama [22] | Weak | + | + | - | + | + | - | + | + | SUL, ATM, AMC, CTX | Cloacal swab | 2013 |
| 11- SE ATCC | Moderate | NR | NR | NR | NR | NR | NR | NR | NR | NR | - | - |
| 12- ST ATCC | Moderate | NR | NR | NR | NR | NR | NR | NR | NR | NR | - | - |

*Active principles tested: Sulfonamide (SUL), Chloramphenicol (CHL), Gentamicin (GEN), Tetracycline (TET), Ampicillin (AMP), Enrofloxacin (ENRO), Ceftazidima (CAZ), Aztreonam (ATM), Amoxacilin + clavulanic acid (AMC), Cefotaxime (CTX), Amoxacilin (AMX), Ceftiofur (CTF), Ciprofloxacin (CIP), Spectinomycin (EST), Sulfafurazole (SOX), Sulfa + trimetoprim (SUT), Streptomycin (SPT), + It has the resistance gene; - It has not the resistance gene; NR: It was not performed; S: It is sensitive to all active principles*

### 2.4 Bacteriophage Isolation and Purification

We prepared a bacterial overlay with Petri plates to isolate the phage from the lysis zone, always using the same host bacteria for their respective phage. We analyzed the morphology of bacteriophages in the plaque by checking the phenotypic characteristics: plaque size differences, clear/cloudy appearance, halo around the lysis plaque, formation of bacterial colonies at the lysis site.

We selected phage plaques and, with a sterile toothpick, transferred them to a new petri dish previously prepared with bacterial overlay. We inoculate by sting countless times and use strips of sterile paper to stretch and spread the phages on the plate. We incubated at 37 ± 1°C for 24 h and repeated this procedure until all bacteriophage plaques were uniform [26].

### 2.5 Bacteriophage Production and Tritation

To produce the phages, we prepared overlay Petri dishes containing the host bacteria. We selected the phages with a sterile toothpick and chopped numerous times. We use sterile strips of paper to evenly spread the phages on the plate and ensure maximum replication [27]. We incubate at 37 ± 1ºC for 18 to 24 h. We added 5 mL of SM buffer [5.8 g NaCl, 2 g MgSO4, 50 mL Tris/HCl (pH 7.5), 1 L distilled water] to each plate and incubated under shaking (90 rpm) at 4°C overnight. We collected the liquid for Erlenmeyer and added 0.584 g of NaCl for each 10 mL of sample, incubated at 4°C for 1 h. We centrifuge 9,000xg, 4°C for 10 min and added 1 g of Polyethylene Glycol 8000 (PEG8000), incubated for 24 h at 4°C with shaking. Centrifuge 9,000xg, 4°C for 10 min, discard supernatant, resuspend pellet with 4 mL SM buffer, add 1 mL chloroform, vortex for 1 min. We centrifuge 9,000xg, 4°C for 10 min, and collect the supernatant for sterile vials.

Serial dilution of the isolated phage stock solution 109 in 900 µL of SM buffer using 100 µL of the phage solution from 10-1 to 10-10 in sterile Eppendorf was performed and rested for 15 min for pre-adsorption of phages. After this time, we added 100 µL of the same host bacterium used in isolation onto previously prepared TSA Agar petri dishes and poured 5 mL of semi-solid medium with diluted phage and incubated at 37 ± 1°C for 24 h [27]. Visible lysis plates were observed between 6h and 24 h, depending on the phage. To determine the phages titers, we calculate the titration for each bacteriophage according to the equation below.

$$\text{Bacteriophage titration (PFU/mL)} = \frac{\text{Number of Lysis Plates x Dilution Factor}}{\text{Bacteriophage Sample Volume (mL)}}$$

### 2.6 Lytic Spectrum and Plaque Assay Efficiency against Different *Salmonella enterica* Serovars

To verify phage infection capacity in its host, we prepared Petri dishes with TSA agar, overlaid with semi-solid medium, and the corresponding host bacterium. We pipetted 5 individual 20 µl drops of phage into the plates and incubated at 37 ± 1°C for 24 h. We observed lysis zones according to halo shape and phage's ability to infect the host. To verify the efficiency of phage infection against the host and other serovars, we serially diluted the concentrated phage to $10^{-8}$, prepared Petri dishes with TSA agar and overlaid with 100 µL of the bacterium in semi-solid medium, micro drops by pipetting 1 drop of 10 µL from each dilution [26]. The bacteriophage titration was adjusted to $10^{9}$ PFU/mL, and the determination of plaque phage infection efficiency was the phage titer relative to *Salmonella enterica* serovar used, compared to the maximum observed titration. We classified plaque efficiency into high (100-10%), medium (1-0.1%), and low (0.01-0.001%) scores according to the phage titer for each serovar.

### 2.7 Molecular Characterization of Bacteriophages

The molecular characterization of bacteriophages was performed in the Federal University of Rio Grande do Sul (UFRGS).

#### 2.7.1 Bacteriophage DNA extraction

The bacteriophage solutions were treated with RNAse (1µg/mL), proteinase K (50 µg/mL) sodium dodecyl sulfate-SDS (0.5%), incubated for 1 h at 56 ± 1°C. From this stage, the phage DNA was extracted using the phenol/chloroform system and ethanol precipitated [28]. DNA integrity was verified by electrophoresis using 0.7% agarose gel and the concentration and purity were determined in the Nanodrop ND-1000 equipment, where the parameters are A 260/280,

which evaluates possible protein contamination, and the values should be <1.8, and A260/230, which assesses possible contamination or residues of organic compounds, where values should be between 2.0-2.20.

#### 2.7.2 Restriction fragment length polymorphisms–RFLP

The methodology uses a combination of two restriction enzymes and nucleic acid digestion. The samples were inoculated on an agarose gel and the RFLP restriction fragments were separated according to their length. Visualization was verified by electrophoresis. To obtain comparable results of phage RFLPs, restriction with *HindIII* and *BamHI* enzymes was performed using the Lane M – *λ HindIII* marker.

#### 2.7.3 Sequencing bacteriophages genomes

Phage DNA was isolated from purified aliquots [28] and DNA libraries were further prepared according to the standard protocol of Nextera XT kit. Sequencing was performed in IlluminaMi-Sequsing a short-read technology and paired libraries (2x150nt). Sequence samples were preprocessed using SeqClean to remove PCR duplicates, contaminants, and sequencing adapters [29]. Besides, the low-quality bases were removed (SeqClean Parameter - quality 0.01-0.01), leaving only bases greater than 120 bp (GRC_Scripts, Seqclean). The genomes were assembled using Newbler and Velvet software, and subsequent analyzes performed on BLAST, PHAST, Virfam, and Geneious software.

#### 2.7.4 Analysis of phage morphology by transmission electron microscopy (TEM)

The analysis of phage morphology by transmission electron microscopy (TEM) was performed in the Center for Microscopy and Microanalysis at the Faculty of Veterinary of the Federal University of Rio Grande do Sul (UFRGS). The morphology of the isolated phages was evaluated by TEM. The volume of approximately 10 µL of samples containing the isolated bacteriophages was pipetted onto a 400 *mesh* grid. The grid was incubated at room temperature for 1 min and excess liquid was removed with filter paper. Viral particles were contrasted with 2% uranyl acetate for 20 seconds and analyzed by electron microscope of JEOL - MET JEM 1200 EX11.

## 3. RESULTS

### 3.1 Isolation and Characteristics of Phenotypic Bacteriophages

#### 3.1.1 Bacteriophage selection after isolation source enrichment

We observed lysis plaques in both isolation sources. For the stool samples of poultry, there were lysis plaques when the hosts *S.* Anatum, *S.* Tennessee, *S.* Rissen, *S.* Enteritidis and *S.* Typhimurium were used. For the samples of wastewater from slaughter in a poultry refrigerator, we observed lysis on the plates containing the hosts *S.* Anatum, *S.* Brandenburg, *S.* Bredeney, *S.* Schwarzengrund, *S.* Infantis, *S.* Rissen, *S.* Lexington, *S.* Panama, and *S.* Typhimurium. Therefore, we selected phages that presented lysis plate parameters, such as turbid/light appearance, halo, and size, and excluded those with a bacterial colony.

#### 3.1.2 Bacteriophage isolation, purification and production

We obtained different phages from each other from the fecal sample of poultry as a source of isolation, but from distinct hosts, which we initially denominate phage UPF_BP1, related to isolation from *S.* Brandenburg (Fig. 1) and as phage UPF_BP2, isolated using *S.* Bredeney (Fig. 2).

#### 3.1.3 Lytic spectrum, plaque assay efficiency and lysis potential of isolated phages against different *Salmonella enterica* serovars

We evaluated the phages ability to infect its hosts, even when diluted. To verify the efficiency of this infection we used serial phage dilution and thus obtained the results shown in Table 2, showing that phage UPF_BP2 was able to act in all *Salmonella enterica* serovars that were confronted.

### 3.2 Genomic Properties

#### 3.2.1 Bacteriophage DNA extraction and RFLP profile

After DNA purification, the DNA concentration was 109.8 ng/µL for phage UPF_BP1, (A 260/280 = 1.86 and A 260/230 = 2.13). For the phage UPF_BP2 the concentration was 23.9

ng/µL and (A 260/280 = 1.81 nm and A 260/230 = 2.02 nm).

The RFLP profile, the phage UPF_BP1 was fragmented, while the phage UPF_BP2 proved to be resistant to *HindIII* and *BamHI* restriction enzymes, and fragmented visualization was not possible.

### 3.2.2 Sequencing bacteriophages genomes

We sequenced the genome and analyzed some features of phages molecular biology. Regarding to phage UPF_BP1 (NCBI Reference Sequence: NC_047875.1), analyzes revealed 71 phage open reading frames (ORFs). Of the 71 ORFs identified, roles were assigned to 40 of them. Besides, 6 ORFs corresponded to the *ea* and *nin* genes.

The excisionase-related gene *xis* showed 100% identity compared to the corresponding protein. The gene involved in abc1 DNA metabolism, which codes for a protein with anti-RecBCD function, was 100% identical to that of the ST104 phage, while the *gp12* (helicase) and *gp13* (primase) genes are related to genome replication and had a 100% identity compared to p22 phage [30].

Genes involved in phage structure and assembly can be divided into coding terminases (*gp2*, *gp3*), capsule gene (*gp40*), DNA injection (*gp45, gp46*), or tail gene (*gp42 e gp43*). The *gp1*, *gp5*, and *gp8* genes showed an identity greater than 99% concerning P22 phage [31]. The terminase, encoded by *gp3*, showed 100% homology with the *Salmonella* phage SETP1, while those encoded by *gp2*showed 100% homology with the *Salmonella* phage ST160 and the *gp56* gene, associated with lysozyme protein, was 75% similar to Phi80 phage [31].

Regarding to phage UPF_BP2 (NCBI Reference Sequence: NC_048649.1), analyzes revealed 70 phage open reading frames (ORFs). Of the 70 ORFs identified, roles were assigned to 26 of them. Genes involved in phage structure and assembly can be divided into coding terminase (*gp54*), capsid gene (*gp51*) or tail gene (*gp31, gp32, gp33, gp40, gp56*). It also has the holina gene (gp30) responsible for opening pores in the cytoplasmic membrane of the target bacteria and endolysin (gp29), which has a proteolytic function of breaking the bacterial cell wall [31,32].

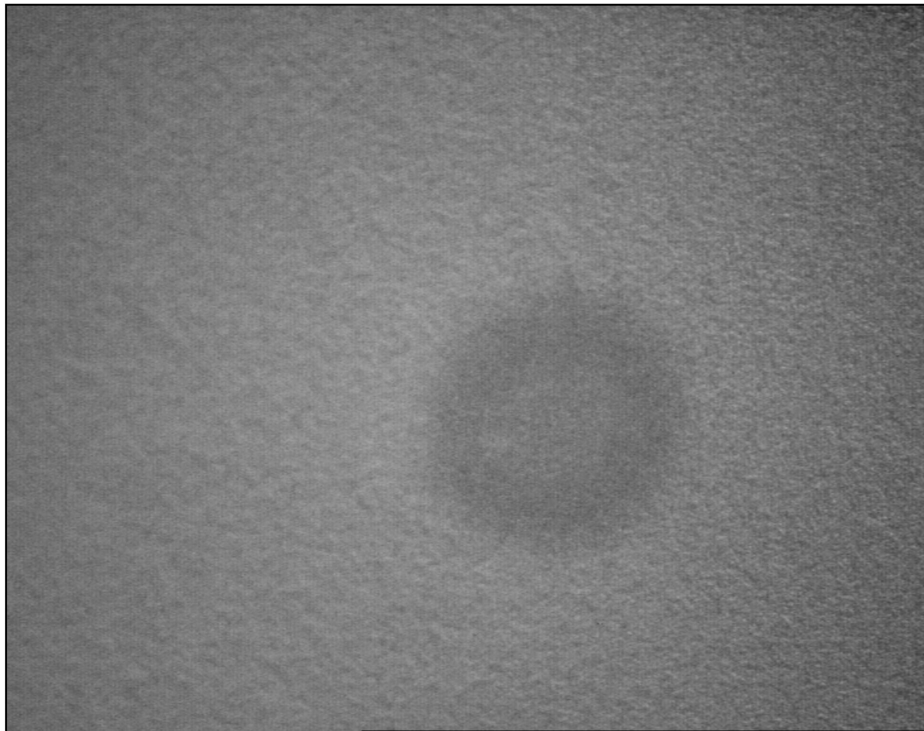

**Fig. 1. Morphology of phage lysis plaque UPF_BP1 in isolation with *Salmonella* Brandenburg**

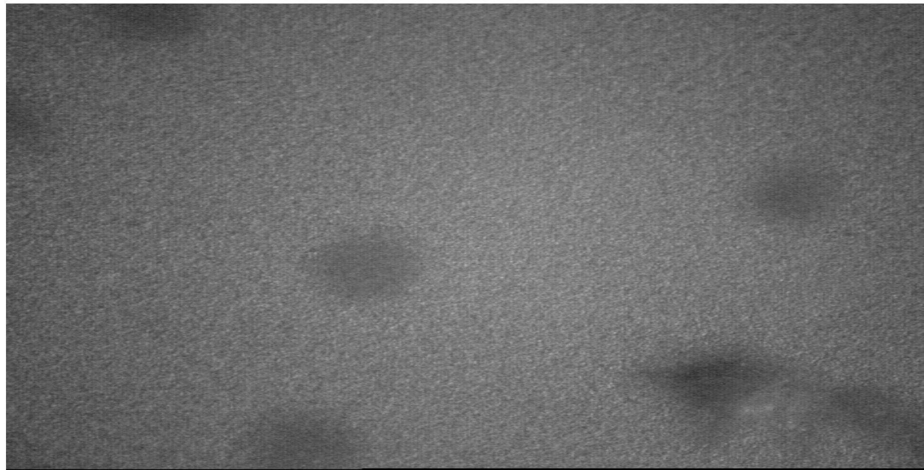

**Fig. 2. Morphology of phage lysis plaque UPF_BP2 in isolation with *Salmonella* Bredeney**

**Table 2. Infection efficiency of isolated phages**

| ***Salmonella enterica* samples** | **Phages** | |
|---|---|---|
| | **UPF_BP1** | **UPF_BP2** |
| *S.* Brandenburg | High | Moderate |
| *S.* Anatum | NI | High |
| *S.* Tennessee | High | High |
| *S.* Agona | High | High |
| *S.* Bredeney | NI | High |
| *S.* Schwarzengrund | High | High |
| *S.* Infantis | NI | High |
| *S.* Rissen | High | High |
| *S.* Lexington | High | High |
| *S.* Panamá | NI | Moderate |
| *S.* Enteritidis | NI | High |
| *S.* Typhimurium | High | High |

*Score: High (>10%), moderate (0.1-1%) and low (< 0.1%); NI- Not infect serovar. Phage UPF_BP1: isolate from Salmonella Brandenburg; Phage UPF_BP2: isolate from Salmonella Bredeney*

Sequencing of the phage UPF_BP1 resulted in a circular, double-stranded DNA genome of 39,902 bp. It showed homology with enterobacterial phage and *Salmonella* phages found in the databases. Sequencing of the phage UPF_BP2 resulted in a circular DNA and genome of 54,894. Sequencing data were entered into the Virfam program, which analyzed the similarities between their genes, their organization and the identification of hypothetical genes with the aforementioned bacteriophages. From these data, it can be determined that the genome of two new hitherto undescribed bacteriophages were identified and sequenced, belonging to the order *Caudovirales*, the phage UPF_BP1 related to the *Podoviridae* Type III family and phage UPF_BP2 related to the *Myoviridae* family, both registered with GenBank under numbers KX776161 and KX826077, respectively.

### 3.3 Morphological Analysis

Morphological analyzes of the phages performed by transmission electron microscopy showed that *Salmonella* phage UPF_BP 1 (Fig. 3A and 3B) have an icosahedral head and short tail (indicated by the arrow). For *Salmonella* phage UPF_BP2 (Fig. 4A and 4B), short-tailed and icosahedral head phages can be identified.

## 4. DISCUSSION

In the present study, bacteriophages were isolated from samples of poultry feces. Twelve *Salmonella enterica* serovars were selected as host bacteria. It is worth mentioning that lysis plates were also observed in wastewater from poultry slaughter when used as a source for

isolating bacteriophages, however, when wastewater was used, lysis plates were observed in most hosts (9/12-75%) when compared to stool samples (5/12-41%). A few reports suggesting that the presence of bacteriophages in sewage could be useful in wastewater treatment [33]. Furthermore, it has been suggested that phages can act as biological tracers of pathogenic bacteria in water and wastewater treatment [34].

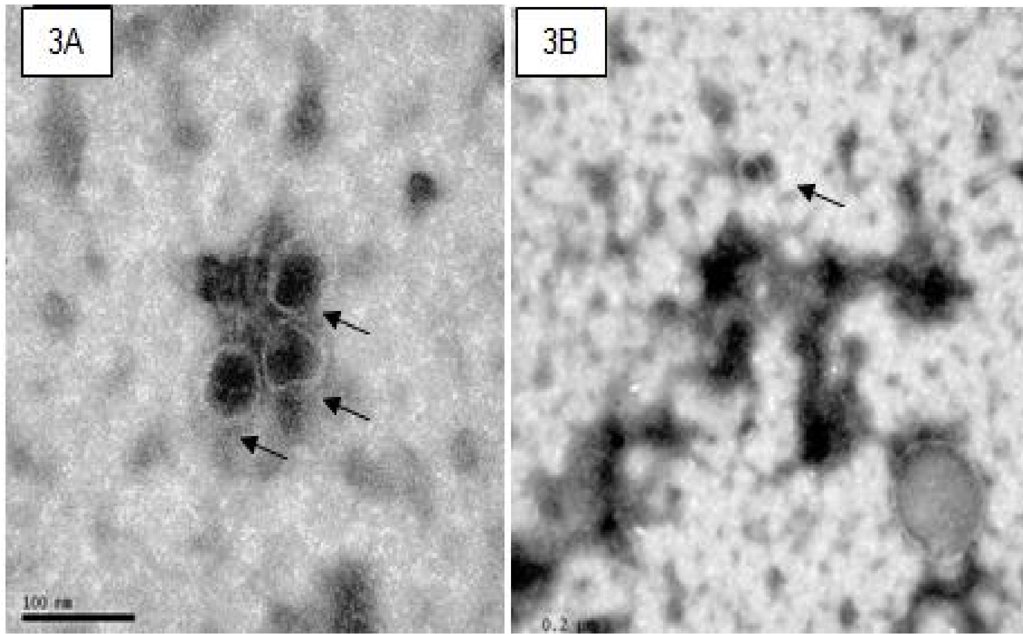


**Fig. 3. Transmission electron microscopy of an aliquot of the *Salmonella* phage UPF_BP1. Arrows indicate the viral particles. Phages with rounded head and short tail. The bars correspond to 100nm (3A) and to 0.2µm (3B)**

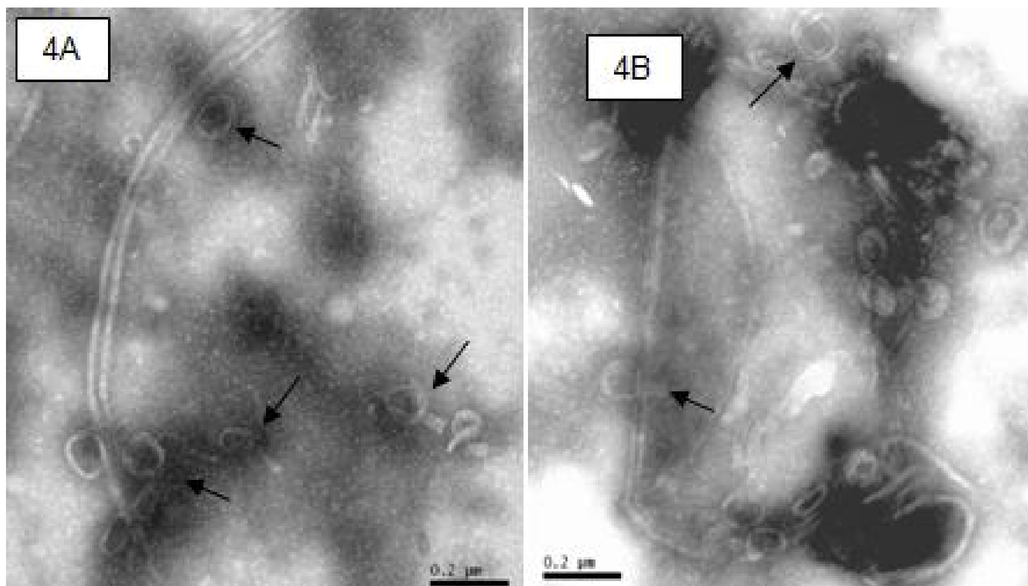


**Fig. 4. Transmission electron microscopy of an aliquot of the *Salmonella* phage UPF_BP2. Arrows indicate the viral particles. (4A) and (4B) - Phages with rounded head and short tail adhered to a bacterial debris. The bars correspond to 0.2µm**

More than 50% of the hypothetical proteins predicted in phage genes with unassigned function are due to a lack of experimental data. As an example, the phage T4, one of the most extensively characterized bacteriophages, whose genome has 168,903 bp, with about 300 probable genes, and almost half of them do not yet have an assigned function [35]. Therefore, identifying their functions is a challenge that must be addressed to increase the knowledge of bacteriophages and the safety level of their applications. In this regard, it should be considered that none of the hypothetical proteins showed a significant resemblance to factors known or involved in bacterial pathogenicity, therefore, they are unlikely to play a relevant role in bacterial virulence [31]. Many of these hypothetical proteins may probably be involved in recognizing and disrupting host metabolism. Thus, they are potential candidates for detection and use in phage therapies [35].

The 6 ORFs corresponded to the *ea* and *nin* genes, which are not essential for bacteriophage function, at least in vitro cultures, but their presence and maintenance suggest that they may confer a selective advantage to the bacteriophage itself when present in other environments [36]. Of the remaining 31 ORFs, they show similarity to the hypothetical coded proteins already described, but their functions have not been determined. Lysogenesis in a phage requires integrase activity, which is encoded by the int gene, and this protein showed 100% similarities compared to the *Salmonella* ST64T bacteriophage homolog [31]. The *cro* gene is directly related to the maintenance between lytic or lysogenic cycle and showed 76% similarity with *Escherichia coli* phage. Those coded by *mnt, arc*, and *ant* are involved in controlling the maintenance of lysogeny [37], and showed high similarity, 93% with P22 phage, 100% with Phi75 phage, and 85% with phage CUS-3, respectively.

The bacteriophage UPF_BP2 expresses the genes for holina and endolysin. The holina which originate pores in the cytoplasmic membrane of the host bacterium which allows the endolysins to reach and degrade the peptidoglycan layer that is the main component of the bacterial cell wall, allowing the produced phages to leave the cell host and reinfect other bacterial cells [32].

The action of endolysins occurs in the final phase of the phage's reproductive cycle, as they are involved in the lytic cycle, in which the phage invades the bacteria, which has its normal functions interrupted in the presence of the virus nucleic acid (DNA or RNA) [32]. Some studies also demonstrate the ability of endolysins to destroy pathogenic bacteria in biofilms and the fact that they are resistant to antibiotics, end up contributing to the fight against many human infections [38].

A study of phages morphology is desirable for comparison with other enterobacterial phages and purposes of identity control during propagation [39]. The morphological analyzes carried out in this study demonstrate that the morphology is in agreement with the sequencing analyzes, demonstrating characteristics similar to other phages belonging to the *Podoviridae* family. The *Salmonella* phage UPF_BP2 show morphological characteristics similar to phages of the *Myoviridae* family, according to the sequencing results. Another fact that can be observed is the presence of bacterial debris, making images darker, making phage responses more difficult. Studies on the morphology of *Salmonella* phages are shown as heads stained with uranium acetate are sometimes positively shown and then appear as deep black, shrunk and usually surrounded by a halo that increases with exposure to the beam [40].

## 5. CONCLUSION

The use of bacteriophages provides new possibilities for the detection and control of pathogenic bacterial agents. The specificity of the target microorganism, few side effects, and the relative ease of phage production make them ideal tools for use as phage therapy. The bacteriophages *Salmonella* phage UPF_BP1 and *Salmonella* phage UPF_BP2 demonstrated lytic action against *Salmonella enterica* tested serovars, feeding our expectations for the development of alternatives for the use of antimicrobials, being possible candidates for use as *Salmonella enterica* biocontrol in foods, animals and in the environment. However, a better general understanding of phage biology is still needed. Recent advances in genome sequencing, comparative genomics, and other genomic studies undoubtedly play an important role in filling this knowledge gap.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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