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Isolation and Morphological Characterization of New Bacteriophages Active against *Campylobacter jejuni*

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Abstract

Campylobacter jejuni (C. jejuni) is a food borne pathogen that causes gastroenteritis worldwide and neurological diseases in humans. Poultry meat is considered the principal source for human infection; in particular when undecooked chicken meals are consumed. Phages against *Campylobacter* spp. could represent innovative weapons to use for reducing pathogen loads in chickens (phage therapy) or to decontaminate broiler meats. For this purpose, new protocols for phage isolation should be promoted in order to detect a more diversified group of *Campylobacter* spp. predators from the environment. Moreover, phage morphological characterization by the use of Transmission Electron Microscope (TEM) could contribute in the process of selection of efficient phage to exploit in phage therapy and meat decontamination for enhancing human health and safety.

Keywords: Campylobacter; Bacteriophages; Phage Therapy; Morphological characterization

Introduction

C. jejuni is a gram-negative thermotolerant micro aerobic pathogen that causes human gastroenteritis worldwide. Poultry is considered the natural reservoir of the bacterium and the animals show no symptom of disease. *C. jejuni* is found in the intestinal contents of most birds and it is frequently isolated from poultry meat at retail. Consumption of undercooked or infected poultry meat is therefore considered the most representative cause of human gastroenteritis [1]. In humans, the infection usually causes self-limiting diarrhoea but can occasionally lead to serious illnesses, such as Guillain-Barre syndrome and reactive arthritis [2]. The majority of human gastroenteritis is attributed to *C. jejuni*, while infection by other members of *Campylobacter* genus is relatively infrequent. The number of confirmed human cases of campylobacteriosis in the EU in 2015 was estimated of about 229,213 and the disease is still the most frequently notified among member States [3].

Moreover, the problematic increase in recent years of antibiotic resistance, both in medicine and agriculture, has lead the interest of scientists towards new weapons to potentially use against pathogenic bacteria. The therapeutic use (phage therapy) of naturally occurring viruses called bacteriophages, that selectively prey and kill bacteria, has the potential to specifically target *Campylobacter* in poultry, thus reducing the number of these bacteria entering the human food chain [4]. Phages have many potential advantages over traditional antibiotics, including specificity for the target bacterial organism, an apparent lack of toxicity or immunogenicity and the relative easiness related to their isolation and harvesting in laboratory facilities. Despite these advantages, development and use of phage therapy has been limited and mostly confined among Eastern Countries since '20s, though the isolation of bacteriophages active against *Campylobacter* has been reported from different sources [5-10].

The purpose of this study was to assess a new protocol for the isolation of phages active against *C. jejuni*. In particular, a specific "pre-enrichment" phase was developed for the detection of weak and low number phages, thus enhancing the probability to isolate these viruses from the environment. Moreover, the use of a mix of *C. jejuni* strains was assessed in order to evaluate the possibility to isolate a more diversified group of phages. In fact, many authors suggested protocols that use only specific sensitive *Campylobacter* strains as hosts, thus potentially limiting the variety of phages to be isolated [5,9,11,12]. Instead, we are aware that the success of the phage therapy is also based on phage cocktails possessing a wide host spectrum in order to target and reduce

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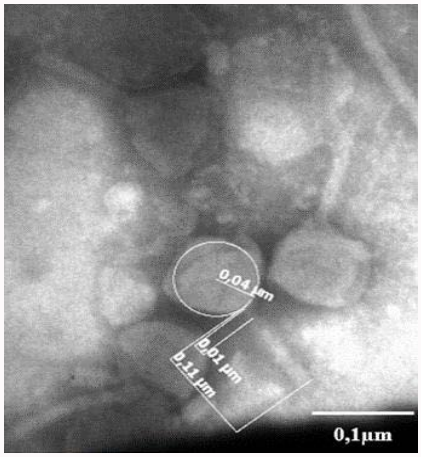

Figure 1: Phage 7 under transmission electron microscope observation (50,000 x).

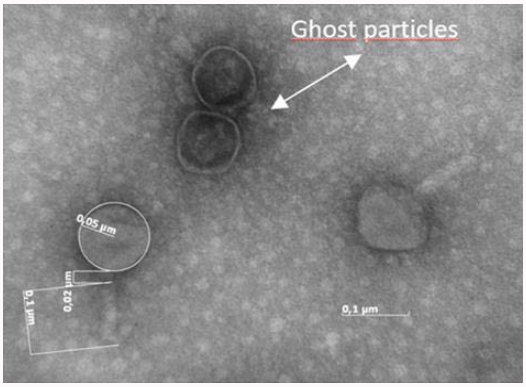

Figure 2: Phage 10 under transmission electron microscope observation (50,000 x).

the diverse populations of naturally occurring *C. jejuni* in poultry [13,14]. Some of the isolated phages were further more subjected to morphological characterization by TEM in order to acquire new important information from their ultrastructure analysis such as body size, physical integrity and genome characteristics (DNA or RNA phage's).

The phages described in this paper will be further assessed for their *in vitro* efficacy against *C. jejuni* strains in order to be selected for potential application in phage therapy and poultry meat decontamination. These approaches could be useful to enhance human safety thus reducing the risk for humans to be infected of campylobacteriosis from consumption of potentially contaminated broiler meat [4].

Materials and Methods

Assessment of a new protocol for phage isolation: phage and host strains, media and culture conditions

Nine strains of *C. jejuni* were used as hosts for phage isolation. Since it is well known that the somatic and flagella antigenic differences among *Campylobacter* strains influence the variability of the phages that can be isolated from the environment [15], the bacterial strains used in this phase have been selected according to different Penner serotypes, PFGE patterns and flagella antigens (Table 1). *C. jejuni* NCTC12662, known to be extremely susceptible to phage infection, has been chosen as host for phage CP220 replication and titration

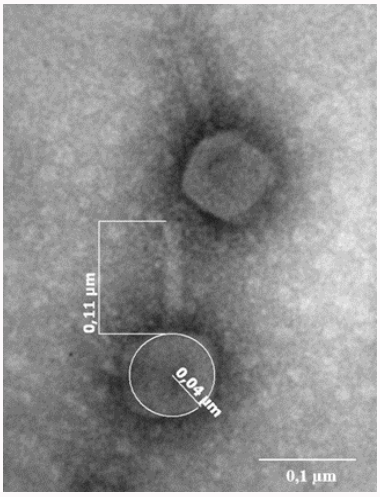

Figure 3: Phage 16 under transmission electron microscope observation (50,000 x).

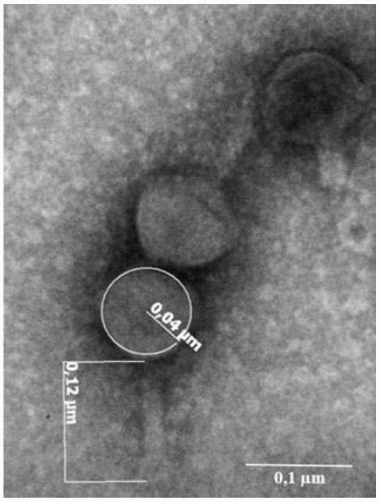

Figure 4: Phage 17 under transmission electron microscope observation (50,000 x).

(Spot Assay). Phage CP220 was used as control in the assays.

For phage isolation, each bacterial strain was seeded separately on Columbia blood agar (Oxoid) and incubated at 42 ± 1°C, in micro-aerophilic conditions (85% nitrogen, 5% oxygen, and 10% carbon dioxide), for 18 h - 20 h. Subsequently, some colonies were transferred to New Zealand Casamino Yeast Broth 1X (10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 1 g/L Casamino Acids, 1 g/L $MgSO_4$, Sigma) supplemented with $CaCl_2$ (1 mm final concentration) and, after reaching an optic density (600 nm) of 0.35, (Eppendorf, Hamburg, Germany) corresponding to about 10^8 colony forming unit (cfu)/ml, the bacterial solution was incubated for 4 h at 37 ± 1°C under micro-arophilic conditions. Then, 200 µl of each culture were mixed together to constitute two different bacterial solutions (pools), each one consisting of field *Campylobacter jejuni* strains plus the NCTC12662 strain. In particular, the host solutions referred to as pool 1 included strains NCTC12662, 252aM/11A, 252bM/11A, IZSAM/25 and 252cM/12A while the pool 2 included strains NCTC12662, 252dM/12A, 252eM/12A, 252fM/12A and 252 gM/12 A. For developing the pre-enrichment phase of this protocol, scalar dilutions (base 10) were prepared from phage CP220 in SM buffer

Table 1: List of *C. jejuni* host strains used for protocol assessment and phage isolation.

C. jejuni	Pennersero type	PFGE type (SmaI)	PFGE type (Kpn)	Fla-SVR
NCTC 12662	HS:5j	-	-	-
252aM/12A	HS:1.44	1	I	Allele 36
252bM/12A	HS:3	2	II	Allele 14
252cM/12A	HS:52	3	III	Allele 222
252dM/12A	HS:57	4	IV	Allele 287
252eM/12A	HS:4,13,16,43,50	5	V	Allele 1284
252fM/12A	HS:5	6	VI	Allele 287
252gM/12A	HS:55	7	VII	Allele 265
IZSAM/25	HS:15	8	VIII	Allele 1638

Table 2: Pre-enrichment phase: phage CP220 replication using *C. jejuni* host strain NCTC12662, host culture pool 1 (mix of *C. jejuni* host strains NCTC12662, 252aM/11A, 252bM/11A, IZSAM/25 and 252cM/12A) and pool 2 (mix of *C. jejuni* host strains NCTC12662, 252dM/12A, 252eM/12A, 252fM/12A and 252gM/12A).

Pre-enrichment phase	Pre-enrichment phase	Phage titre overall increase (log)
Phage CP220 (10^3 pfu/ml)+NCTC12662	3×10^7	4
Phage CP220 (10^3 pfu/ml)+Pool 1	3×10^6	3
Phage CP220 (10^3 pfu/ml)+Pool 2	3×10^8	5

(Tris-HCl with NaCl, $MgSO_4$ and 0.01% gelatin) until reaching the titer of 10^3 plaque forming unit (pfu)/ml. Then the pre- enrichment phase was assessed by using both host culture pools separately, following this procedure: 4 ml of 10^3 pfu/ml of phage were added to 1 ml of NZCYM 5X with 5 µl of $CaCl_2$ 1M and 50 µl of host pool 1 and pool 2, separately. At the same time, a pre-enrichment step was set up by using only NCTC12662 reference strain, as control. After incubation for 18 h to 24 h at 37°C in micro aerophilic conditions and agitation (VWR, USA) at 125 Revolutions Per Minute (rpm), 1% chloroform solution (Sigma-Aldrich) was added to the cultures, following centrifugation (Eppendorf, Hamburg, Germany) at 4500 g for 15 min. The supernatants were filtered through 0.45 nm filters and CP220 phage was titrated using the Spot Assay [16] technique. This experiment was replicated three times and the results were expressed as the mean number of the phage titre values obtained.

Isolation of bacteriophages, purification and host spectrum evaluation

One-hundred-ninety-eight samples were analyzed for the isolation of bacteriophages active against *C. jejuni*. They were represented by 103 cloacals wabs, 51 fresh feces, 32 boot socks and 12 water samples from the cooling systems of poultry farms. The choice of these samples derived from the postulate that phages are more present in those places was their hosts are abundant [11].

The 9 *C. jejuni* strains (Table 1) were harvested and used to constitute pool 1 and pool 2 host culture broths. Four mL of each filtered sample (0.45 µm) and phage CP220 (control) were separately added to 1 ml of NZCYM 5X with 5 µl of $CaCl_2$ 1M and 50 µl of pool 1 and pool 2 broth cultures and subjected to the pre-enrichment phase as described before. Phage detection and evaluation of host spectrum were performed using the Spot Assay technique [16], with some modifications. Briefly, 500 µl of each bacterial strain culture were added individually to 4 ml of NZCYM soft agar (NZCYMB supplemented with 0.6% of Agar, Sigma), as described before. The presence of phages against *C. jejuni* was evaluated by spotting 10 µl of each sample (filtered with filters 0.45 µm) and pre-enriched suspensions on the plates of NZCYM agar seeded with hosts and incubated for 24 h to 37 h ± 1°C in microaerophilic conditions. In case of phage detection (presence of plaques), plaques were subjected to three-step purification. Briefly, for each purification step, the plaques were individually picked up from the agar and eluted into 2 ml of SM buffer [0.05 M TRIS, 0.1 M NaCl, 0.008 M $MgSO_4$, 0.01% (weight in volume) gelatin, pH 7.5] for 8 h to 9 h at room temperature; then the supernatant was filtered with 0.45 µm filters. Subsequently, 1 ml of host broth culture was mixed with 1 ml of the supernatant left for 15 minutes at 37 ± 1°C in aerophilic conditions, to allow phages to attach on the bacterial cells. At the end of the incubation, the culture was added to 4 ml of NZCYM soft agar, poured on a plate of NZCYM agar and incubated at 37 ± 1°C in micro-aerophilic conditions for 48 h. After incubation, the plate was coated with 5 ml of SM buffer. The soft agar was gently fragmented with a sterile loop and left to elute for 6 h at room temperature under gentle agitation (60 rpm). The eluated suspension was then filtered with 0.45 µm filters and subjected to titration as described before. This procedure was repeated 3 times. Isolated and purified bacteriophages were centrifuged for 2 h at 40,000 g at 4°C (Beckman JS-21, JA20 rotor) and the pellets were re-suspended in 2 ml of SM buffer until reaching a final titer of 10^8 pfu/ml. In particular, puages Φ 7, Φ 10, Φ 16 and Φ 17 were selected for having showed the best lytic activity pattern and the broadest lytic spectrum (data not shown) and they were subjected to further concentration and purification step with caesium chloride for TEM observation and morphological characterization. In particular, caesium chloride was added to a ≥ $\log_{10}$ 10 PFU ml^{-1} bacteriophage suspensions to a final concentration of 0.75 gr ml^{-1} [17]. Then, phages were subjected to centrifugation at 264,000 g at 4°C for 24 h (Beckman TL-100 ultracentrifuge with TLA 100.3 rotor). Formation of a thin blue band following centrifugation indicated the location of concentrated bacteriophages. The blue band suspension was recovered by piercing the centrifuge tube with a hypodermic needle at a level just below the blue band itself. Residual of caesium chloride was removed by centrifugation of the phage suspension with a Microcon 30,000 Da molecular weight cut off column (Millipore) at 6,500 g for 10 minutes. The retained phage particles were washed twice with 100 µl SM buffer by centrifugation at 6,500 g for 10 minutes. The column was than inverted and the bacteriophages were eluted in 100 µl SM buffer by centrifugation at 6,500 g for 10 minutes

and stored at 4°C in sterile tube.

Phage morphological characterization by transmission electron microscopy

Morphological characteristics of Φ 7, Φ 10, Φ 16 and Φ 17 were assessed with the TEM EM 900 (Zeiss, Hamburg, Germany). Phages were prepared to their highest achievable titre (10^8 pfu/ml) in SM buffer and filtered (0.45 µm), fixed onto 200-mesh copper grids coated with carbon-stabilizer formvar and treated for negative staining with 2% phosphotungstic acid. The grids were examined between 12,000 x and 80,000 x magnification. The images were produced with AxioCam MRm and analysed by AxioVision, Release 4.6 programs (Zeiss, Hamburg, Germany) [18].

Results and Discussions

Phage CP220 increased its titer of 3 logs when using the pool 1 of host cultures for its replication, reaching values of 3×10^6 pfu/ml. With Pool 2, phage CP220 increased its count by 5 logs, reaching the value of 3×10^8 pfu/ml. With the only sensitive strain NCTC12662, phage CP220 increased its titer by 4 logs, reaching the count of 3×10^7 pfu/ml (Table 2). The 18.18% of samples (36/198) was found positive to the presence of phages. Among them, 80.55% (29/36) were fresh faeces and 19.44% (7/36) were cloacal swabs. The phages from 15 samples showed evident lytic activity and ability to propagate. Among these, 2 phages (Φ 7 and Φ 16), both isolated from fresh faeces, showed lytic activity versus the totality of the 9 *C. jejuni* host strains tested.

The results from the use of this new protocol showed that *Campylobacter* strains mixed in pool did not negatively influence the ability of phages to replicate during the pre-enrichment phase. In fact, the phage titers achieved by using host pool 1 and 2 fully met our expectations, resulting in a successful phage replication demonstrated by an increase of its titer with both host strain pools. The pre-enrichment phase of our method can be successfully used to amplify very low numbers of phages present in a sample, thus enhancing the sensitivity of the protocol itself. Noteworthy, a large number of the previously identified and characterized bacteriophages are isolated using *C. jejuni* NCTC12662 as indicator strain due to its high sensitivity towards phages [5,9,11]. Indeed, almost all *Campylobacter* phages isolated up to now could recognize similar host's receptors and consequently the phage collections available may thus not represent the phage diversity needed to target the different populations of *Campylobacter* expressing diverse surface structures. Therefore, this experimental protocol could represent a faster, less expensive and useful means for isolating a more diversified group of phages from the environment than those detectable with other already published protocols [5,9,11,12,]. From the ultrastructure analysis, the four phages observed were all constituted of icosahedral-isometric heads and long contractile tails with visible sheaths. They were located among the *Caudovirales* order, *Myoviridae* family [19], Group A [20], thus belonging to double stranded DNA viruses. Our findings corroborate what is already described in literature. In fact, most of the phage's isolated from the environment are reported to belong to *Myoviridae* family while only few phages to *Siphoviridae* [11] and *Podoviridae* family [21]. In particular, Φ 7 showed a total length of about 190 nm with head of 80 nm of diameter, neck of 10 nm and tail of 100 nm (Figure 1); Φ 10 showed a total length of about 220 nm with head of 100 nm of diameter, neck of 20 nm and tail of 100 nm (Figure 2); Φ 16 showed a total length of about 190 nm with head of 80 nm of diameter, neck and tail of 110 nm (Figure 3); Φ 17 a total length is about 200 nm with head of 80 nm of diameter, neck and tail of 120 nm (Figure 4). In general, *Campylobacter* phages are reported to possess icosahedral heads, long contractile tails and double stranded DNA, with head diameters ranging from 80 nm to 140 nm, and tail lengths range from 95 nm to 120 nm and the morphological findings of the phages isolated in this paper are in agreement with these statements [11]. Moreover, the morphological findings from TEM analyses were useful for comparing and further selection of bacteriophages to employ in phage therapy [18]. Within our phage samples, TEM also enabled us to distinguish between "full" infective virus particles from empty "ghost" particles (Figure 2), represented by viruses after loss of their genetic material, potentially as consequence of stress factors (e.g. heat, UV radiations, high pressures) [22]. In particular, lytic activity in empty phages is performed by cell wall degrading enzymes (lysis from without), but virions cannot replicate [22,23]. Findings that derive from a viral morphological analysis are useful for phage characterization but also for their further *in vivo* applications.

The phages isolated and characterized in this paper, and in particular Φ 7, Φ 10, Φ 16 and Φ 17, will be further assessed for their *in vitro* efficacy for selecting a more limited number of phages for future experiment on meat decontamination and *in vivo* phage therapy trials.

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