Applied Microbiology
Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Method for bacteriophage isolation against target *Campylobacter* strains

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Keywords
Bacteriophage, *Campylobacter coli*, *Campylobacter jejuni*, isolation, method, poultry.

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2009/0670: received 14 April 2009, revised 4 November 2009 and accepted 6 November 2009

doi:10.1111/j.1472-765X.2009.02774.x

Abstract

Aims: Poultry meat is considered a major source of *Campylobacter*. This micro-aerobic bacterium is commonly responsible for foodborne illness. This work focuses on the isolation of *Campylobacter coli* lytic bacteriophages (phages) against target *C. coli* strains.

Methods and Results: A method involving the enrichment of free-range chicken samples in a broth containing the target *C. coli* strains and salts ($CaCl_2$ and $MgSO_4$) was used for phage isolation. This method allowed the isolation of 43 phages that were active against 83% of the *C. coli* strains used in the isolation procedure. Approximately 65% of the phages were also effective against *Campylobacter jejuni* strains.

Conclusions: The use of target pathogens in the phage isolation step improves the likelihood of detecting and isolating phages for the control of these specific strains.

Significance and Impact of the Study: This technique will be valuable in the context of phage therapy for enriching for phages that are active against specifically identified strains of bacteria, for example from a food poisoning outbreak or epidemic strains resistant to multiple antibiotics. In these situations, using the conventional methods for searching for bacteriophages active for these particular strains can be a time-consuming, if not an unsuccessful process. Using the isolation method described in this manuscript, the particular strains can be added to the enrichment broth increasing the probability of finding phages against them. Therefore, it will shorten the time needed for seeking phages able to lyse target strains, which in most of the cases, because of the rapid increase in antimicrobial-resistant bacteria, is of crucial importance.

Introduction

Campylobacter is the main cause of enteric illness in the United States and in several member states of the EU (Friedman *et al.* 2000; Lindqvist *et al.* 2001; Samuel *et al.* 2004), and poultry meat products are considered the major source of campylobacteriosis (Jacobs-Reitsma 2000; Shane 2000). Because these organisms are enteric commensals, they are very difficult to control in poultry, and therefore they are present in the derived products, where they are pathogenic to humans (Jacobs-Reitsma 2000). This situation is made worse by recently introduced restrictions on the use of antibiotics in animal production. Phage therapy offers a promising alternative for controlling these pathogens.

Studies of phage therapy for use against *Campylobacter* have focused mainly on *C. jejuni* because it is the most commonly isolated *Campylobacter* species (Tam *et al.* 2003). *Campylobacter coli* has been considered the second most frequently isolated *Campylobacter* species but its

importance has been greatly underestimated (Tam *et al.* 2003). There has been a decrease in *C. jejuni* and an increase in *C. coli* recorded in broiler chickens (Avrain *et al.* 2003; El-Shibiny *et al.* 2005), and it is recognized that adult birds carry higher numbers of *C. coli* than young birds (Petersen *et al.* 2001). The control of *C. coli* in broilers is therefore of extreme importance. Furthermore, it has been suggested that *C. coli* is more resistant to some antimicrobials than is *C. jejuni* (Korolik *et al.*1998). Aarestrup *et al.* (1997) demonstrated that the occurrence of resistance for the 16 antimicrobial agents tested was generally high among *C. coli* isolates compared to *C. jejuni* isolates.

One of the most important issues in phage therapy is the ability to obtain phages capable of infecting the causal pathogen, hence the isolation step is a critical element. The isolation of *Campylobacter* phages is considered difficult because of the fastidious growth requirements of the host bacteria. This paper describes an improved method for the isolation of *C. coli* phages.

Materials and methods

Campylobacter strains

The 12 strains of *C. coli* used in this study were isolated from poultry and poultry products. They were tested for lysogeny (see method below). Three clinical strains of *C. jejuni* were included in the lytic spectrum study (Table 1). All of the strains were *flaA*-typed according to the method described by Nachamkin *et al.* (1993). This is a rapid and cost-effective method (Wassenaar and Newell 2000) that is based on the fact that the *flaA* gene shows regions of conservation surrounding regions of variability. Therefore, primers can be designed for polymerase chain reaction (PCR), and their products can be used for restriction fragment length polymorphism.

The incubation of all agar plates and broths was at 42°C for 18 h, under micro-aerobic conditions (5% O_2, 5% H_2, 10% CO_2, 80% N_2).

Selection of nonlysogenic Campylobacter strains.
Campylobacter strains were screened for lysogeny by using mitomycin C as inducing agent. Briefly, 5 ml of fresh broth growth media (NZCYM broth, N6905; Sigma Aldrich, St Louis, MO) was inoculated with 50 μl of an overnight culture of the strain to be tested for lysogeny. The cultures were incubated at 42°C under micro-aerobic conditions. When they reached the exponential growth phase, mitomycin C was added to a final concentration of 0·5 μg ml^{-1}. The absorbance was measured at 600 nm each hour for 8 h. The cultures were centrifuged (3000 ***g***, 12 min, 4°C), and the supernatant filtered through a membrane filter of 0·45 μm of pore size. A 10-μl aliquot of each of the treated suspensions was spotted onto bacterial lawns prepared individually from each of the 12 strains in NZCYM media (N6905; Sigma Aldrich) supplemented with 0·6% of agar (A6686; Sigma Aldrich) and incubated. The strains that produced a zone of clearing around the spot were omitted from the study. This selection procedure was repeated on three separate occasions.

Isolation of phages

Finely chopped intestines from 12 six-month-old free-range chickens were used for the isolation of phages, according to two different methods. In the first experiment, 10 g of chicken samples was added to SM buffer (0·05 mol l^{-1} Tris–HCl [pH 7·5], 0·1 mol l^{-1} NaCl, 0·008 mol l^{-1} MgSO4) and incubated for 24 h at 4°C. The suspension was centrifuged (8600 ***g***, 10 min), and the aqueous phase was treated with chloroform: one volume per four volumes of supernatant and centrifuged (8600 ***g***, 10 min). It was then used for phage detection (see method below). In the second experiment, the chicken samples (10 g) were mixed with a suspension (50 μl) of each of the 12 *C. coli* strains [grown individually for 18 h in Bolton Broth (CM0983B; Oxoid, Unipath, Basingstoke, UK) supplemented with selective supplement of antibiotics (Oxoid, SR0208E) and 5% of lysed horse blood (Oxoid, SR0048)] and 10 ml of fresh broth, supplemented with 400 μg ml^{-1} of $CaCl_2$ and 400 μg ml^{-1} of $MgSO_4$. This mixture was vortexed, incubated (42°C, micro-aerobic conditions, 18 h) and treated with chloroform as described previously.

Phage detection

A drop of each phage sample (10 μl) was added to lawns of each of the 12 strains of *Campylobacter* and the plates incubated. Lysis zones were scraped, suspended in 100 μl of SM buffer and replated on a lawn of the *Campylobacter* strain from which they were originally isolated. Serial dilutions were made to obtain single phage plaques that were propagated three times by this method to ensure purity of the phage.

The isolated phages were named as phiCcoIBB, representing the host *C. coli* strain (Cco) and the Institute for Biotechnology and Bioengineering (IBB) where the phages were isolated. The phage name is followed by a number given to each particular phage.

Lytic spectra

Each phage was tested against each *C. coli* and *C. jejuni* strains (Table 1). Ten microlitres of phage isolate was applied to each bacterial lawn. The plates were incubated,

Table 1 Lytic spectra of the isolated phages against *Campylobacter coli* and *Campylobacter jejuni* strains

		C. coli strains												*C. jejuni* strains			
FlaA type		A							B			C		D	E	F	Total strains infected (%)
Phage hosts	Phages*	A11	8907	8908	8911	8909	8910	A12	A15	A3	A2	A4	A18	2140	3820	8024	
A15	1	+	−	−	−	−	−	−	+	+	+	−	−	+/−	−	+/−	40
	2	+	+	−	+	+	+	−	+	+	+	−	−	+/−	−	+	67
	5	+	−	−	−	−	−	−	+	+	+	−	−	−	−	−	27
A3	6	+	−	+/−	+	+	+/−	−	+	+	+	−	−	−	−	−	60
	7	+	+/−	+/−	+	+	+/−	−	+	+	+	−	−	+	−	−	67
	8	+	+/−	+/−	+	+	+/−	−	+	+	+	−	−	−	−	−	60
	9	+	+/−	+/−	+	+	+/−	−	+	+	+	+	−	+/−	+/−	−	80
	10	+	+/−	+/−	+	+	+/−	−	+	+	+	−	−	−	−	−	60
8907	11	+	+	−	−	−	−	−	+	+	+	+	−	+	+	+	60
	12	+	+	+	+/−	+	+−	+	+	+	+	+	−	+	+	+	93
	13	+	+	+/−	+/−	+	+/−	−	+	+	+	+	−	−	−	+/−	73
	14	+	+	+/−	+/−	+	+/−	−	+	+	+	+	−	+	−	−	73
8910	23	+	−	+/−	+/−	+	+	+/−	+	+	+	+	−	−	−	−	67
	24	+	+	+/−	+/−	+	+	+/−	+	+	+	+	−	−	−	−	73
	25	+	−	+/−	+/−	+	+	−	−	+/−	+	−	−	−	−	−	47
	26	+	−	+/−	+/−	+	+	−	+	+/−	+	−	−	−	−	−	53
8911	27	+	−	−	+	−	−	−	+	+	+	−	−	+	+	+	53
	28	+	−	−	+	−	−	−	+	+	+	+	−	+	+	+	60
	29	+	−	−	+	−	−	−	+	+	+	−	−	+/−	+/−	−	47
	30	+	−	−	+	−	−	−	+	+	+	−	−	−	+/−	+	47
	31	+	−	−	+	−	−	−	+	+	+	−	−	−	+	−	40
A4	32	+	+/−	+/−	+	+	+/−	−	−	+	−	+	−	+	−	−	60
A11	33	+	+	+	+	+	+	+	+	+	+	+	−	+/−	+	+	93
	34	+	+	+	+	+	+	+	+	+	+	+	−	−	+/−	+	87
	35	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	93
	36	+	−	−	−	−	−	−	+	+	+	−	−	−	−	+/−	33
	37	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+/−	93
	38	+	+	+	+	+	+	−	+	+	+	+	−	+	−	−	73
A2	39	+/−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	27
	40	+/−	−	−	−	−	−	−	+	+	+	−	−	+	+	+	47
	41	+/−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	20
	42	+/−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	27
	43	+/−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	27

Clear lysis areas were marked as (+), turbid lysis areas are represented as (+/−) and absence of lysis was marked as (−).
*Isolated phages phiCcoIBB1-phiCcoIBB43 are represented by the numbers 1–43.

and the appearance of clear zones around the point of application was recorded as the ability to lyse that strain. Three independent experiments of this procedure were performed.

Characterization of phages by Transmission Electron Microscopy (TEM)

Phages were purified by PEG (polyethylene glycol) according to the procedure described by Sambrook and Russell (2001). Phage suspensions were centrifuged at 25 000 ***g*** for 60 min and washed twice in ammonium acetate (0·1 mol l^{-1}, pH 7·0). Sediments were deposited on copper grids with carbon-coated Formvar films, stained with 2% (w/v) uranyl acetate (pH 4·2) and studied in a Philips EM 300 electron microscope operating at 60 kV. Magnification was monitored with catalase crystals (Polaron Electron Opticals Ltd, London, UK).

Results

The first phage isolation experiment was performed without the use of an enrichment method. This procedure did not allow the isolation of phages from the chicken samples, using the *C. coli* strains tested, as no lysis areas were observed (data not shown). Therefore, an alternative methodology was tested in which salts and the target hosts were incubated with the chicken samples (enrichment procedure). All the 12 *C. coli* strains were used in this enrichment as they were shown to be nonlysogenic.

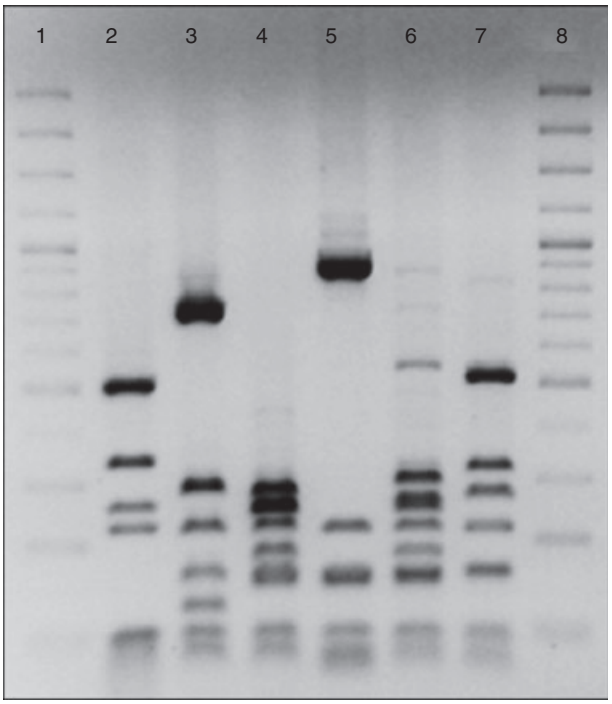

Figure 1 Restriction fragment length polymorphism patterns of the flagellin gene flaA digested with DdeI. Lanes are as follows: 1. Ladder 100 bp (Fermentas, GeneRuler 100bp Plus); 2–7. FlaA Type A–F, of the Campylobacter strains used for the lytic spectra; 8- Ladder 100 bp (Fermentas, GeneRuler 100bp Plus).

This method allowed the isolation of 43 *C. coli* phages (data not shown) from six chicken samples. Of the 12 *C. coli* strains used for phage isolation, only two (A12 and A18) were unable to propagate any of the phages present in the samples.

Of the 43 phages isolated, 10 were discarded because the zones of lysis they produced on their host strain were very turbid. Thus, only the remaining 33 phages were assessed for their lytic spectra (Table 1).

Some of the phages, mainly those isolated using the same *C. coli* host strain, had similar lytic spectra: phiCcoIBB_6 and phiCcoIBB_8 isolated via *C. coli* A3, and phiCcoIBB_33 and phiCcoIBB_34 isolated via *C. coli* A11 (Table 1). Two phages were found to have similar lytic spectra, which had been isolated via different *C. coli* host strains: phiCcoIBB_14 (host 8907) and phiCcoIBB_24 (host 8910). These two hosts are of the same *flaA* type (Fig. 1). In general, strains of different *flaA* types exhibited different susceptibility to the isolated phages. However, some strains with the same *flaA* type showed different lytic patterns, as did strains A12 and 8910 of *flaA* type A. Strains A11, A2 and A3 were able to propagate all of the isolated phages. Conversely, *C. coli* strain A18 was unable to propagate any of the 33 isolated phages. Of the 33 phages, 64% were able to propagate in at least one of the *C. jejuni* strains tested, and 24% of the isolated phages were able to propagate in all of the *C. jejuni* tested. Phages phiCcoIBB35 and phiCcoIBB37 which were isolated via host A11 and also phage phiCcoIBB12 isolated via host 8907 showed the broadest lytic spectra, being able to lyse 93% of the *Campylobacter* strains tested. Therefore, these phages were selected to be characterized by TEM, exhibiting similar structures and sizes, each possessing an icosahedral head and a contractile tail with tail fibers typical of phages from the *Myoviridae* family (Fig. 2).

Discussion

Relatively few researchers have been successful in isolating *Campylobacter* phages, and the most probable reason for this is inadequate isolation methodology. In the first attempt to isolate *Campylobacter* phages, we used a methodology previously reported by several authors (Connerton *et al.* 2004; Atterbury *et al.* 2005; Loc Carrillo *et al.* 2005). This method is based on the incubation of chicken samples overnight at 4°C in SM buffer without the addi-

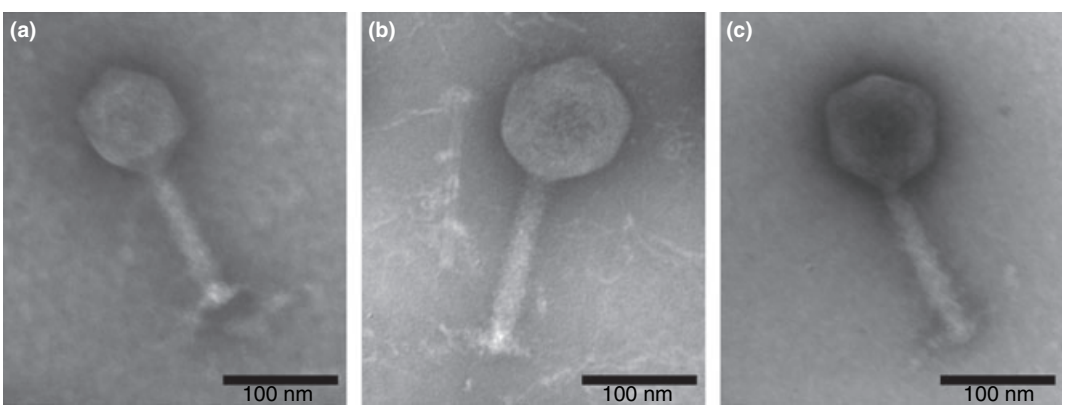

Figure 2 Transmission Electron Microscopy images of phages (a) Phage phiCcoIBB35 (b) Phage phiCcoIBB37 (c) phage phiCcoIBB12.

tion of *Campylobacter* strains. As no bacterial hosts were added to the preparation and as the low temperature does not allow phage propagation (Atterbury *et al.* 2003a), this procedure may have prevented the recovery of phages from the chicken samples in concentrations sufficiently high to ensure the infection in plating stage. The method implemented in our study increased the likelihood of detecting and isolating phage present in very low numbers by using a selective phage-enrichment technique. The samples were incubated with strains of *Campylobacter* to amplify the phage. It is important to assure that these strains are nonlysogenic because in the case of lysogenic strains, the prophage can escape from the original host and can integrate into a different one (transduction), leading to the transfer of virulence genes and those mediating resistance to antibiotics. Such phages are therefore inappropriate candidates for phage therapy. By selecting just the nonlysogenic strains for the enrichment broth, the probability of isolating this type of phages is avoided.

In the method described, the enrichment broth also contained $CaCl_2$ and $MgSO_4$, as there is evidence that these salts enhance the attachment of the phages to the bacterial hosts (Salama *et al.* 1989; Aarestrup *et al.* 2007). Phages are more likely to be found in samples where the levels of *Campylobacter* colonization are higher, as more hosts are available for phage propagation (Atterbury *et al.* 2003b). We therefore selected samples likely to contain high numbers of *Campylobacter*. Free-range chickens are exposed to the outdoor environment, so the likelihood of them becoming colonized by *Campylobacter* is greater (Hald *et al.* 2001). The enrichment method developed and used in this study isolated 43 *Campylobacter* phages from free-range chickens, and these were active against approximately 85% of the *C. coli* strains used in the isolation step. Some of the phages, namely phage phiCcoIBB12, phiCcoIBB35 and phiCcoIBB37 showed broad lytic spectra and were able to lyse not only the *C. coli* strains but also the clinical isolates of *C. jejuni* of different *flaA* types. These phages are considered good candidates for phage therapy. According to the TEM characterization, they were classified as members of the *Myoviridae* family, which has been reported in the literature for the majority of *Campylobacter* phages (Atterbury *et al.* 2003b; Connerton *et al.* 2004; Hansen *et al.* 2007; Loc Carrillo *et al.* 2007). A detailed physiological and genetic characterization of these phages as well as their *in vivo* performance to control the levels of *Campylobacter*-contaminated poultry is being evaluated.

Overall, the method described not only improves the chances of isolating phages by seeding the samples with strains of *Campylobacter* isolated from chicken, but in so doing isolates phages that can be used subsequently to target those specific strains of *Campylobacter*. Thus, in the context of phage therapy, a particular pathogen that is causing a flock illness can be isolated using the present method and used to seed chicken samples to isolate specific phages against that particular bacterium. Those phages can then be used for treating the infected flock.

Acknowledgements

The authors acknowledge the European Commission under the FP-6-2003-Food-2-A to the project 2005-7224 for the financial support and the Portuguese Science Foundation (FCT) through the grant SFRH/BD/23484/2005.

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