Utilization of bacteriophages to combat Campylobacter


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ABSTRACT: Campylobacter infections are amongst the most frequently encountered foodborne bacterial infections around the world. Handling and consumption of raw or undercooked poultry products have been determined to be the main route of transmission. The ability to use phages to target these bacteria has been studied for more than a decade and although we have made progress towards deciphering how best to use phages to control Campylobacter associated with poultry production, there is still much work to be done. This review outlines methods to improve the isolation of these elusive phages, as well as methods to identify desirable characteristics needed for a successful outcome. It also highlights the body of research undertaken so far and what criteria to consider when doing in-vivo studies, especially because some in-vitro studies have not been found to translate into phage efficacy in-vivo.

KEYWORDS: Tamarindus indica, Staphylococcus aureus, MDR

1. Historical overview about Campylobacter spp.:

It is a well-known fact that poultry appeared to be a significant source of Campylobacter and chicken was found to be heavily intestinal carriers of Campylobacter when compared with other food animals (Humphrey et al., 2007).

These variations in Campylobacter spp. prevalence might be due to differences in hygienic conditions, cross-contamination that may occur during de-feathering, eviscerating, and some other environmental factors such as the temperature of water in the scalding tank (Hagos et al., 2021).

The prevalence of Campylobacter spp. in bovine meat was 11.90%. This was comparable to the finding reported from a previous study done by (Salihu et al., 2009) (12.9%) in Nigeria, and (Taremi et al., 2006) (10%) in Iran. However, it was higher than the findings reported by (Dadi L. et al., 2008) (6.2%) in Ethiopia (Nonga et al., 2010); (5.6%) in Morogoro, Tanzania; and (Vanderlinde et al., 1998) (0.8%) in Australia. Food of animal origin has been incriminated for being the main source of Campylobacter infection in humans (Oberhelman et al., 2000).

Since raw meat from beef is widely consumed in the country; the occurrence of Campylobacter in meat increases the likelihood of the pathogen transmission to humans. The present finding was lower than studies conducted by (Salihu et al., 2009) and (Thépault et al., 2018) who reported a prevalence of 69.1% and 22%, respectively. One of the most likely hypotheses to explain the discrepancies is the differences in protocols used for the detection of thermophilic Campylobacter, and especially the absence of an enrichment step for the isolation of thermophilic Campylobacter in (Cha’tre et al., 2010).

2 General description of Genus Campylobacter

Campylobacter spp. are Gram-negative, non-spore forming bacteria and are members of the family campylobacteraceae. The Genus Campylobacter comprises 17 species and 6 subspecies (Nachamkin, 2007; Silva et al., 2011). The two species most commonly associated with human disease are C. jejuni and C. coli. C. jejuni accounts for more than 80% of campylobacter-related human illness, with C. coli accounting for up to 18.6% of human illness. C. fetus has also been associated with foodborne disease in humans (Gurtler et al., 2005; FDA 2012).
The growth and survival of *Campylobacter* spp. depends on a variety of factors. *Campylobacter* spp. are sensitive to environmental conditions, such as temperature, availability of water and oxygen; and have limited capacity to survive environmental stresses. *Campylobacter* spp. grow in the 30-45°C. At 32°C, *C. jejuni* may double its number in approximately 6 h (Forsythe, 2000). *Campylobacter* spp. do not multiply at temperatures below 30°C, such that the number of *Campylobacter* spp. will not increase in foods held at room temperature (20-25°C) (Park, 2002).

Members of the genus campylobacter are long spiral forms, curved, atypical Gram negative rods with size ranging from 0.2 to 0.8 μm wide and 0.5 to 5 μm long (Halablab et al., 2008). Campylobacter cells transform from spiral form to coccoid morphology with less motility when aged or exposed to air for prolonged time periods (ISO, 2006). They are usually motile by means of a single polar unsheathed flagellum at one or both ends, but may also lack flagella. The uni-polar flagellum gives campylobacter characteristic cork-screw motility (Song et al., 2004).

3. Campylobacter infection in humans and poultry

Mäesaar et al. (2014) examined 606 poultry meat samples at retail level and 380 broiler chicken caecal samples at slaughterhouse level in Estonia for presence of *Campylobacter* spp. of the results showed that 20.8% of the poultry meat and 39.2% of the caecal samples were found positive for *Campylobacter* spp.

Torralbo et al. (2014) collected 2221 cloacal swabs and 747 environmental swabs from 291 broiler flocks from broiler farms in Andalusia (southern Spain). The prevalence of campylobacters in individual birds was 38.1%, and the flock prevalence was 62.9%. Flocks were predominantly infected by *C. jejuni* and *C. coli* but were also infected.

*Campylobacter* species represent one of the most common causes of bacterial diarrheal illness worldwide. According to the United States Centers for Disease Control, there are about 1.3 million cases of *Campylobacter* infection each year in the United States alone. This leads to an economic cost between $1.3 to 6.8 billion dollars annually in the United States (Kaakoush et al., 2015). Campylobacter infection is associated with the consumption of raw milk, undercooked poultry, and contaminated water. Patients typically experience a self-limited diarrheal illness lasting 5 to 7 days. Immunocompromised and elderly patients are at the highest risk for morbidity, mortality and prolonged illness. Despite having treatment and eradication modalities in place in animal reservoirs, there has been a dramatic increase of cases in developed and underdeveloped regions of the world (Kaakoush et al., 2015).

4. Antimicrobial resistance

The un-controlled use of antibiotics in animal husbandry, for treatment and growth promotion has led to an increase in antibiotic resistant campylobacter infections in humans (Iovine, 2013). Therefore, an understanding of the antibiotic resistance mechanisms in *C. jejuni* is needed to provide proper therapy both to the veterinary and human populations (Iovine, 2013).

In addition to intrinsic resistance mediated by efflux (Jeon et al., 2011), antibiotic exclusion [via the major outer membrane porin (MOMP) (Page et al., 1989), lipooligosaccharide and possibly capsule (Jeon et al., 2011)] also contribute to intrinsic resistance (Figure 1).

![Figure 1: Summary of major antibiotic resistance mechanisms in campylobacter (Jeon et al., 2011).](https://bfszu.journals.ekb.eg/journal)

5. Campylobacter species virulence factors

Specific virulence mechanisms are not clearly demonstrated for *Campylobacter* species up till now, probably owing to the paucity of pathogenesis resemblance between campylobacters and the other bacteria (Dasti et al., 2010). Bacterial motility, adherence, invasion ability and toxins production have been described as virulence factors for *Campylobacter* spp. (Dasti et al., 2010, and Bhunia, 2018). (Figure 2) (Van Vliet, 2001) as following:
Figure (2): Diagram demonstrates the virulence attributes of Campylobacter species taken from a previous report (Van Vliet, 2001). IL-8: interleukin-8, NF-Kb: nuclear factor kappa-light-chain-enhancer of activated B cells

6. Characterization of Campylobacter phages

The use of phages to control pathogenic bacteria has been investigated since they were first discovered in the beginning of the 1900s. Over the last century we have slowly gained an in-depth understanding of phage biology including which phage properties are desirable when considering phage as biocontrol agents and which phage characteristics to potentially avoid. Campylobacter infections are amongst the most frequently encountered foodborne bacterial infections around the world. Handling and consumption of raw or undercooked poultry products have been determined to be the main route of transmission. The ability to use phages to target these bacteria has been studied for more than a decade and although we have made progress towards deciphering how best to use phages to control Campylobacter associated with poultry production, there is still much work to be done. This review outlines methods to improve the isolation of these elusive phages, as well as methods to identify desirable characteristics needed for a successful outcome. It also highlights the body of research undertaken so far and what criteria to consider when doing in-vivo studies, especially because some in-vitro studies have not been found to translate into to phage efficacy in-vivo (Janež N. et al., 2013)

The best sources to isolate phage are from environments where the host is highly prevalent. Campylobacter is ubiquitous in temperate environments but favors the intestine of all avians, where they colonize the gut as a commensal organism (Newell and Fearnley, 2003). By isolating the Campylobacter strains from the same location as their phage, there is a higher probability of having a host susceptible to the phages sought. Failing the possibility of isolating ‘native’ hosts, possessing a highly susceptible strain such as C. jejuni NCTC 12662, which is known to be sensitive to most C. jejuni and C. coli phage isolates, is an alternative solution (Connerton et al., 2004; Loc Carrillo, 2005; Owens et al., 2012).

Once isolated and purified, phages must undergo a variety of genotypic and phenotypic tests necessary to identify them as suitable candidates for use as biocontrol agents. Determining the lytic activity of a phage is the most common characterization, because it can help determine if the phage should be tested further. Phages possessing broad lytic spectra are often highly desirable, although phages capable of lysing bacterial strains that are less susceptible to a wide variety of phages are also deemed beneficial. Lytic activity of a Campylobact er phage should be assessed with a panel of reference Campylobacter strains that include C. jejuni and C. coli, as well as wild-type isolates that reflect the environment under study (Hansen et al., 2007), other to determine how virulent the phage is towards the different strains.

6.1.1. Morphological characteristics

A slightly more technical but relatively fast characterization method commonly used is determining the structure of phages. Phages can be examined using transmission electron microscopy (TEM) (Ackermann, 2001) and more recently using atomic force microscopy (Kuznetsov et al., 2011). It is advisable to have as high a titer of phage suspension as possible, with a minimum titer of c. 108 PFU/mL necessary to locate phage on the surface of a carbon-coated copper grid. Phage suspensions may be concentrated using standard molecular protocols such as ultrafiltration membranes, polyethylene glycol (PEG) precipitation, or cesium chloride
equilibrium gradients (Sambrook and Russell, 2001). Simple preparations for examining Campylobacter phage under TEM are highlighted by (Atterbury et al. (2003a), and Owens et al. (2012)).

6.1.2. Protein profiling

Only two protein profiles of Campylobacter phage have been published to-date (Timms et al., 2010; Hammerl et al., 2011). (Timms et al. (2010) used precast gels. They loaded their purified phage suspension (c. 1010 PFU/mL) to gradient gels using the manufacturer's SDS sample and gel running buffers, and followed the manufacturers' instructions. The gels were run at 200 V for 35–50 min, and stained with colloidal coomassie blue. Protein bands were excised and digested with trypsin, before undergoing electrospray ionization followed by tandem mass spectrometry (MS/MS). Both research groups used the Mascot Deamon web server database to analyze their peptide sequences.

6.1.3. Genomic characterization

Pulsed-Field Gel Electrophoresis has been used extensively to determine the genome size of most Campylobacter phages characterized to-date (Sails et al., 1998; Atterbury et al., 2003a). It is recommended to use at least a 109 PFU/mL suspension of phage for genome size determination, and higher titers for restriction endonuclease analysis. Once embedded in an agarose block, the protein-based structures of the phage are degraded using proteinase K and a detergent (e.g., lauryl sarkosyl). Proteinase K is then inactivated by washing the agarose block at 55 °C and the washed block can be stored in TE buffer at 4 °C for no more than 3 months. For restriction digest analysis, a number of restriction enzymes have been used, although most have failed to produce a restriction profile (Sails et al., 1998; Hwang et al., 2009; Owens et al., 2012).

Electrophoretic parameters used to analyze the PFGE profile are dependent on the specified fragment sizes, and PFGE apparatus manufacturers such as Biorad provide helpful guidelines in their equipment manuals. As size standards the MidRange I PFG Marker by New England Biolabs or lambda concatemer have been successfully used. Analysis at the genomic level was first performed for the set of 16 Campylobacter typing phages (Sails et al., 1998). The majority of Campylobacter phages possessing icosahedral heads and contractile tails isolated so far may be grouped according to their genome sizes (Sails et al., 1998; Connerton et al., 2011).

Preparation of genomic DNA for sequencing requires highly purified, high titer phage suspensions. A variety of protocols have been used to isolate Campylobacter phage DNA, as has the technology to generate DNA libraries, including use of the Roche's 454 Genome Sequencer FLX system (Timms et al., 2010; Hammerl et al., 2011; Carvalho et al., 2012); proprietary technology by Fidelity Systems (Kropinski et al., 2011); and shotgun sequencing (Timms et al., 2010). The extraction methods used to obtain Campylobacter phage DNA plays an important part in the information obtained. Proteins, which have been found to be tightly bound to phage DNA, tend to be removed during the phenol extraction procedure along with a significant amount of (N80%) DNA. Separation of DNA from proteins has also proved problematic with the Qiagen genomic DNA purification kit columns (Kropinski et al., 2011). To add, phage genome sequencing has proved difficult due to their refractory properties to restriction enzyme digestion and an inability to amplify DNA with regular Taq polymerase. It may be worth noting that due to the small number of phage genomes sequenced so far, and the limited bioinformatics data available to identify and annotate genes, sequencing a phage does not guarantee to determine if the phage carries ‘unknown’ genes coding for lysogeny, or ones that can promote a bacterium’s virulence or

7. Interaction between Campylobacter phages and their hosts

Since phage genomic sequencing is still in its infancy stage, it is important to study the phage-host interactions through both in-vitro and in-vivo studies in order to understand development of resistance. (Holst Sorensen et al. (2011) used the well-characterized Campylobacter jejuni NCTC11168 strain to select for a phage-resistant mutant, in order to determine the receptor site for Campylobacter phage F336. By using periodic or proteinase K, they were able to elucidate that phage binding was due to a carbohydrate moiety rather than an outer membrane protein on the surface of the phage-resistant mutant strain. With the aid of high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) analysis of intact bacterial cells, they found that the difference between the susceptible wild type and resistant strain was a modification of the capsular polysaccharide's (CPS) hyper variable O-methyl phosphoramidate structure. This structure has been found to be highly abundant in C. jejuni strains although it is unknown how a defect within this structure affects the virulence of the resistant-mutant strain (Holst Sorensen et al., 2011). Scott et al. (2007a) used in-vivo studies to investigate how Campylobacter phage CP34 predation upon C. jejuni HPC5 populations in the avian gut, would affect the development of phage-resistant mutants. Three phenotypes were discovered from the strains isolated: resistance to phage, poor colonisers of the chicken intestine, and producers of a functional M-like phage, although these were not seen with in-vitro kinetics studies. These phenotypes resulted from intra-genomic inversions between Mu-like prophage sequences present in the parental strain. Interestingly, the reintroduction of these phenotypes to chickens in the absence of phage predation resulted in bacterial genomic rearrangements that lead the population to regain their competency at colonizing the chicken gut as well as revert back to being sensitive to phage (Scott et al., 2007a). The intriguing differences between results obtain
from in-vitro and in-vivo kinetic studies of phage-host interactions can be highlighted by the work published by Loc Carrillo et al. (2005). A particular C. jejuni strain (HPC5) was tested for its development of phage resistance to two different Campylobacter phages (CP8 and CP34) both in-vitro and in-vivo. In-vitro studies showed 8% and 11% of the colonies tested became resistant to successive CP8 and CP34 phage infections, respectively. In contrast, only 4% of colonies recovered from the CP34 phage treated in-vitro studies gained resistance. Furthermore, isolates from the in-vitro studies were not found to revert back to the sensitive phenotype after five consecutive subcultures. Kinetic differences between these two phages and the target bacteria were also found to have considerably different behaviors between their in-vitro and in-vivo environments, as highlighted below (Loc Carrillo et al., 2005).

7.1 Use of phages to control Campylobacter in live birds and food products

To-date, there have been a few studies looking at the feasibility to use Campylobacter phages to control these pathogenic bacteria both at the farm-level and on the processed carcass. Once a phage has been characterized and deemed suitable for applications as an antibacterial agent, there will be a number of in-vivo experiments necessary to turn theory into practice.

7.1.1. Use in live birds

Control Campylobacter in the intestine of experimentally colonized birds. Wagenaar and colleagues were one of the first to investigate the application of a single Campylobacter phage type in live broilers as well as test the effect of a two-phage treatment. They postulated that the addition of a second phage might reduce the rate of phage-resistant mutants developing, although the presence of such mutants was not tested for. The authors state that the efficacy of the combination of phages was comparable to their individual effects seen in in-vitro studies, to determine that no antagonistic effect would occur (Wagenaar et al., 2005). Loc-Carrillo and colleagues studied two Campylobacter phages (CP8 and CP34) in an in-vivo model, based on in-vitro efficacy studies. Birds pre-colonized with two different wild-type Campylobacter strains resulted in different outcomes after being treated with phage CP8. A significant drop in the population of C. jejuni HIC8 colonizing the ceca, after 1 day of treatment, was seen compared to the relatively unaffected HPC5 population. However, when phage CP34 was used, the treatment produced different results to phage CP8 against the C. jejuni HPC5 colonizing the ceca, where the drop in bacterial load was not only lower but also maintained for up to day 5 post treatment. Interestingly, the in-vivo results were contrary to in-vitro results and the ability of phage CP8 in killing C. jejuni HPC5 in-vitro was not reproduced in the treated chickens (Loc Carrillo et al., 2005), most likely due to the vastly different environments the bacterial population encountered between the chicken gut and as homogeneously grown planktonic cells in a flask. El-Shibiny et al. (2009) conducted similar in-vivo studies with a wild-type C. coli OR12 strain that was used to colonize the birds and treated with the same phage dosages. Only the highest dose of 109 PFU reduced the Campylobacter counts in the ceca by 1–2 Logs within the first 2 days (El-Shibiny et al., 2009). Carvalho and colleagues tested the efficacy of a phage cocktail on chicks colonized by C. jejuni or C. coli. The phages chosen had different complimentary lytic spectra. Phage treatment delivered through the feed appeared to produce a slightly higher reduction of C. coli when compared to administration by oral gavage. To minimize the number of birds culled over the whole study, fecal and cloacal swab samples were used to enumerate the number of Campylobacter bacteria excreted by the chicks. Investigating the rate of phage resistance development in Campylobacters passaged through the chick guts, they found 6% of the isolates were resistant to phage despite not being exposed to the phage treatment, in comparison to 13% of isolates that showed phage resistance after being exposed to phage treatment. After passaging the resistant strains back through the chicken gut, they found 86% of the colonies that were previously phage-resistant without exposure to phage had reverted back to being sensitive, whereas only 54% of the colonies that were phage-resistant after being exposed to the phage treatment reverted back to their sensitive phenotype (Carvalho et al., 2010).

7.1.2. Use in food products

The use of phages to control the level of Campylobacter contamination on the surface of chicken carcasses has also been investigated by a number of research groups. Two of the studies used chicken skin tainted with known concentrations of susceptible Campylobacter cells (Goode et al., 2003; Atterbury et al., 2003), while another used raw and cooked beef products with high and low bacterial loads treated with high and low phage titers (Bigwood et al., 2008). In a later study involving polynomial modeling, Bigwood and colleagues calculated that an MOI of 105 would be required to kill at least 25% of a susceptible bacterial population on the surface of food within a 2 h period (Bigwood et al., 2009).

Summary

Campylobacter spp. has become one of the most challenging bacteria to eradicate in the past few decades. This bacterium possesses exceptional surviving abilities and can persist in extreme conditions such as the gut and human gastrointestinal system as a result of its ability to withstand an alkaline milieu. In dentistry
Campylobacter spp. is one of the main bacteria associated with chronic apical periodontitis in failed gastrointestinal treatments.

Despite the fact that endodontic infections have a polymicrobial nature, the gastrointestinal environment may favor and support the survival of one species, which is commonly Campylobacter jejuni and Campylobacter coli. Campylobacter jejuni and Campylobacter coli is actually seldom present in primary endodontic infections, in cases of postendodontic treatment with apical periodontitis, failed cases are approximately nine times more likely to harbor Campylobacter jejuni and Campylobacter coli than cases of primary infections. Campylobacter jejuni and Campylobacter coli which isolated chicken intestine usually carry virulence factors related to adhesions and biofilm formation, which facilitate its colonization of different gastrointestinal sites and enable it to cause severe infections. Moreover, isolates from gastrointestinal infections may carry certain antibiotic resistant determinant that has the ability to be transferred to other pathogenic bacteria in biofilm communities. Campylobacter jejuni and Campylobacter coli biofilms enable it to resist traditional antibacterial rinsing solutions such as chlorhexidine or sodium hypochlorite and adapt themselves and grow in the presence of calcium hydroxide. Campylobacter jejuni and Campylobacter coli form biofilm in between gastrointestinal parts without being affected by treatment. The increasing number of antibiotic-resistant Campylobacter jejuni and Campylobacter coli biofilm formation, together with the failure of antiseptic rinsing or antibacterial dressing to combat them, require alternative methods to be developed and executed. The use of lytic bacteriophages is one of the most promising techniques that are exploring to fight multidrug resistant Campylobacter jejuni and Campylobacter coli and their biofilms.

REFERENCES


