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The Use of Bacteriophages to Control *Campylobacter jejuni* from Chickens

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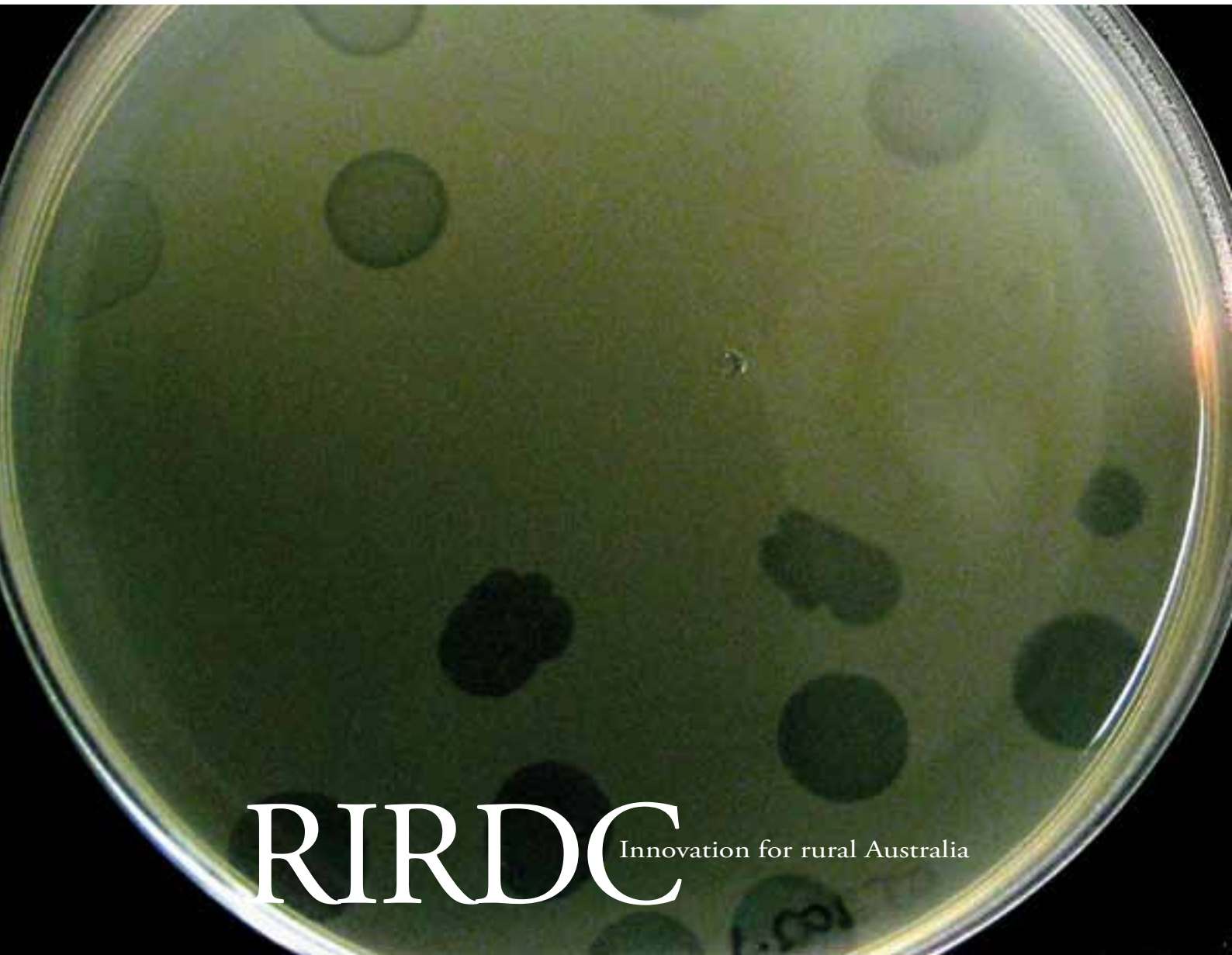

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The Use of Bacteriophages to Control *Campylobacter jejuni* from Chickens

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Foreword

Campylobacter jejuni is an organism of significant public health concern. It is reportedly the most common cause of diarrhoeal disease in humans in developed countries, and common sources are live poultry and poultry products. Therefore, further measures are sought to either prevent or control infection of chickens with this organism, or to decrease its presence on poultry meat. One proposed adjunctive control measure is the use of naturally occurring bacteriophages. This study has investigated the presence and characteristics of bacteriophages active against *C. jejuni* in South Australian poultry, and their ability to lyse strains of *C. jejuni* under laboratory conditions. While *C. jejuni*-active bacteriophages were successfully isolated in quite large numbers from free range egg layers and broilers, and shedded egg layers, the presence of such bacteriophages could not be detected in any samples from indoor commercial broilers, despite successful isolation of the associated *C. jejuni* bacteria. For this reason, this study also compared *C. jejuni* isolates from a range of indoor and free range poultry sources, to ascertain whether the differences in bacteriophage ecology may be due to differences in the host strains, including the presence or absence of prophage sequences.

This type of information is useful to those involved in food safety. No previous studies have been conducted on bacteriophages from Australian poultry. It is also of interest to persons concerned with the differences, at a microbiological level, between poultry reared under indoor commercial conditions versus free range. The importance of this report is that it provides information on the presence and characteristics of bacteriophages from South Australian chickens and their relationship to wild type *C. jejuni* strains.

Although this study demonstrates that bacteriophages can be isolated that are capable of lysing multiple *C. jejuni* strains under laboratory conditions, much more work needs to be done to determine why naturally occurring bacteriophages cannot be isolated from commercial indoor poultry and to better understand the obviously complex ecology of *Campylobacter* bacteriophages. Until these issues are resolved no conclusions can be drawn as to whether bacteriophages represent a useful adjunct treatment to reduce *Campylobacter* contamination of chicken meat.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Chicken Meat R&D program, which aims to which aims to improve the efficiency of chicken meat production; enhance the quality and safety of chicken meat products and improve the image of chicken meat products; help to protect a sustainable production environment; and improve industry's competitiveness, both domestically and internationally.

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Craig Burns
Managing Director
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Abbreviations

ϕ	bacteriophage(s)
ATCC	American Type Culture Collection
bp	base pairs
C. coli	*Campylobacter coli*
CFU/ml	colony forming units per millilitre
CJ	*Campylobacter jejuni*
C. jejuni	*Campylobacter jejuni*
CJIE	*Campylobacter jejuni* insertion element
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra acetic acid
kbp	kilobase pair
NCTC	National Collection of Type Cultures
PCR	polymerase chain reaction
PFU/ml	plaque forming units per millilitre
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
TAE	tris-acetic EDTA
TE	tris EDTA
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
v/v	volume per volume
w/v	weight per volume

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Executive Summary

What the report is about

This study investigated *Campylobacter jejuni* and its associated wild type bacteriophages. In particular, it looked at the presence and characteristics of bacteriophage active against *C. jejuni* from South Australian poultry farms.

Who is the report targeted at?

Those persons with an interest in the bacterial ecology of *Campylobacter*, and also those concerned with typing methods for *C. jejuni*. It is also aimed at industry members who have an interest in the distribution of *C. jejuni* and its associated bacteriophages.

Where are the relevant industries located in Australia?

Meat chicken production and processing is concentrated in NSW and Victoria with significant production in Queensland and less in South Australia and Western Australia. Production and processing, while previously centred around the capital cities is now extending into regional areas.

The chicken meat industry is highly vertically integrated, with ownership concentrated in two large integrated national companies.

Background

Bacterial food-borne infections, of animal origin, account for the majority of reported cases of human gastrointestinal disease. Worldwide, over 90 percent of reported bacterial food-borne illness in humans can be attributed to either *Campylobacter* or *Salmonella* species. Of these two organisms, *Campylobacter* is the most common bacterial cause of diarrhoeal disease in humans. *Campylobacter* is a commensal organism in many warm-blooded animals, including all domestic livestock. A significant traceable source of human infection is poultry. Whilst current methods available to control *Campylobacter* have a documented effect, further reductions would be of benefit.

One proposed adjunctive control measure for *Campylobacter* is the use of bacteriophages. Bacteriophages are viruses that are able to infect and kill bacterial cells and they will only multiply when susceptible bacteria are present. The emergence of antibiotic resistant bacteria has, in recent times, renewed Western research interest in the possibilities of using bacteriophages to control bacteria, such as *Campylobacter*. Bacteria that are resistant to antibiotics will still be bound and lysed by bacteriophages, as the infection mechanisms for bacteriophages are completely different to those of any available antibiotics. Research has been carried out on the use of bacteriophages as direct therapy or for prevention of bacterial infections in numerous animal species, with animals also being used as models for human disease. Results of these studies show considerable promise as to the potential success of bacteriophages as targeted antimicrobial agents. It is these results, along with their inherent capabilities, which indicate that bacteriophages may have the potential to be used as antibacterial agents, in this case to target *Campylobacter*, specifically *C. jejuni*. Compared to other bacterial species, research into the use of *Campylobacter* bacteriophages is somewhat limited, but the results of available studies show considerable promise.

Aims/objectives

The project aimed to produce a safe, non-chemical environmentally sustainable way to reduce colonisation of chickens with *C. jejuni.* The projected deliverable was a panel of bacteriophages and phage products effective against *C. jejuni*.

Methods used

The methodology was a mixture of classical microbiological methods, culture, bacteriophage extraction and purification, and microscopy, together with modern molecular methods such as PCR.

Results/key findings

The research suggests that the ecology of *Campylobacter* bacteriophages and their bacterial hosts is far more complex than that of other species. Whilst bacteriophages active against *Escherichia coli* could be readily isolated from samples from poultry reared under traditional indoor commercial conditions, the same was not observed for *Campylobacter* bacteriophages. Despite the presence of *Campylobacter* bacteria in samples from indoor commercial chickens, no bacteriophages could be isolated. This was in contrast to samples obtained from free range broilers and egg layers, from which bacteriophages were readily isolated. The observation of this complex ecology correlates with the known fastidious nature of *Campylobacter* bacteria and phenotypic changes observed due to very minimal changes in growth conditions. These results also correlate with published literature that suggest free range poultry as a source of bacteriophages, and report a difference between isolation rates of bacteriophages in chickens raised in free range versus organic conditions.

This work represents the first Australian study of *Campylobacter* bacteriophages isolated directly from industry samples. Bacteriophages successfully isolated and purified showed similar phenotypic properties, including similar structure as observed under Transmission Electron Microscopy. The structure is similar to *Campylobacter* bacteriophages isolated and reported from overseas studies. These bacteriophages showed lytic activity against a wide range of wild type *C. jejuni* strains *in vitro*. Observed sensitivity was as high as one in three isolates being sensitive to certain bacteriophages. The amount of lysis, however, was inconsistent. It is thought that the bacteriophages may not be entirely lytic and possess some lysogenic potential, which is undesirable for any bacteriophages to be used as bacterial control agents. A distinct difference was also observed in sensitivity between isolates from different poultry sources.

Based on the above observations, work was conducted to determine if there was any similarity between isolates obtained from different sources. Restriction fragment length polymorphism (RFLP) of the *flaA* gene suggested that isolates from one company showed similar profiles, compared to those of other companies. There was also an apparent difference observed between the *C. jejuni* strains from indoor commercial poultry and free range chickens. Leading on from this work, PCR was also used to try and identify the presence of prophage sequences integrated into the genome of the *C.jejuni* strains from this study. This is the first known work using PCR to identify prophage insertions in *Campylobacter*. Prophage can confer resistance to lysis from bacteriophages and was postulated as a possible reason for the differences in observed sensitivity to the bacteriophages. This was determined to be unlikely as the cause in this case. Prophages were readily identified in the isolates tested, however, and there was a tendency for more prophages to be identified in strains of indoor commercial poultry origin, which is suggested as a cause of the apparent absence of lytic bacteriophages.

Isolated bacteriophages were shown to be able to lyse *C. jejuni* under laboratory conditions and so the potential to provide a panel of bacteriophages demonstrating lytic activity was achieved. However, the phenotypic characteristics of the lysis shown by individual bacteriophage preparations shows some inconsistency, which appears to relate to small changes in the growth conditions of the bacterial host. A statistically significant reduction of *C. jejuni* growth on artificially contaminated skin was shown only in one experiment.

The inability to detect bacteriophages in broiler chickens reared under traditional indoor commercial conditions needs to be better explained before the project is taken any further. Also, the relationship between *C. jejuni* bacteriophages and their hosts needs to be better understood. Use of bacteriophages could play a beneficial role in reducing *Campylobacter* contamination of chicken meat, as this has not been disproven, but it has been identified that much more needs to be done before any such strategy could be implemented.

Implications for relevant stakeholders

Bacteriophages active against *Campylobacter* appear to have a far more complex ecology than bacteriophages of other species. This suggests that their use as a control mechanism would be limited until this relationship can be better understood.

Differences in the ability to isolate bacteriophages from chickens raised in traditional indoor commercial and free range conditions, observed differences in the *flaA* RFLP profiles of *C. jejuni* strains from these sources, and differences in sensitivity to bacteriophages suggests that that rearing conditions have a significant influence on the molecular epidemiology and bacteriophage ecology. Further studies should be conducted to more accurately determine the molecular epidemiology and characteristics of *Campylobacter* in chickens from all different rearing conditions. This is the first molecular epidemiological study of *Campylobacter* from meat chickens in Australia.

Prophage sequences appear to be widespread in *C. jejuni* strains. There appears to be consistency in the prophage sequences identified in strains from the same source. A larger study could be conducted to determine whether prophage identification can be used as a *Campylobacter* typing technique.

Recommendations

At this stage recommendations arising from this work can only encourage further research to determine why the interactions between *C. jejuni* and its bacteriophages is so different from the interactions seen with other bacteria and their bacteriophages.

- More work is needed to investigate why we, and other research groups, find it so difficult to isolate *C. jejuni* bacteriophages from indoor meat chickens.

- The issue of whether the bacteriophages isolated are truly lytic bacteriophages or whether they are temperate/lysogenic needs to be resolved, as does the complex bacteriophage-host relationship.

Introduction

Summary

Campylobacter is the most common bacterial cause of diarrhoeal disease in humans in developed countries. A significant traceable source of human infection is poultry (Butzler 2004; Corry & Atabay 2001; Keener *et al.* 2004; Wagenaar *et al.* 2006). *C. jejuni* and *C. coli* account for the majority of human infections, with *C. jejuni* being the more common of the two, accounting for up to 95 percent of all human infections (Butzler 2004; Dingle *et al.* 2005; Loc Carrillo *et al.* 2007; Ng *et al.* 1997). Whilst current methods available to control *Campylobacter* have a documented effect, further reductions are sought (Keener *et al.* 2004).

One proposed adjunctive control measure for *Campylobacter* is the use of bacteriophages. Bacteriophages are viruses that are able to infect and kill bacterial cells (Doyle & Erickson 2006; Huff *et al.* 2003; Matsuzaki *et al.* 2005), and they will only multiply when susceptible bacteria are present (Brüssow 2005; Stave & Teaney 2006). The emergence of antibiotic resistant bacteria has, in recent times, renewed Western research interest in the possibilities of using bacteriophages to control bacteria, such as *Campylobacter*. Research has been carried out on the use of bacteriophages as direct therapy or for prevention of bacterial infections in numerous animal species, with animals also being used as models for human disease. Results of these studies show considerable promise as to the potential success of bacteriophages as targeted antimicrobial agents. It is these results, along with their inherent capabilities, which indicate that bacteriophages may have the potential to be used as antibacterial agents, in this case to target *Campylobacter*, specifically *C. jejuni*.

Campylobacter

Campylobacter species are curved, S-shaped or spiral Gram-negative rods, which occur primarily as commensal organisms in humans and many domestic animals. They are characterized as being oxidase and catalase positive, and generally microaerophilic, growing in atmospheres containing 3–15% O_2 and 2–10% CO_2. *Campylobacter* species do not ferment or oxidise carbohydrates. They have a single polar unsheathed flagellum at one or both ends of the cell, which facilitates their characteristic corkscrew-like motility. They have particularly fastidious growth requirements and behaviour *in vitro*. *Campylobacter jejuni* belongs to the thermophilic group of *Campylobacters*, along with *C. coli*, *C. lardis* and *C. upsaliensis*. The thermophilic species grow at 42-43^{o}C and 37^{o}C, but not at 25^{o}C (Snelling *et al.* 2005). This study is concerned only with *C. jejuni*, as it accounts for the majority of human *Campylobacter* infections (Butzler 2004; Dingle *et al.* 2005; Loc Carrillo *et al.* 2007; Ng *et al.* 1997).

Campylobacter in poultry

The source of initial exposure to *Campylobacter* species in poultry is usually unidentifiable. Once a bird is exposed, however, the gastrointestinal tract is rapidly colonized and provides optimal growth conditions for the bacteria (Snelling *et al.* 2005). Flocks usually become infected at around three weeks of age, and a large percentage of a flock will become lifelong excreters, within a short period of time following the initial exposure, with *C. jejuni* and *C. coli* the primary colonizers (Atterbury *et al.* 2003b; Corry & Atabay 2001). High levels of the organism (10^6–10^7 CFU/g) are then present in the caecal contents and faeces (Corry & Atabay 2001).

Although biosecurity measures can reduce *Campylobacter* colonization, they do not eliminate the risk altogether. The effects of controlling *Campylobacter* in individual poultry flocks is somewhat negated by the effects of cross contamination during transport, and subsequent abattoir processing–scalding, plucking and evisceration (Corry & Atabay 2001). Washing and the use of chlorine reduces the levels of contamination but does not entirely eliminate the risk of contamination of the final product.

Although *Campylobacter* is inactivated rapidly at normal cooking temperatures, inadequate cooking temperatures and improper handling of raw poultry products, combined with the low infectious dose necessary to cause an infection, means that any remaining organisms on poultry products pose a risk to human health.

Bacteriophages

Early last century, the concept of using host-specific bacteriophages to control bacteria emerged. Bacteriophages, also referred to as phages, are viruses that infect bacterial cells. They are the most numerous life forms on earth. The global bacteriophage population is estimated to be more than 10^{30} (Dabrowska *et al.* 2005; Hanlon 2007). In theory, bacteriophages are relatively inexpensive, certainly less expensive than developing new antibiotics, and easy to source (Bull *et al.* 2002; Kutter 1997; Matsuzaki *et al.* 2005).

Bacteriophages are usually found in the present of their host bacteria (Hanlon 2007; Higgins *et al.* 2005). In general, bacteriophages are very specific and only able to infect particular species, even strains, of bacteria (Hanlon 2007). They are only capable of infecting bacterial cells: there is no evidence that they are able to interact with other cells (Hanlon 2007; Higgins *et al.* 2005). A bacteriophage can only attach to a bacterial cell with a specific cell surface receptor (Doyle & Erickson 2006; Hanlon 2007). Once a bacteriophage attaches to a susceptible cell, it may undergo one of two different life cycles: virulent or temperate. A virulent bacteriophage will enter the cell and use the cell's own mechanisms to multiply. Once these mechanisms are exhausted, the cell lyses and hundreds of copies of the original bacteriophage are released (Doyle & Erickson 2006; Hanlon 2007). Temperate bacteriophages do not lyse the host cell. Instead, the DNA of a temperate bacteriophage integrates into the genome of the bacterial cell. A bacterial cell with integrated temperate bacteriophage DNA is said to be in a state of lysogeny. The integrated genome itself is referred to as a prophage. When the host bacterium replicates, the bacteriophage genome is also replicated (Hanlon 2007; Parisien *et al.* 2007). Prophages can encode for genes that change the phenotype of the host bacterium. This is known as lysogenic conversion (Boyd *et al.* 2001; Porwollik & McClelland 2003). The presence of prophage can also confer immunity to infection by other lytic and lysogenic bacteriophages (Baumler *et al.* 1998).

Bacteriophage genomics and classification

The morphology of bacteriophages is variable, but most have an icosahedral head (capsid) and a tail. The nucleic acid (DNA or RNA) of the bacteriophage is contained within the head (Hanlon 2007; Parisien *et al.* 2007). Bacteriophages have coevolved along with their specific bacterial hosts. Like other viruses, they are reliant on the energy production and biosynthesis of their cell host. Of these, only a relative few have been entirely sequenced (Brüssow & Hendrix 2002; Brüssow *et al.* 2004; McGrath *et al.* 2004). Bacteriophages exhibit enormous genetic diversity, but there is also evidence of many gene similarities. The current classification scheme is based on morphology and the type of genome–single-stranded or double-stranded, RNA or DNA. Genome size is variable, ranging from barely 4 kb to 600 kb in length. Over 95 % of reported bacteriophages possess a tail structure, but it is unknown as to whether this is a true representation of the percentage of tailed bacteriophages, or rather due to sampling bias. Nevertheless, more is known about the tailed bacteriophages than any other bacteriophages. Many genetically diverse bacterial species have been shown to have associated tailed bacteriophages. Of the known tailed bacteriophages, these are clusted into groups (Brüssow & Hendrix 2002; Hendrix 2003). Tailed bacteriophages belong to the order *Caudovirales*. This order currently has three recognized families–*Myoviridae, Siphoviridae* and *Podoviridae*–all of which are classified based on their tail morphology. The *Myoviridae* have long contractile tails, *Siphoviridae* possess a long non-contractile tail, and *Podoviridae* a short non-contractile tail. In tailed bacteriophages the genome encodes for DNA packaging, head, tail and tail fibres, DNA replication, transcription regulation and lysis genes. In the smaller genome bacteriophages, these are the primary functions, but bacteriophages with larger genomes are observed to have more encoded genes, which can interfere more with the

cellular functions of the bacterial host. The function of many genes that have been sequenced from bacteriophage genomes have yet to be determined, and the significance of the similarities and differences in the genomes of sequenced bacteriophages are also unknown. Temperate bacteriophages are known to encode for certain bacterial toxins, via insertion of prophage DNA into the host genome. One such example is cholera toxin (Brüssow & Hendrix 2002). Temperate bacteriophages can undergo lytic cycles and during this process can pick up bacterial host genes including virulence genes and antibiotic resistance genes and transfer them to a new bacterial host (horizontal gene transfer) (Brabban *et al.* 2005). For this reason it is important to ensure that bacteriophages selected for phage therapy are lytic bacteriophages and not temperate bacteriophages. It is thought that strictly virulent bacteriophages may have a different evolution than temperate bacteriophages. Theoretically, virulent bacteriophages never become prophages. Many of these bacteriophages hydrolyse the DNA of their host after infected the cell. It is therefore considered that horizontal gene exchange, which is readily exhibited by temperate bacteriophages, is less likely to occur (Hendrix 2003).

Preventative and therapeutic use of bacteriophages in animals

Early bacteriophage investigations showed that they were able to kill bacteria and appeared to have the potential to control bacterial infection, but the results of early research were unconvincing (Keener *et al.* 2004). After the discovery of antibiotics, Western research into bacteriophages was halted, but clinical research and use of bacteriophage therapy has continued in Eastern Europe and the former Soviet Union. Dedicated bacteriophage research centres still exist in Tbilisi, Georgia and in Wroclaw, Poland (Kutter 1997), but only limited amounts of this research is accessible to Western researchers. Antibiotic resistance has renewed Western interest in the possibilities of using bacteriophages to control bacteria.

Many studies have investigated the use of bacteriophages to control various bacterial species. Most Western research has so far focused on the effects of bacteriophages on Gram-negative bacteria, but some work has also been published regarding Gram-positive pathogens (Hanlon 2007; Matsuzaki *et al.* 2005). Reports have been published looking at bacteriophages application in cattle, sheep, pigs, chickens, fish and crustaceans. Research has also been conducted on the use of bacteriophages to control the foodborne pathogen–*Listeria monocytogenes*–on the surface of food products (Leverentz *et al.* 2003). Two commercial bacteriophage spray products have also recently been released–one for the control of *L. monocytogenes* on ready-to-eat meat and poultry products (Lang 2006) and another to control the zoonotic bacterium–*E. coli* O157:H7–at cattle abattoirs (ElAmin 2007).

Bacteriophage research in chickens

In chickens, *E. coli* is a cause of respiratory infection, known as colibacillosis, which results in significant mortalities and carcass condemnation. The efficacy of bacteriophages to prevent infection has been studied. Huff et al. (2002) inoculated broilers with mixed cultures of *E. coli* and bacteriophages, which had been combined 24 hours prior to inoculation. Inoculation was directly into the airsac. The broilers that received the bacteriophages had significantly lower mortality, compared with a control group. A combination of 10^4 CFU/ml of *E. coli* with 10^8 PFU/ml of bacteriophage resulted in the most significant decrease in mortality (85% mortality in the control group compared with no mortality in the phage-treated group). A separate experiment where the same bacteriophages were administered via the drinking water, before and after *E. coli* inoculation, showed no effect on the course of infection (Huff *et al.* 2002).

The efficacy of treating *E. coli* with intramuscular (IM) injection of bacteriophages has also been investigated. *E. coli* was inoculated into the airsac of the birds, and bacteriophages were injected IM immediately following bacterial inoculation. The highest titre (10^8 PFU/ml) of one of the bacteriophages used in the experiment (SPR02) resulted in a significant decrease in bird mortality. The importance of the multiplicity of infection (MOI) was also demonstrated in this study. This is the ratio of the number of bacteriophages per bacteria cell. A MOI of 10^4 (10^8 PFU of bacteriophage with 10^4 CFU of *E. coli*) was most successful, resulting in complete treatment efficacy. The required MOI

reported in bacteriophage studies in animals is highly variable (Huff *et al.* 2006). The efficacy of IM injection was also compared to that of an aerosol spray of bacteriophages. The bacteriophages were administered, via aerosol spray, prior to inoculation of *E. coli* into the thoracic air sac. There was a significant reduction in mortality, even when the bacteriophages were administered 3 days before inoculation with E. coli (Huff *et al.* 2005). Bacteriophages administered via aerosol spray immediately following inoculation with *E. coli* significantly reduced mortality, but if administration of bacteriophages was delayed to 24 or 48 hours following initial inoculation, there was no decrease in mortality. In contrast, IM injection administered up to 48 hours after inoculation still significantly decreased mortality (Huff *et al.* 2003).

Huff *et al.* (2004) have also studied the efficacy of enrofloxacin (a fluoroquinolone antibiotic) combined with bacteriophage therapy to treat colibacillosis. Bacteriophages were administered IM as a single injection immediately after the birds were challenged with *E. coli*. Enrofloxacin was administered in the drinking water at 50 ppm for 7 days. The most significant reduction in mortality was achieved with the combination of enrofloxacin and bacteriophage therapy (Huff *et al.* 2004).

Prior to the work of Huff *et al.*, Barrow *et al.* (1998) studied the effects of bacteriophage administration on *E. coli* septicaemia and meningitis in chickens. IM injection of a combination of *E. coli* and bacteriophages (into separate muscles), at a 1:1 ratio, resulted in no morbidity or mortality. An intracranial inoculation with *E. coli* was also performed, with concurrent IM injection of bacteriophages. Significant reductions in morbidity and mortality were observed with 10^6–10^8 PFU/ml of bacteriophages. If bacteriophage administration was delayed until the development of signs of disease, a significant reduction in mortality could still be achieved (Barrow *et al.* 1998).

An organism in chickens of concern to human health is *Salmonella enterica* serovar Enteritidis. Higgins *et al.* (2005) have demonstrated experimentally that bacteriophage treatment (10^8 PFU/ml), either applied to rinse water or directly to chicken carcasses prior to rinsing, reduced levels of S. Enteritidis recoverable from carcass rinse water.

Bacteriophages and *Campylobacter*

Campylobacter bacteriophages have been isolated from the chicken intestinal tract, and Atterbury *et al.* (2005) observed that in birds carrying bacteriophages the recovery of *Campylobacter*, and the number of strains, was reduced. They also observed that bacteriophages active against *Campylobacter* species can survive processing, and are present on retail poultry products (Atterbury *et al.* 2003b; Atterbury *et al.* 2005). The effect of applying bacteriophages to artificially inoculated sections of chicken skin has been analysed. The highest titre of bacteriophages used (10^7 PFU/ml) significantly reduced the numbers of *Campylobacter* isolated (Atterbury *et al.* 2003a). Goode *et al.* (2003) also demonstrated a reduction in *Campylobacter* isolation from bacteriophage-treated chicken skin (10^6 PFU/cm^2).

The application of bacteriophages to directly decrease *Campylobacter* colonization of the chicken gastrointestinal tract has also been studied. Bacteriophages active against *Campylobacter* were administered orally in an antacid suspension to 25-day-old broilers, which had been inoculated with *C. jejuni* at 18-20 days of age. Twenty-four hours following treatment, the isolation of viable *Campylobacter* was reduced in one or more of three intestinal sampling sites, but counts began to increase at 72 hours post-bacteriophage administration (Loc Carrillo *et al.* 2005). Wagenaar *et al.* (2005) have also studied the effect of *Campylobacter* bacteriophages on *C. jejuni* colonization in young broilers. Bacteriophages as a preventative and therapeutic modality were assessed. In the therapeutic group, *C. jejuni* counts were initially decreased post-bacteriophage treatment. After 5 days, counts reached a plateau, but were still lower than the control group. In the preventive group, *C. jejuni* colonization was delayed. After a week, the counts had stabilized at a level comparable with the therapeutic group (Wagenaar *et al.* 2005).

Objectives

The key objective is to determine a better understand of the presence and characteristics of bacteriophages in South Australian chickens and whether they can be used to lyse *C. jejuni* by:

- Isolating and characterising bacteriophages from various samples from chickens.
- Determining the lytic activity of a panel of isolated wild type bacteriophages against a wide range of *C. jejuni* strains.

The deliverables from this project were to be a panel of bacteriophages and phage products effective against *C jejuni*.

The hypothesis for this research project was that naturally occurring bacteriophages can be sourced, which are capable of lysing multiple wild-type strains of *C. jejuni* from chickens, and may be able to reduce the carriage of *C. jejuni* on retail poultry products, and subsequently in live chickens.

Methodology

Isolation and identification of Campylobacter

Campylobacter strains were isolated from fresh faecal samples on *Campylobacter* agar base (Karmali) (Oxoid, CM0935) with *Campylobacter* selective supplement (Karmali) (SR0167E), and propagated on Columbia agar (Oxoid, CM0331) supplemented with 5 % horse blood. Karmali agar was found to give the best isolation rate of *Campylobacter* with less contamination and better growth. All isolates were grown at 42^{o}C, under microaerophilic conditions. Isolates were stored in glycerol snap freeze medium, in cryo vials, at -80^{o}C.

Initial identification was completed via phase contrast microscopy and biochemical methods–oxidase, catalase and hippurate hydrolysis.

Multiplex PCR

Multiplex PCR was used to identify isolates to species level. All reactions were carried out in a total volume of 25 µL. Each multiplex PCR tube contained 0.2-0.3 mM deoxynucleoside triphosphate; 5 µL of 5 x reaction buffer, 2 mM $MgCl_2$; 0.5 µM *C.jejuni* primers; 1 µM *C. coli* primers; 1 µM 23S rRNA primers or 0.5 µM 16S rDNA primers; 1.25 U of *Taq* DNA polymerase, and 1 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 µL. DNA amplification was carried out in a MyCycler thermocycler (Bio-Rad). *C. jejuni* NCTC 12662 and *C. coli* NCTC 11366 were used as positive species controls.

23s rRNA primers (Wang *et al.* 2002):

23S-F 5'-TATACCGGTAAGGAGTGCTGGAG-3'
23S-R 5'-ATCAATTAACCTTCGAGCACCG-3'

16S rDNA primers (Persson & Olsen 2005):

16S-F 5'-GGAGGCAGCAGTAGGGAATA-3'
16S-R 5'-TGACGGGCGGTGAGTACAAG-3'

C. jejuni hipO primers (Wang *et al.* 2002):

CJ-F 5'-ACTTCTTTATTGCTTGCTGC-3'
CJ-R 5'-GCCACAACAAGTAAAGAAGC-3'

C.coli glyA primers (Wang *et al.* 2002):

CC-F 5'-GTAAAACCAAAGCTTATCGTG-3'
CC-R 5'-TCCAGCAATGTGTGCAATG-3'

For reactions including the 23S rRNA primers–denaturation at 95°C for 6 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 30 sec, with a final extension at 72°C for 7 min. Reactions including the 16S rDNA–denaturation at 94°C for 6 min, followed by 35 cycles of denaturing at 94°C for 50 sec, annealing at 57°C for 40 sec and extension at 72°C for 50 sec, and finally 72°C for 3 min.

PCR products were run on a 1.5 % agarose gel, containing 0.25 µg/mL ethidium bromide, for 60-90 minutes. A 100 bp DNA marker was included on the gel for approximation of PCR product size.

Bacteriophage isolation

Various samples were sought from abattoirs and farms of both indoor commercial and free range chicken companies. Each sample was suspended in salt of magnesium (SM) buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4$ and 0.01 % w/v gelatine) – 1:10 (w/v) faeces/litter/intestinal contents, 1:5 (w/v) visceral rinse/scald tank water/sewage water – then shaken overnight, at 4^{o}C, on an orbital shaker. The supernatant was removed and centrifuged at 4000 g for 20 minutes, at 4^{o}C. Four millilitres of supernatant was removed and filtered through a 0.45 µM Millipore membrane filter. This direct buffer isolation method was used as *Campylobacter* does not grow well in clear broth, which is commonly used in bacteriophage enrichment methods for other bacterial species. This method was proven to successfully isolate *Campylobacter* bacteriophages by re-extraction of *C. jejuni* ϕ NCTC 12675 from artificially infected litter samples.

Bacteriophage detection

The filtrate was tested for the presence of bacteriophages using a traditional plaque assay plate technique, using NZCYM agar (1.5 % agar base, 0.7 % semi-solid overlay). The filtrate was spotted onto a lawn of the indicator strain *C. jejuni* NCTC 12662. Typical plates are illustrated in Figures 1 and 2.

Bacteriophage purification

If lysis was present at a filtrate spot on the plate this was extracted using a sterile 1 µL culture loop and resuspended in 200 µL of SM buffer. Overlay plates were then redone, but with 10 µL of the bacteriophage suspension included in the overlay. Individual bacteriophage plaques were then extracted from the plates using a sterile 500 µL pipette tip and resuspended in 200 µL of SM buffer. Plating out and plaque extraction, of each individual bacteriophage-positive sample, were repeated a further two times.

Determination of bacteriophage titre

Each bacteriophage suspension was diluted to give a dilution factor of 10^{-1} and serial diltions made to a dilution factor of 10^{-10}. A 10 µL droplet of each dilution, starting with the undiluted sample, was spotted onto an NZCYM overlay plate containing *C. jejuni* NCTC 12662, in duplicate. The numbers of individual plaques present at each spot were counted and the number of plaque forming units (PFU) per ml (bacteriophage titre) was calculated from the total dilution (*Figure 3*).

Bacteriophage amplification and propagation

Five millilitres of SM buffer was pipetted onto the surface of a pre-incubated overlay plate, with the bacteriophage suspension in the overlay, and the plate shaken overnight at 4^{o}C, on an orbital shaker. The SM buffer was then removed from the surface of the plate using a 10 mL syringe, and filtered through a 0.45 µM filter. Multiple plates were prepared for each bacteriophage suspension and the SM buffer plate wash combined post-filtration.

Spectrum of activity of bacteriophages *in vitro*

Bacteriophages were selected for further testing based on having been successfully purified and then showing potential lytic activity on spot overlay plates, using control strain NCTC *C. jejuni* 12662, as opposed to appearing obviously lysogenic, with incomplete lysis. A control bacteriophage was also included from the National Collection of Type Cultures – NCTC ϕ 12675. The 241 wild type

C. jejuni isolates collected and speciated during the course of this study were tested for their sensitivity a panel of 55 bacteriophages, plus the control bacteriophage.

Electron microscopy

Bacteriophage samples were prepared to their highest achievable titre ($10^7 – 10^{10}$ PFU/ml) in SM buffer. Ten microlitres of bacteriophage suspension was applied to the surface of a 200-mesh copper grid and stained with 1 % aqueous phosphotungstic acid (pH 6.5), then examined via transmission electron microscopy at up to 92,000x magnification.

Genomic DNA extraction and restriction enzyme digestion

Bacteriophage genomic DNA was extracted using a phenol/chloroform extraction method and the DNA resuspended in 50 µL of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). Genomic bacteriophage DNA was analysed by restriction enzyme digestion. The enzymes used were *Eco*RI, *Hind*III, *Sma*I, *Dra*I, *Sau*3AI and *Hha*I. Digests were separated on agarose gel electrophoresis.

Bacteriophage stability at variable pH and temperature

All bacteriophage suspensions were stored in SM buffer, at 4°C, until required. Two bacteriophage suspensions (ϕCJ011 and ϕCJ048) were tested for their stability at different pH and temperature over a six week period. The buffer was adjusted separately to pH 2.5, 5.5, 7.5 and 9.0, and then used to dilute the bacteriophage suspensions to 10^7 PFU/ml. Two millilitre aliquots of each pH preparation were stored at 25°C, 37°C and 42°C. The bacteriophage titre of each preparation was determined after 24 hours and then at 7 day intervals for a period of six weeks. Bacteriophage suspension ϕCJ048 was also tested for stability in acid over 24 hours, using a similar method, except that the pH of the buffer was adjusted to pH 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0, and the bacteriophage titre determined at regular intervals over 24 hours.

Application of bacteriophages to retail chicken meat

Bacteriophage suspension ϕCJ051 was used in single bacteriophage suspension experiments, at titre of 10^6 PFU/ml. ϕCJ002, ϕCJ016, ϕCJ032 and ϕCJ048 were also used in experiments in combination with ϕCJ051. Each suspension was prepared individually in SM buffer (pH 7.5), to a titre of 10^6 PFU/ml, and combined in equal volumes. Experiments were conducted separately using *C. jejuni* NCTC 12662 (experiment 2), and a combination of CJ0097, CJ0186, CJ0207, CJ0211 and CJ0224 (experiment 1) as infecting strains. 5g pieces of raw chicken meat, with the skin on, were infected with *C. jejuni*. Five hundred microlitres of the bacteriophage suspension (10^6 PFU/ml) was pipetted on to the skin surface of 'treated' samples, whereas for untreated samples 500 µL of sterile SM buffer was pipetted on to the skin surface. One group of samples were stored at -20°C overnight. All other storage was at 4°C for the duration of the experiment. In all experiments the levels of *Campylobacter* present were determined at 24 hours intervals post-inoculation for a period of 5 days. Re-isolation of bacteriophages was attempted 1 hour and 120 hours post-inoculation.

Re-isolation of *Campylobacter*

Campylobacter was recovered by stomaching chicken pieces in maximum recovery diluent and isolating with Karmali agar.

Re-isolation of bacteriophages

Bacteriophages were recovered by stomaching chicken pieces with 20 ml of SM buffer and then using the plaque assay method.

Multiple indicator strains of *Campylobacter jejuni*

It was postulated that bacteriophages could not be isolated from indoor commercial chicken samples owing to an inherent resistance to the universal indicator strain *C. jejuni* NCTC 12662. Therefore, 18 wild type strains of *C. jejuni* were selected based on their levels of sensitivity to previously isolated bacteriophages, plus two control strains–*C. jejuni* NCTC 12663 and *C. jejuni* NCTC 11168. These were used as isolating strains and fresh samples were collected from indoor commercial poultry facilities and processed for bacteriophages.

Restriction fragment length polymorphism (RFLP)

The RFLP profiles of 174 *C. jejuni* strains were determined and compared, as it was thought that decreased sensitivity of *C. jejuni* strains from individual companies may be due to the presence of similar or clonal isolates.

Reactions were carried out in 25 µL volumes. Each reaction contained 0.2 mM deoxynucleoside triphosphate; 5 µL of 5 x reaction buffer; 2 mM $MgCl_2$; 0.5 µM *flaA* primers; 1.25 U of *Taq* DNA polymerase; and 1 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 µL. DNA amplification was carried out in a MyCycler thermocycler using an initial denaturation step at 94^{o}C for 1 min, followed by 35 cycles of denaturation at 92^{o}C for 30 sec, annealing at 55^{o}C for 1 min 30 sec and extension at 72^{o}C for 2 min 30 sec, and finally 72^{o}C for 5 min. *C. jejuni* ATCC 33560 was used as a positive control. PCR products were run on a 1.0 % agarose gel, containing 0.25 µg/ml of ethidium bromide, for 60 minutes to check for successful amplification of the *flaA* gene. The PCR product was then digested with *Dde*I and run on a 2.0 % agarose gel, 10x11cm in size, containing 0.25 µg/ml ethidium bromide, and run at 100 V for 90 minutes in 1 x TAE buffer. Gel images were inputted into the BioNumerics program and compared using a Dice comparison, with limits set at 3.0 %, ensuring the *C. jejuni* ATCC 33560 control digests were considered 100 % similar within these limits.

flaA primers (Nachamkin *et al.* 1993):

fla-F	5’-GGATTTCGTATTAACACAAATGGTGC-3’
fla-R	5’-CTGTAGTAATCTTAAAACATTTTG-3’

Campylobacter jejuni insertion elements

It was postulated that the presence of prophage could explain differences in sensitivity between *C. jejuni* isolates from different sources. Primer sequences for three separate *Campylobacter jejuni* insertion elements were chosen: cje0215, cje0270 and cje1471 (Clark & Ng 2008). PCR reactions were carried out in 25 µL volumes with each set of primers separately. Each reaction contained 0.2 mM deoxynucleoside triphosphate; 5 µL of 5 x reaction buffer; 2 mM $MgCl_2$; 0.5 µM of the appropriate forward and reverse cje primers; 1.25 U of *Taq* DNA polymerase; and 1 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 µL. DNA amplification was carried out in a MyCycler thermocycler using an initial denaturation step at 95^{o}C for 6 minutes, followed by 30 cycles of denaturation at 95^{o}C for 1 minute, annealing at 50^{o}C for 1 minute (53^{o}C for cje1471), and extension at 72^{o}C for 1 minute, and finally 72^{o}C for 7 min. PCR products were run on a 1.5 % agarose gel at 100 volts, containing 0.25 µg/ml of ethidium bromide. A 100bp ladder was included for approximation of band fragment sizes.

CJIE primers:

cje0215-F	5'-GCG AGT GAA GGC AAA AG-3'
cje0215-R	5'-TTC TCC ATA GCA AGT GAT AAA C-3'
cje0270-F	5'-TTC ACC GCA AAG ATA AAA CTA A-3'
cje0270-R	5'-ACT ATA ATA TCA GCT GGG GAA CTA-3'
cje1471-F	5'-CCG CAA ATG AAA CCG AAC AA-3'
cje1471-R	5'-GCC ATA ACC CAA AGC AGG ATT-3'

Results

Campylobacter isolation and identification

A total of 317 *Campylobacter* isolates were collected during the course of this study (*Table 1*). All isolates were of poultry origin, sourced either directly from chickens or from facilities housing or processing live chickens. Eighty six isolates were obtained from a previous project in Queensland (group 2), 148 isolates were obtained from a previous project at the University of South Australia (group 1), and 83 isolates were isolated during the course of this study (group 3). The majority of fresh faecal samples collected were positive for *Campylobacter* growth on selective media. All isolates were initially identified as *Campylobacter* and speciated using the outlined biochemical methods. Using multiplex PCR, 241 of these isolates were speciated as *C. jejuni* and 76 as *C. coli*.

Table 1 *Campylobacter* isolates speciated during the course of this study

Company	Group	Category	*C. jejuni* isolates	*C. coli* isolates
company A (Qld)	group 2	indoor commercial	39	NA
company B (Qld)	group 2	indoor commercial	15	NA
company C (Qld)	group 2	indoor commercial	32	NA
company 1	group 1 (148 isolates); group 2 (22 isolates)	indoor commercial	107	63
company 2	group 3	indoor commercial	9	0
company 3	group 3	free range broilers	16	6
company D	group 3	free range egg layers	6	2
company E	group 3	free range egg layers	4	4
company F	group 3	free range egg layers	5	1
company G	group 3	shedded egg layers	8	0
TOTAL			**241**	**76**

A table showing the *Campylobacter* isolates obtained during the course of this study, where they were isolated from, including the rearing conditions of the chickens, whether the isolates were speciated as *C. jejuni* or *C. coli*, and which isolate 'group' they are assigned to for the purposes of this study. (*Isolate Group 1*–from a previous project at the University of South Australia, all from company 1; *Isolate Group 2*–from a previous project in Queensland, from companies A, B and C; *Isolate Group 3*–isolated during the course of this study, from a range of companies.)

A comparison of the percentage of *C. jejuni* compared to *C. coli* isolates is not relevant for the Queensland isolates, as only isolates that had been previously identified as *C. jejuni* were obtained. No *C. coli* isolates were obtained. For the isolates that were isolated during the course of this study the percentage of *C. jejuni* and *C. coli* isolates was 74.7 % and 25.3 %, respectively. For the company 1 isolates from the previous study, 62.8 % were *C. jejuni* and 37.2 % *C. coli*. This correlates well with reports that the percentage of *C. coli* isolated from poultry ranges anywhere from 6 to 50 % of all *Campylobacter* present (El-Shibiny *et al.* 2007), but is usually isolated less frequently than *C. jejuni*.

Of the isolates isolated during this study, the percentage of *C. coli* in the free range *Campylobacter* isolates was 21.3 %, and in from indoor commercial birds 33.4 %, despite some studies suggesting that the percentage of *C. coli* isolated from free range birds is higher (El-Shibiny *et al.* 2007). However, the small numbers may mean the percentages are not an accurate reflection of actual colonization percentages. Only the *C. jejuni* isolates were selected for use in further research.

In conclusion, the results of this work correlate well with the reported levels of *Campylobacter* infection in poultry. Both *C. jejuni* and *C. coli* were readily isolated from indoor commercial broilers, free range broilers and free range egg layers. There was no appreciable difference in the isolation rates between the different sources, but this may be due to sample size.

Bacteriophage isolation

Owing to the less fastidious nature of *Escherichia coli* compared with *Campylobacter*, this organism was chosen as an experimental model for initial testing of the bacteriophage isolation technique. Bacteriophages active against the *E. coli* strain used were readily isolated from the poultry samples tested (shed litter samples from indoor commercial poultry farms), as determined by the presence of lysis on overlay agar plates (*Figure 1*). Bacteriophages active against *C. jejuni* could not be successfully isolated from the same samples.

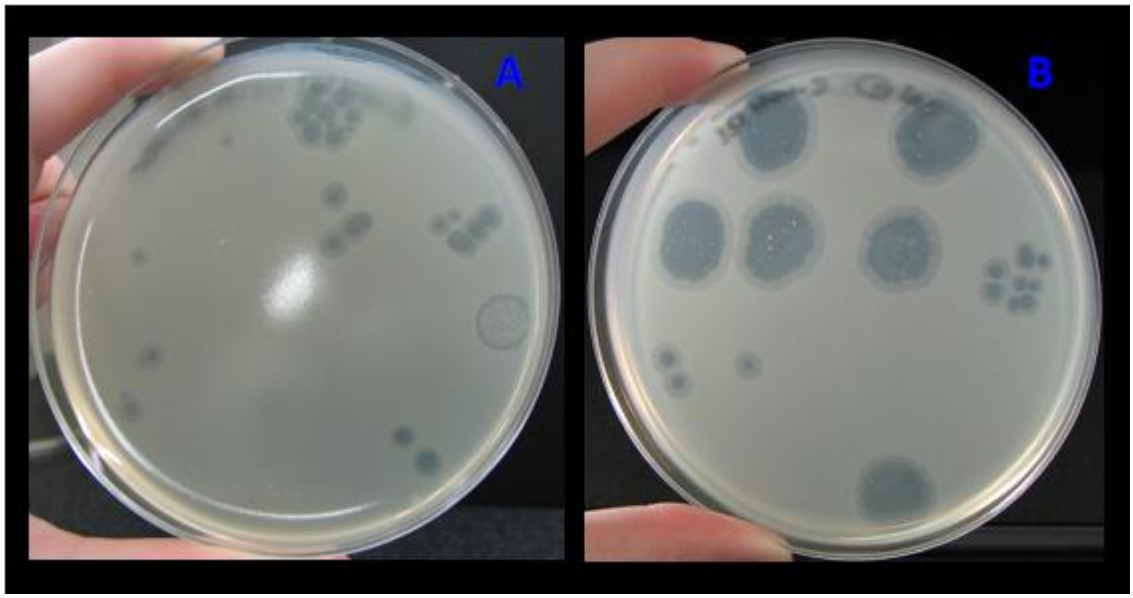

Figure 1 *Escherichia coli*-active bacteriophages on plaque assay plates

This is a typical result showing positive results for *E. coli*-active bacteriophages in processed samples from chicken shed litter. Bacteriophage lysis is evident as areas of clearing on the turbid lawn of the plate. A) Multiple areas positive for bacteriophage lysis, from different samples spotted onto the bacterial lawn. B) Individual purified bacteriophage sequentially diluted by a factor of 10 and spotted onto the bacterial lawn, to give individual bacteriophage plaques.

C. jejuni ϕ NCTC 12675 was used to artificially infect previously *C. jejuni* bacteriophage negative chicken shed litter samples and could be successfully re-isolated using the described method, even when the infecting titre was very low. Chicken samples types processed for *C. jejuni* bacteriophages were: shed litter, faeces, gut contents, visceral rinse and scald tank water. Other samples processed were: sewage water, and duck, sheep, ferret and cattle faeces. No bacteriophages could be isolated from broilers reared under traditional indoor commercial conditions or from any non-poultry samples.

This is consistent with reports obtained via email correspondence with research groups in both the UK and The Netherlands. Both groups reported extreme difficulty or complete lack of isolation of *C. jejuni* bacteriophages from indoor commercial poultry. They also reported observation of the much more complex behaviour of *C. jejuni* bacteriophages compared to other species. It was under the recommendation of the UK research group that samples were sought from free range poultry.

A total of 661 individual samples were processed looking for bacteriophages active against *C. jejuni*– 130 were positive. All positive samples were from poultry sources, specifically free range egg layers and broilers, and other egg layers. Eight separate sampling sites yielded bacteriophage-positive samples: 1 processing plant (processing primarily free range broilers), 2 free range broiler farms, 4 free range egg layer farms, and 1 shedded egg layer farm (with comparatively lower implemented biosecurity measures compared to indoor commercial broiler facilities). The highest yield of bacteriophage-positive samples (63.3 %) was obtained from a free range egg layer farm, which was the most geographically isolated from the rest of the farms tested. The lowest isolation rate (4.4 %), out of the eight bacteriophage-positive sources, was from a broiler farm with younger (29-day-old) birds. Using farm-only sample data, the percentage isolation in free range broilers versus egg layers is 30.0 % and 46.8 %, respectively, but the free range broilers sampled directly on-farm represent a smaller sample group (50) compared to the free range egg layers (156).

Figure 2 *Campylobacter jejuni* bacteriophages on a plaque assay plate

This is a typical result showing positive results for *C. jejuni* bacteriophages in processed samples from chickens. Bacteriophage lysis is evident as areas of clearing on the turbid lawn of the plate.

One hundred and twenty *C. jejuni* bacteriophage-positive samples were selected for purification. Of these, 73 were successfully purified–55 showed potential lytic activity and were selected for further studies.

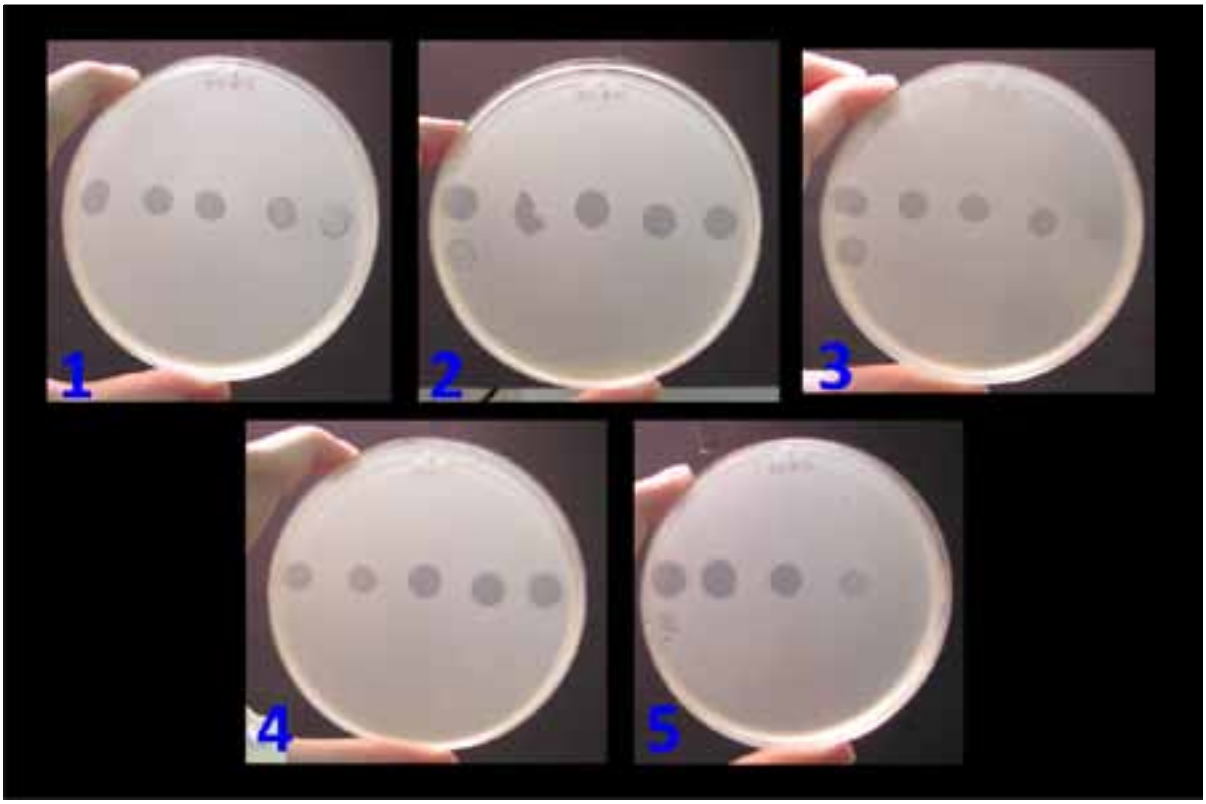

Figure 3 Bacteriophages sequentially diluted and spotted on plaque assay plates to determine titre

All bacteriophages spotted on isolating strain *C. jejuni* NCTC 12662. 1) ϕCJ039; 2) ϕCJ052; 3) ϕCJ048; 4) and 5) ϕCJ032 with the aim of generating discrete plaques at lower dilutions.

Table 2 All samples tested for *C. jejuni* bacteriophages during this study

Sample source	Bacteriophage positive	Bacteriophage negative	TOTAL
Free range egg layers	73	83	156
Free range broilers	46	96	142
Indoor commercial poultry	0	237	237
Other	11	58	69
Non-poultry (other species)	0	49	49
Environmental	0	8	8
TOTAL	130	531	**661**

Summary of all samples processed during the course of this study to isolate *C. jejuni* bacteriophages. Sample sources are shown on the left. Total number of samples processed from each source is shown, as are the numbers of samples that were positive versus negative for *C. jejuni* bacteriophages.

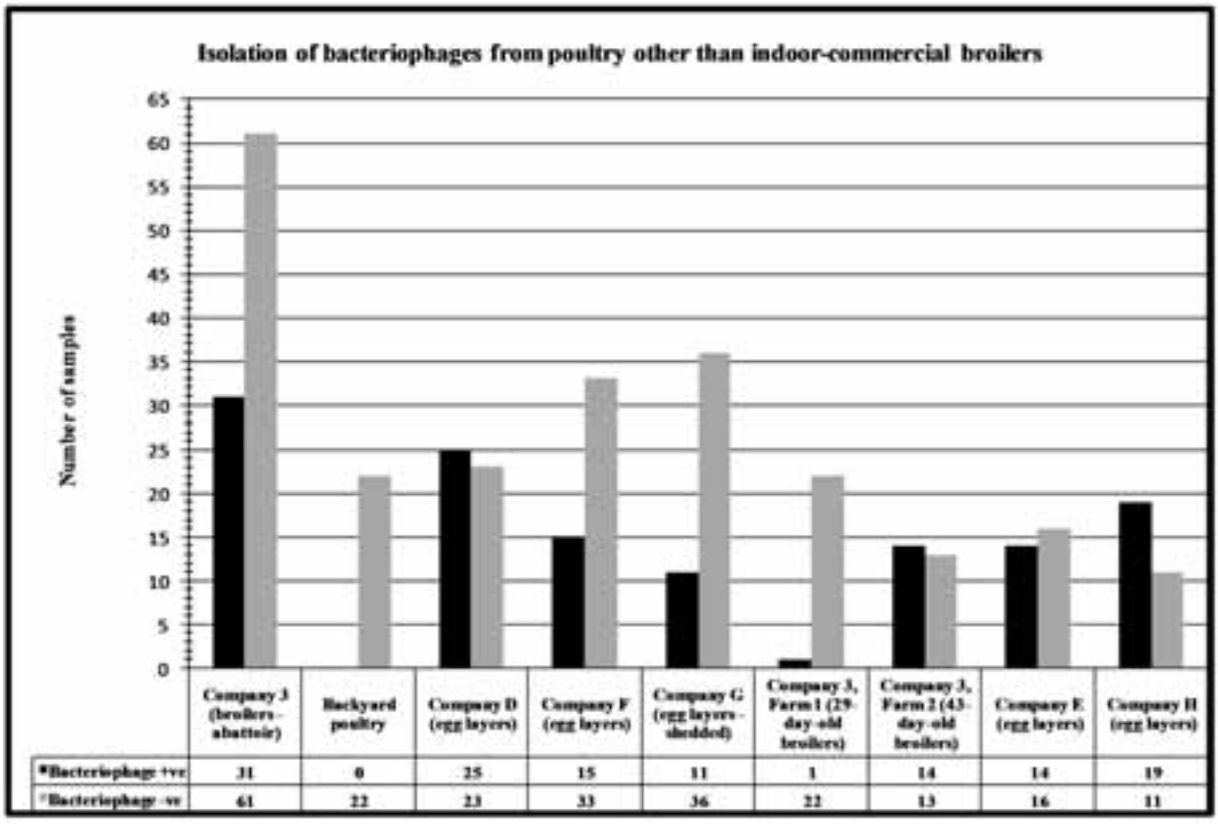

Figure 4 Bacteriophage isolation from poultry sources other than indoor commercial broilers

Graph showing the distribution of positive and negative bacteriophage samples from all poultry sources tested, other than those from broilers raised under traditional indoor commercial conditions. All sources tested not included in this group were negative for any bacteriophages. The numbers of samples positive for *C. jejuni* bacteriophages shown in this graph represent all positive samples recorded in this study.

Characterisation of bacteriophages

Campylobacter sensitivity to bacteriophages

A total of 241 *C. jejuni* isolates were tested for sensitivity to the 55 purified bacteriophage suspensions, plus a control bacteriophage (ϕ NCTC 12675). Sensitivity to a bacteriophage was determined by the presence of lysis on an overlay agar plate. Lysis was graded subjectively: negative/no evidence of lysis (-), minimal lysis (+), moderate lysis (++) or strong lytic response, with completely clear lytic plaques (+++). According to this scale 239 isolates showed at least minimal lysis from one or more bacteriophage suspensions. Significant lysis, however, was classified as only moderate (++) or strong (+++) lysis. Seventy percent of isolates (169) showed significant sensitivity to at least one bacteriophage, including the control bacteriophage. Excluding the control bacteriophage, the overall percentage of sensitive isolates was lower at 59 %. There was a difference in significant sensitivity between the three groups of isolates: including the control bacteriophage group 1 – 69 %, group 2 – 77 %, and group 3 – 63 %; excluding the control bacteriophage group 1 – 46 %, group 2 – 76 %, and group 3 – 55 %.

As discussed, there was some inconsistency in the appearance of plaques on overlay plates. Different agars were trialled to try and produce more uniformity in appearance. No combination produced

appreciably better, results. Hence NZCYM was selected for all testing. Differences in the phenotypic appearance of the lysis is thought to be due to small changes in the growth conditions of the host bacteria, which correlates with the fastidious nature of *Campylobacter* spp.

Table 3 *C. jejuni* isolate sources tested for significant sensitivity to the panel of 25 bacteriophages

Company	Category	Significant sensitivity (%)
company A (Qld)	indoor commercial	**74** (74)
company B (Qld)	indoor commercial	**80** (87)
company C (Qld)	indoor commercial	**75** (75)
company 1	indoor commercial	**48** (67)
company 2	indoor commercial	**44** (56)
company 3	free range broilers	**88** (88)
company D	free range egg layers	**67** (67)
company E	free range egg layers	**0** (75)
company F	free range egg layers	**40** (60)
company G	shedded egg layers	**25** (25)

All *C. jejuni* isolates tested for significant sensitivity to the bacteriophage panel. The type of chicken industry or rearing conditions of each company/source is shown. The number in bold represents the number of isolates from the source that were sensitive to the bacteriophage panel excluding the control bacteriophage (ϕ NCTC 12675). The number in brackets is the significant sensitivity including the control bacteriophage. Significant sensitivity to a bacteriophage was determined subjectively as ++ or +++ lysis on a plaque assay.

Individual bacteriophage suspensions showed a wide variation in activity against *C. jejuni* strains. The control bacteriophage ϕ NCTC 12675 showed the highest level of activity, with 38.0 % of isolates showing sensitivity. Amongst the wild type isolates ϕCJ052 exhibited widest range of activity, with 32.5 % of isolates sensitive. Consistent with the difference in sensitivity between the three groups of isolates, individual bacteriophage suspensions showed varying activity between groups. Bacteriophages isolated from birds from company H showed the highest levels of activity for isolate groups 2 and 3, but lower levels of activity in group 1. Bacteriophages ϕCJ001, ϕCJ002, ϕCJ003, ϕCJ011 and ϕCJ016, and ϕCJ048 and ϕCJ050-055 showed similar activity patterns in numerous, but not all, isolates tested. A final panel of 25 bacteriophage suspensions was chosen based on their activity against the *C. jejuni* isolates, and to represent a cross-section of isolation sources.

Table 4 Final bacteriophage panel selected from this study

Bacteriophage	Source	Sensitive isolates (%)
ϕCJ001	company E	20.3
ϕCJ002	company E	18.3
ϕCJ002	company E	19.5
ϕCJ011	company E	18.7
ϕCJ015	company 3	12.0
ϕCJ016	company 3	27.8
ϕCJ018	company 3	10.0
ϕCJ030	company D	12.5
ϕCJ032	company D	14.0
ϕCJ033	company D	12.0
ϕCJ035	company D	12.9
ϕCJ039	company F	14.9
ϕCJ040	company F	20.3
ϕCJ041	company F	12.9
ϕCJ042	company F	11.6
ϕCJ043	company F	15.4
ϕCJ044	company F	16.6
ϕCJ045	company F	10.4
ϕCJ048	company H	23.8
ϕCJ050	company H	21.7
ϕCJ051	company H	31.3
ϕCJ052	company H	32.5
ϕCJ053	company H	24.2
ϕCJ054	company H	23.8
ϕCJ055	company H	25.0

Final panel of 25 *C. jejuni* bacteriophages selected based on their activity against the *C. jejuni* isolates, and to represent a cross-section of isolation sources. The percentage of isolates that showed significant sensitivity to each bacteriophage suspension is shown.

Electron microscopy

Five bacteriophages were chosen to be examined via transmission electron microscopy, as representatives of 5 different isolation sources. All bacteriophages examined were seen to be tailed bacteriophages, belonging to the order *Caudovirales*, and determined to likely belong to the *Myoviridae* family, having contractile tails with a sheath and a central tube. Bacteriophages ϕCJ001, ϕCJ016, ϕCJ039 and ϕCJ053 were observed to possess an isosahedral head/capsid, a short neck, tail,

and a terminal absorption structure consisting of a base plate and short tail fibres. The same structures were present for bacteriophage ϕCJ032, with the exception of tail fibres, which were replaced by a terminal bleb. These are similar to the few reported images available of other *Campylobacter* bacteriophages.

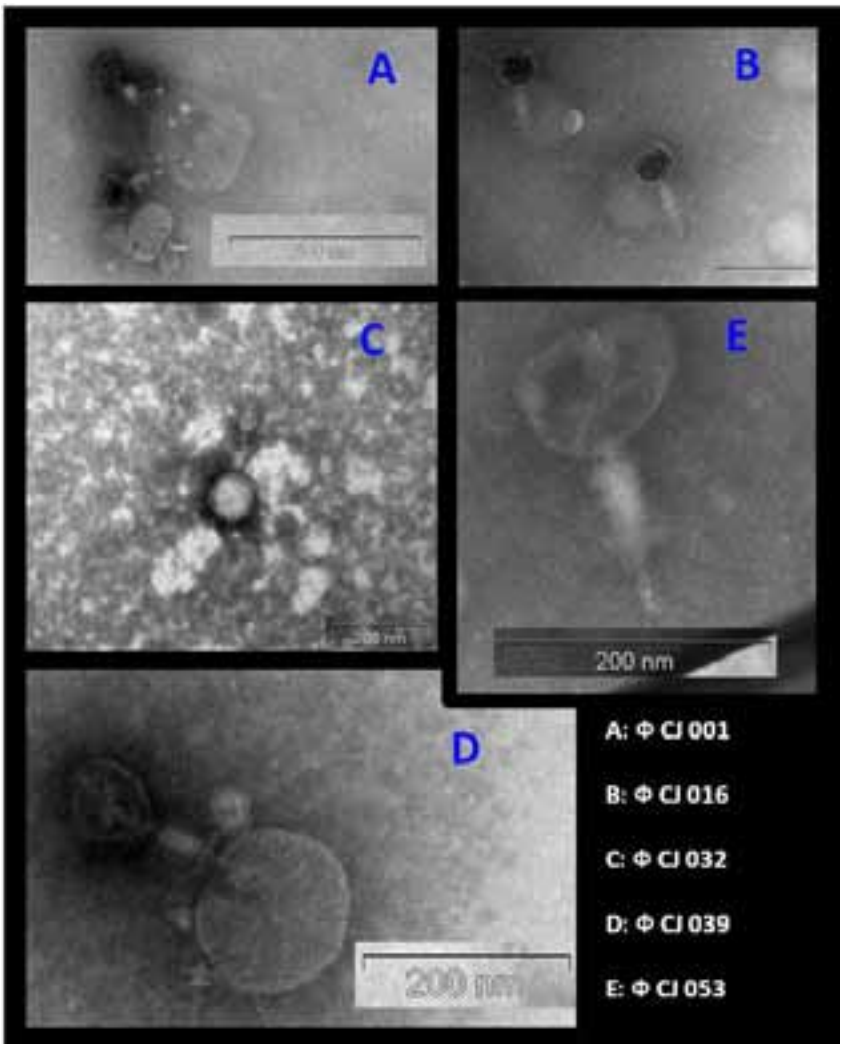

Figure 5 Bacteriophages as examined by transmission electron microscopy (TEM)

Five bacteriophages, from 5 different isolation sources, were stained with 1% phosphotungstic acid and examined via TEM at up to 92,000x magnification. They were determined to belong to the *Myoviridae* family.

Genomic DNA

Genomic DNA was successfully extracted from a representative group of the bacteriophage panel. Restriction enzyme digestion has indicated that the genome size is large–greater than 100kbp–and AT-rich. Work is still being conducted to more specifically determine genome size, as a restriction enzyme has not yet been identified which is able to cut the genome into the desired number of fragments for genome size approximation. Different restriction profiles have been observed, indicating the bacteriophages are distinctly different and not duplicates of the same bacteriophage.

Bacteriophage survival at different pH and temperature

The titre of bacteriophages under various pH and temperature conditions was observed over a period of 6 weeks, and specifically under acid conditions over a short (7.5 hour) period. The inconsistencies in plaque appearance dependant on host behaviour posed some concerns in interpreting the results over time, however trends were observed. The bacteriophages were seen to become non-viable at pH 2.5 and 3.0 very quickly. Less reduction was observed at pH 5.5 to pH 9.0, except at 42^{o}C, where no bacteriophages could be identified at any pH after 2-3 weeks. The bacteriophages appeared to be able to survive, at least at low titres, for a number of hours between pH 3.0 and pH 4.5, but large reductions in titre were demonstrated. The storage method used during this study (pH 7.5, 4^{o}C) was demonstrated as the most suitable choice, as the bacteriophage titre was the most consistent under these conditions.

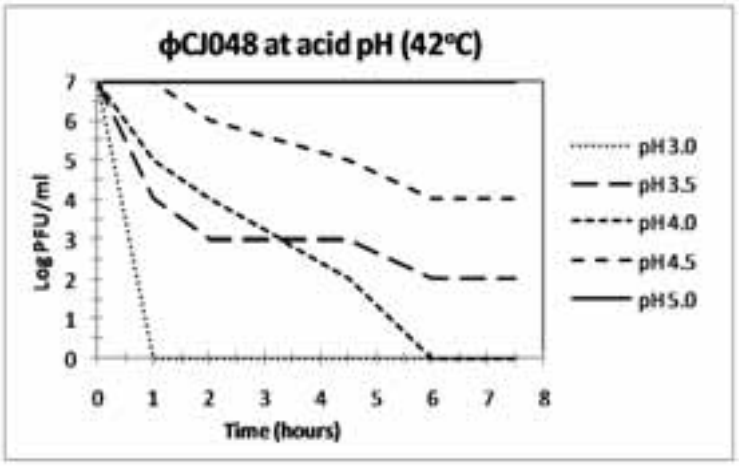

Figure 6 Bacteriophage (ϕCJ048) titre at various acid pH over 7.5 hours

Observed titres of bacteriophage ϕCJ048, stored at 42^{o}C, at different pH–3.0, 3.5, 4.0, 4.5 and 5.0–over a period of 7.5 weeks. Titres are reported as a log function of PFU/ml.

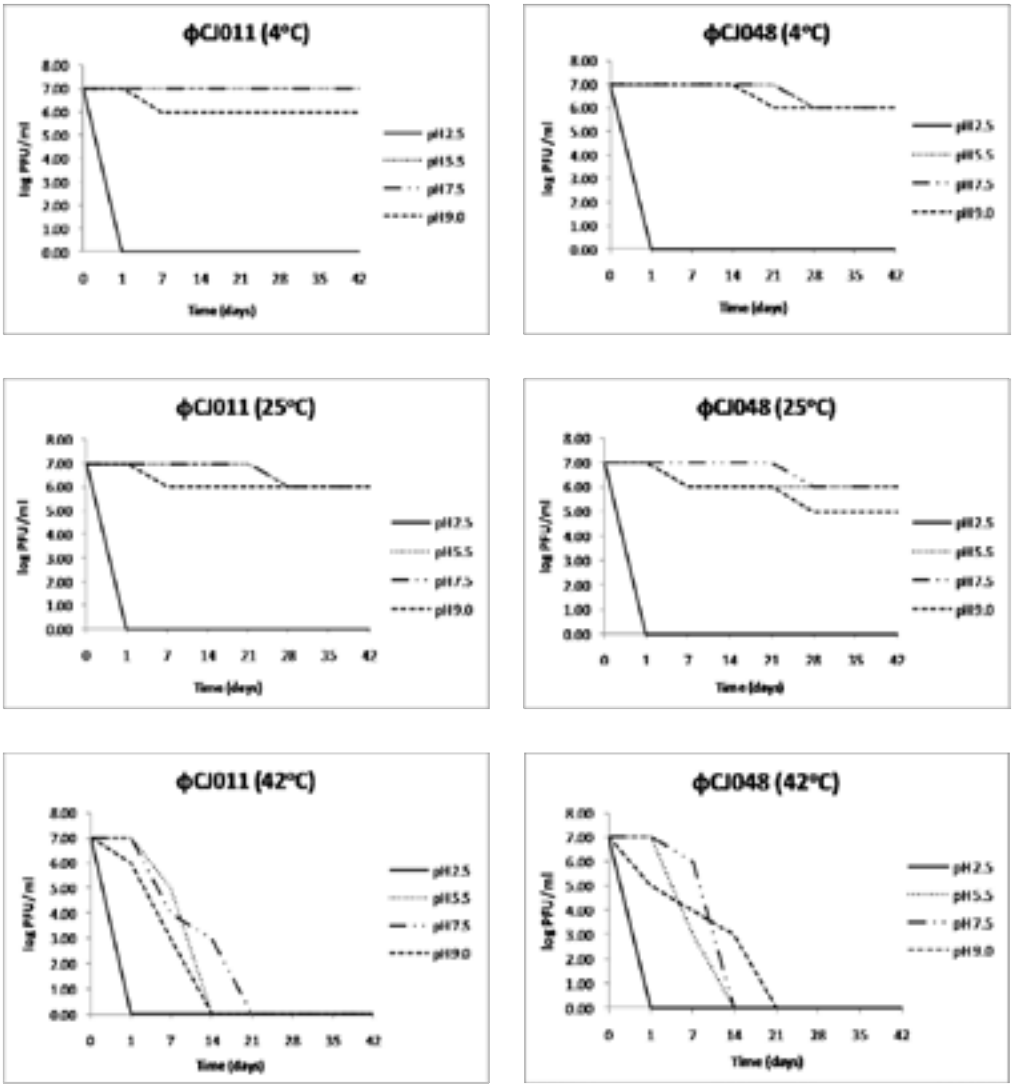

Figure 7 Bacteriophage titres at different pH and temperature over a 6 week period

Observed titres of bacteriophages ϕCJ011 and ϕCJ048 stored at 4°C, 25°C and 42°C, at different pH–2.5, 5.5, 7.5 and 9.5–for a period of 6 weeks. Titres are reported as a log function of PFU/ml.

Bacteriophage application to chicken meat

In experiment 1, using a single bacteriophage (ϕCJ051), when comparing all untreated samples to bacteriophage treated samples, there was a statistically significant decrease in *C. jejuni* growth from the samples in the treated group ($p < 0.05$). However, the reduction represented less than one $\log_{10}$ reduction. In experiment 2, no statistically significant decrease was seen in the bacteriophage treated groups. The only significant reduction in experiment 2 was seen with pre-freezing the samples ($p < 0.001$), but this was seen in all samples that were pre-frozen, not just the bacteriophage treated group, and was a far greater reduction than was seen with bacteriophage application alone, which is consistent with another report of *Campylobacter* bacteriophage application to chicken meat (Atterbury *et al.* 2003), where freezing reduced *Campylobacter* counts comparable to or greater than bacteriophage application alone.

Bacteriophage properties

Wild type *C. jejuni* isolation strains

Eighteen wild type *C. jejuni* strains were selected based on their sensitivity to bacteriophages that had already been isolated. Two NCTC strains were also used–the original isolating strain *C. jejuni* NCTC 12662 and *C. jejuni* NCTC 11168. Samples were processed in the same way as all previous samples, however instead of just using *C. jejuni* NCTC 12262 as an indicator strains, all 20 isolates were used. 137 samples were collected to test the theory that the lack of isolation of bacteriophages from broilers reared under traditional indoor commercial conditions was due to a resistance of the indicator strain to any bacteriophage present. All processed samples were tested against the 20 different isolates. No bacteriophages were isolated in this manner. This indicates that the lack of isolation of bacteriophages from chickens raised under indoor commercial conditions is not due to inherent resistance of the indicator strain (*C. jejuni* NCTC 12662) to any bacteriophages present.

The *C. jejuni* isolates chosen were:

- CJ0009
- CJ0010
- CJ0014
- CJ0018
- CJ0101
- CJ0106
- CJ0115
- CJ0120
- CJ0140
- CJ0184
- CJ0196
- CJ0199
- CJ0202
- CJ0206
- CJ0213
- CJ0223
- CJ0227
- CJ0231
- *C. jejuni* NCTC 12662
- *C. jejuni* NCTC 11168

Restriction fragment length polymorphism

One hundred and seventy four *C. jejuni* isolates were processed for their restriction fragment length polymorphism (RFLP) profile, via restriction enzyme digestion of the flagellin (*fla*) A gene. Isolates were analysed in the BioNumerics program, using a Dice comparison with limits set at 3.0 %. Those isolates with restriction patterns equal to or greater than 70 % similar were considered one *fla* group. Isolates with 100 % similar *fla* restriction patterns were considered sub-groups. Those isolates less than 70 % similar were considered to belong to separate *fla* groups. Isolates that were not 100 % similar, but exhibited greater than 70 % similarity were given arbitrary letters to distinguish them as closely related isolates (i.e. more than 70 % similar to other group members). Using this analysis method, six main *fla* groups were identified.

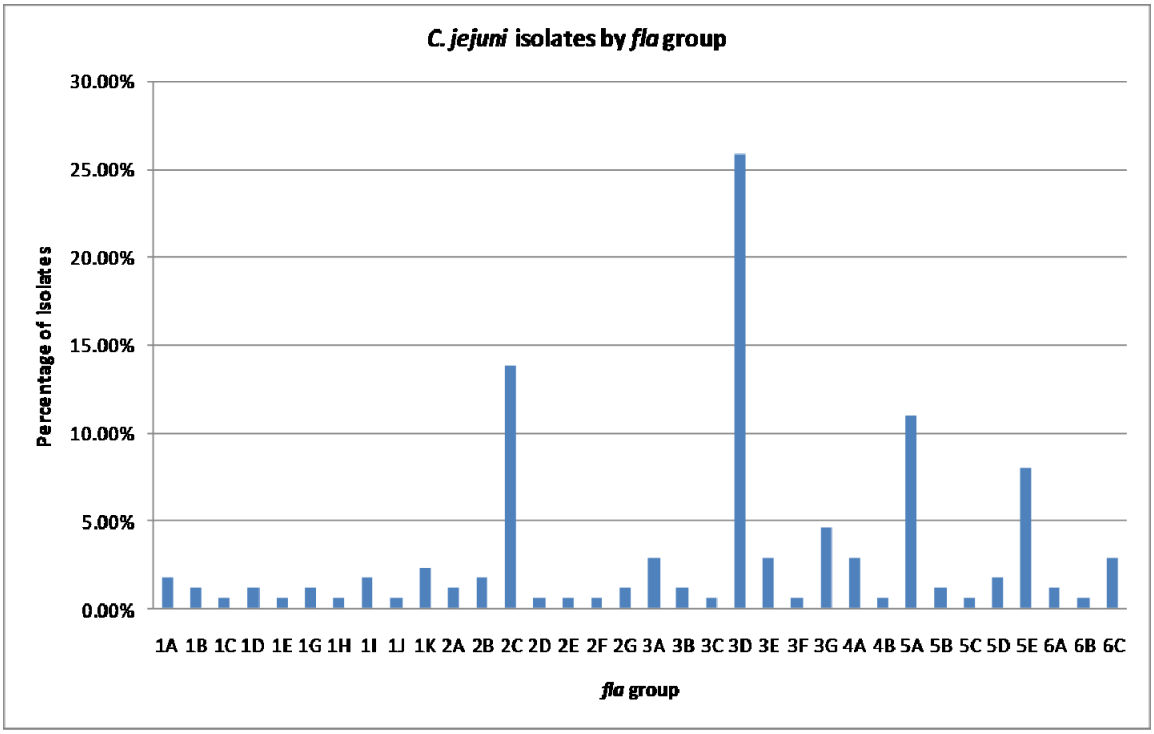

Figure 8 *fla* groups identified by RFLP of *C. jejuni* isolates

174 *C. jejuni* isolates were processed by restriction fragment length polymorphism. This graph shows the distribution of these isolates amongst the different *fla* groups identified.

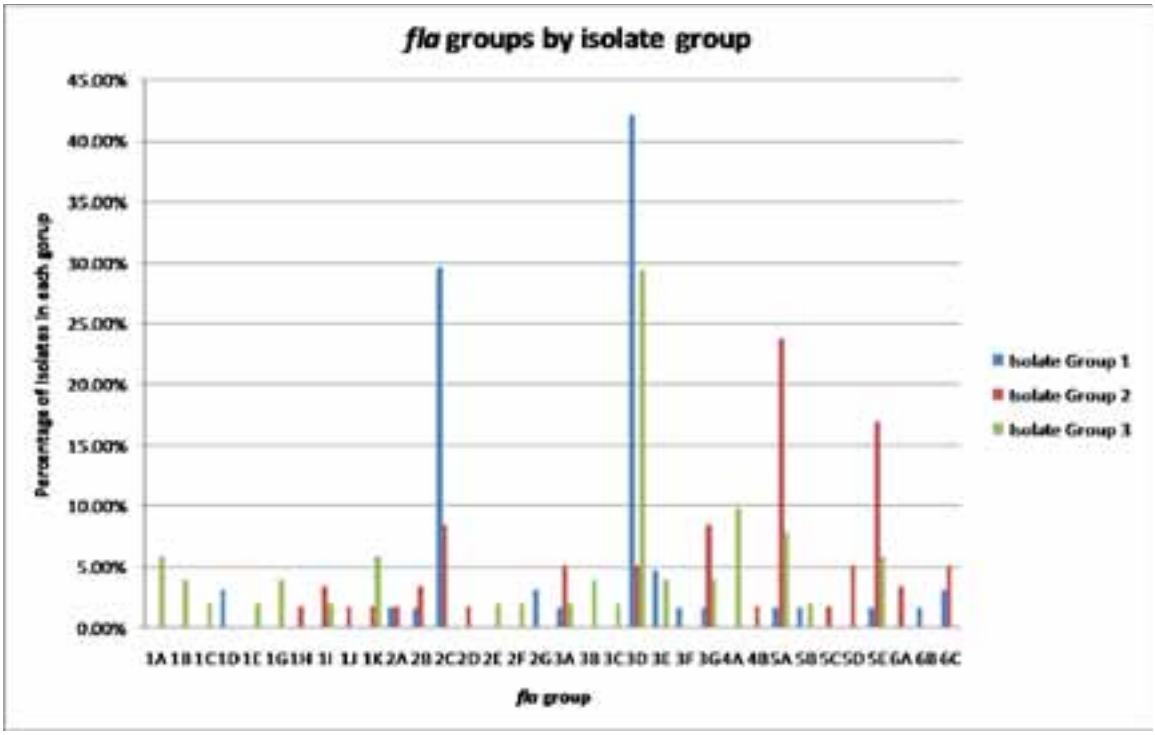

Figure 9 *fla* groups identified, shown by isolate group

C. jejuni isolates from the three separate groups of isolates obtained for this study and their distribution amongst the different *fla* groups identified. (*Isolate Group 1*–from a previous project at the University of South Australia, all from company 1; *Isolate Group 2*–from a previous project in Queensland, from companies A, B and C; *Isolate Group 3*–isolated during the course of this study, from a range of companies.)

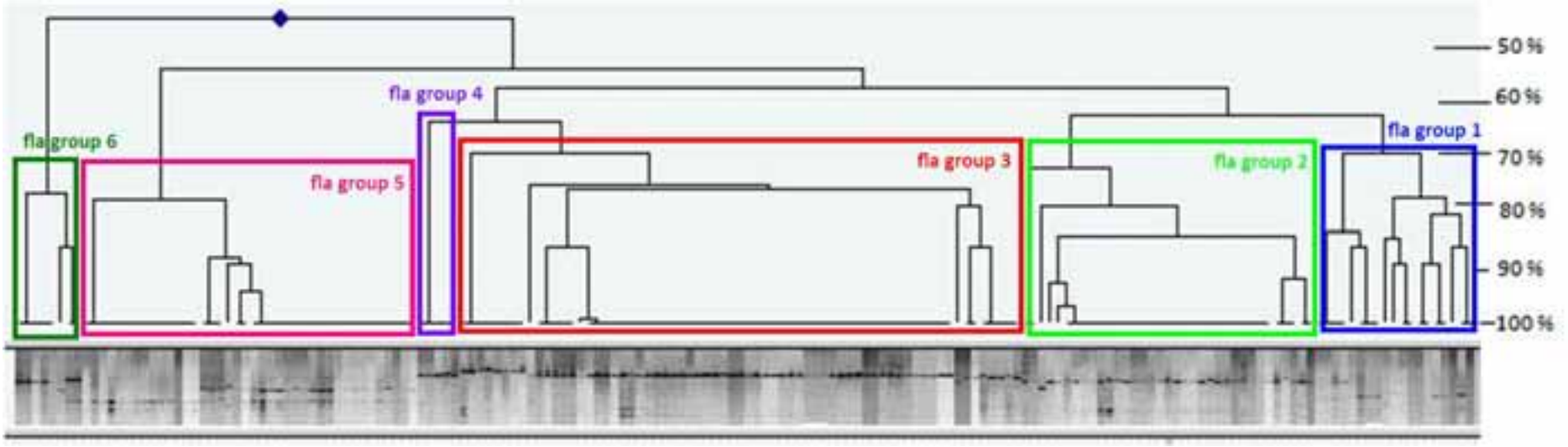

Figure 10 RFLP of *C. jejuni* isolates–BioNumerics output

The output from BioNumerics program comparing the *Dde*I RFLP profiles of the *flaA* gene of 174 *C. jejuni* isolates, plus the control strain *C. jejuni* ATCC 33560, and the relationship between the isolate profiles. The percentage similarity of the isolates is shown on the right, which was used to determine different *Fla* groups.

Table 5 *fla* groups identified via RFLP, shown by isolate group

fla group	Isolate group 1	Isolate group 2	Isolate group 3	TOTAL	*fla* sub-groups
1	2	5	13	20	A-K
2	23	9	2	34	A-G
3	33	11	23	67	A-G
4	0	1	5	6	A-B
5	3	28	8	39	A-E
6	3	5	0	8	A-C

174 *C. jejuni* isolates were processed for their *flaA* RFLP profile. This table shows the numbers of isolates in each *fla* group and the numbers of *C. jejuni* isolates from each isolate group belonging to the individual *Fla* group. The sub-groups identified are listed in the column on the right. (As in Figure 9: *Isolate Group 1*–from a previous project at the University of South Australia, all from company 1; *Isolate Group 2*–from a previous project in Queensland, from companies A, B and C; *Isolate Group 3*–isolated during the course of this study, from a range of companies.)

Four main clusters of isolates were identified–*fla* groups 2C, 3D, 5A and 5E. Of 174 isolates analysed, 102 produced *flaA* gene restriction patterns belonging to these four groups–45 isolates belonged to *fla* group 3D, 24 to *fla* group 2C, 19 to *fla* group 5A, and 14 to *fla* group 5E. The majority of group 1 isolates belonged to *fla* groups 2C and 3D (29.7 % – 2C; 42.2 % – 3D). Of 59 group 2 isolates, 23.7 % belonged to *fla* group 5A, and 16.9 % to *fla* group 5E. The majority of group 3 isolates (29.4 %) belonged to *fla* group 3D. Whilst sensitive and insensitive isolates were distributed across all *fla* groups, the clustering of isolates from the same source within individual *fla* sub-groups is suggestive of similarity between isolates from the same source. Multiples of the same or similar isolates from one source, which are insensitive to bacteriophages, may explain apparent lower bacteriophage sensitivity percentages in isolate group 1.

C. jejuni insertion elements

C. jejuni isolates from each isolate group were processed to try and identify prophage sequences that have previously only been reported in one paper and were screened for using Southern blots and not PCR (Clark & Ng 2008). Prophage can confer resistance in bacteria to other similar bacteriophages. It is reported that these prophage identified in *C. jejuni* strains cannot be induced to come out of the bacterial genome (Clark & Ng 2008). Work was conducted during this study to try and induce temperate bacteriophages (prophage) to come out of the genome of numerous *C. jejuni* isolates using mitomycin C. This work was unsuccessful, but it was thought that the genome may still contain prophages that cannot be induced.

One hundred isolates were processed by PCR using the primers reported for cje0215, cje0270 and cje1471–38 group 1; 25 group 2; 37 group 3–plus three control strains–*C. jejuni* NCTC 12262, *C. jejuni* NCTC 11168, and *C. jejuni* ATCC 33560. The only strain reported as being previously tested was *C. jejuni* NCTC 11168, which was reported as negative for all insertion elements/prophage (Clark & Ng 2008).

Table 6 Number of prophage sequences observed in the tested *C. jejuni* isolates

Prophage identified	0	1	2	3
Group 1	17	10	9	2
Group 2	13	7	5	0
Group 3	25	8	4	0

100 wild type *C. jejuni* isolates were screened by PCR for the presence of 3 different *Campylobacter jejuni* insertion elements or 'prophages'. This table shows the numbers of prophage elements identified in isolates from each group.

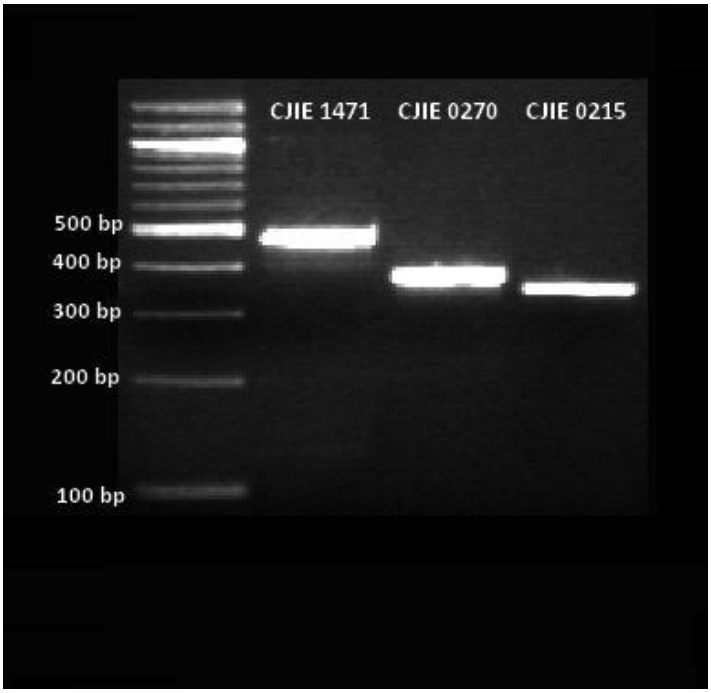

Figure 11 *Campylobacter jejuni* insertion elements (CJIE)

CJIE fragments amplified by PCR and run on a 1.5 % agarose gel by electrophoresis, stained with ethidium bromide, and observed under UV light. Expected product sizes–CJIE 1471=475bp, CJIE 0270=373bp, CJIE 0215=315bp. A 100bp DNA marker is shown on the left, with specific DNA fragment sizes for reference.

All the control strains tested were identified as negative for the 3 prophage insertion elements. Overall, 45 isolates were identified as having at least one prophage insertion. Only 2 isolates were identified as having all 3 prophage insertions. CJIE 0215 was present in 21 isolates, CJIE 0270 in 29 isolates and CJIE 1471 in 16 isolates. Only 2 isolates had only CJIE 0215–most isolates with CJIE 0215 were identified as also having CJIE 0270. Ten isolates had only CJIE 0270. Fourteen isolates had only CJIE 1471. The percentage of strains in which prophage insertions were identified was lower in the group of isolates actually isolated during the course of this study, compared to the other two groups. This was significant in comparison to group 1 ($p < 0.01$). There was no significant difference between the other groups. This is the only group that included a number of free range strains–this group contained only these free range strains and a number of isolates from company 1, which is the source of all isolates in group 1. Twenty three of the 37 isolates in this group were from various free range sources. Of these, 8 contained at least one prophage sequence. It is postulated that difference in the distribution of

prophage in isolates between free range and indoor commercial *C. jejuni* isolates may explain the difference in the ability to isolate bacteriophages from the two different sources. A larger sample group of free range isolates has been sourced from another concurrent project at the University of South Australia to be processed looking for these same three prophage insertion elements. Results are not available at this time. Whether the bacteriophages isolated during the course of this study are lysogenic and have the potential to become prophage is also still under investigation.

An additional observation from this work has been that some isolates from the same source appear to have similar or the same insertion elements. This may indicate that these prophage sequences could represent a suitable typing method, at least for *C. jejuni*.

Summary of Results

The project achieved the aim of isolating a range of bacteriophages capable of lysing C. *jejuni* both in laboratory cultures and on artificially contaminated chicken meat. It should be noted that in line with experiences reported by other groups that freezing plus bacteriophage was much more effective in reducing *Campylobacter* counts than use of bacteriophages alone. However, because it was not possible to isolate bacteriophages from commercial indoor chickens a full evaluation of the usefulness of bacteriophage in reducing *Campylobacter* contamination on chicken meat was not possible. Much effort has been put into trying to determine why bacteriophages can readily be isolated from free-range chicken but not commercial indoor chickens. So far the solution eludes us. Lack of consistency in cultural behaviour and colonial morphology of *Campylobacter* makes it difficult to be sure if some bacteriophages are lytic or temperate. If temperate bacteriophages are used there is some risk (which is yet to be evaluated in *Campylobacter*) of transfer of antibiotic or virulence genes from one *Campylobacter* to another. The difficulty in assuring the lytic status of bacteriophages and the inability to isolate bacteriophages from indoor chickens led to deferment of the proposal to investigate presence of tail-like bacteriocins in *C jejuni* bacteriophages. The bacteriophage isolates obtained were stable at 4°C and 25°C but not at 42°C over a longer period, and they were adversely affected by acid pH.

Overall the project provides preliminary data that suggests bacteriophage could play a role as an adjunctive control but much more work needs to be done on the ecology of *C. jejuni* bacteriophages as this is clearly much more complex than the ecology of bacteriophages associated with other enteric bacteria.

Implications

- Bacteriophages active against *Campylobacter* appear to have a far more complex ecology than bacteriophages of other species. This suggests that there use as a control mechanism would be limited until this relationship can be better understood.

- Differences in the ability to isolate bacteriophages from chickens raised in traditional indoor commercial and free range conditions, observed differences in the *flaA* RFLP profiles of *C. jejuni* strains from these sources, and differences in sensitivity to bacteriophages suggests that that rearing conditions have a significant influence on the molecular epidemiology and bacteriophage ecology. Further studies should be conducted to more accurately determine the molecular epidemiology and characteristics of *Campylobacter* in chickens from all different rearing conditions.

- Prophage sequences appear to be widespread in *C. jejuni* strains. There appears to be consistency in the prophage sequences identified in strains from the same source. A larger study could be conducted to determine whether prophage identification can be used as a *Campylobacter* typing technique.

Recommendations

At this stage recommendations arising from this work can only encourage further research to determine why the interactions between *C. jejuni* and its bacteriophages is so different from the interactions seen with other bacteria and their bacteriophages.

- More work is needed to investigate why we, and other research groups, find it so difficult to isolate *C. jejuni* bacteriophages from indoor meat chickens. The ecology of *C. jejuni* bacteriophages appears to be much more complex than the ecology of bacteriophages associated with other enteric bacteria.

- The issue of whether the bacteriophages isolated are truly lytic bacteriophages or whether they are temperate/lysogenic needs to be resolved, as does the complex bacteriophage-host relationship.

- Although outside the remit of this project, it could be worthwhile investigating if prophages could be used to develop a typing system for *C. jejuni*.

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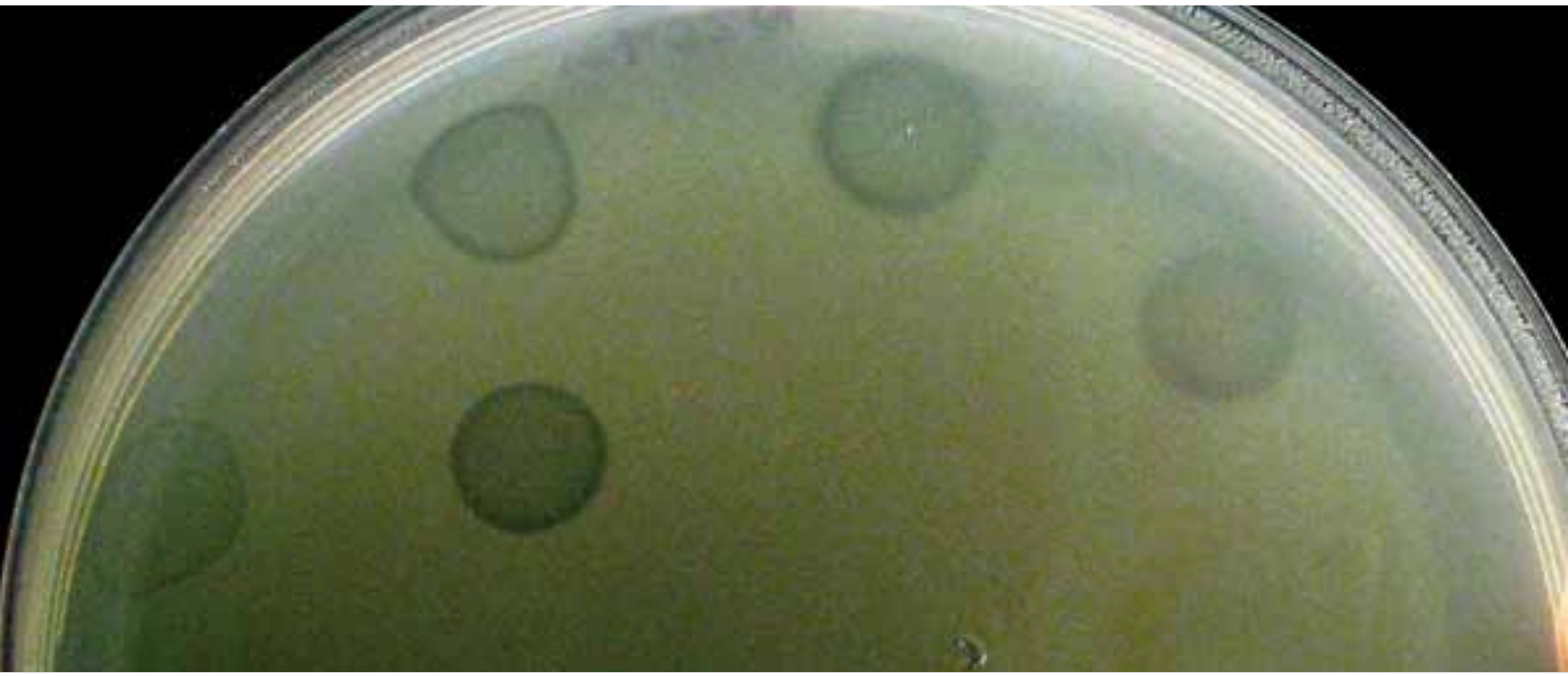
The Use of Bacteriophages to Control *Campylobacter jejuni* from Chickens

by M.D Barton, M.W. Heuzenroeder and J. Owens

Publication No. 11/005

Worldwide, over 90 percent of reported bacterial food-borne illness in humans can be attributed to either *Campylobacter* or *Salmonella* species. Of these two organisms, *Campylobacter* is the most common bacterial cause of diarrhoeal disease in humans.

One proposed adjunctive control measure for *Campylobacter* is the use of bacteriophages. Bacteriophages are viruses that are able to infect and kill bacterial cells and they will only multiply when susceptible bacteria are present. The emergence of antibiotic resistant bacteria has, in recent times, renewed Western research interest in the possibilities of using bacteriophages to control bacteria, such as Campylobacter. This study investigated *Campylobacter jejuni* and its associated wild type bacteriophages.



Cover photo: *Campylobacter jejuni* bacteriophages on a plaque assay plate

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