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ANTIBIOTIC RESISTANCE DETERMINANTS OF AUSTRALIAN *CAMPYLOBACTER JEJUNI* & *CAMPYLOBACTER COLI* ISOLATES

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ABSTRACT

Campylobacter species are the most common cause of foodborne disease in Australia and many countries throughout the World. Although campylobacteriosis is usually self-limiting, severe cases and those in the young, elderly and immunocompromised require antibiotic therapy. Antibiotic resistant *Campylobacter* isolates however may prolong illness and increase the risk of invasive disease. Antibiotic resistance in *Campylobacter* is thought to have arisen through the selective pressure of exposure to antimicrobial agents in veterinary medicine or animal husbandry, leading to the acquisition and dissemination of antibiotic resistance determinants, and genetic elements that harbour such genes, amongst isolates.

Little was known about tetracycline and trimethoprim resistance in Australian campylobacters, including the presence of resistance genes and associated genetic elements. Aims of this study were therefore to identify in Australian *Campylobacter jejuni* and *Campylobacter coli* isolates i). Tetracycline resistant determinants and associated genetic elements, ii). Trimethoprim resistant determinants and associated genetic elements, and iii). Integron like structures and associated genetic elements.

High-level tetracycline resistance was observed in 46 *C. jejuni* and *C. coli* isolates, with MICs ranging from 32 to >256μg/ml. All isolates examined harboured the *tetO* gene, confirming that tetracycline resistance in Australian campylobacters is also due to the previously reported TetO determinant. While several studies have described a significant role for plasmids in tetracycline resistance, this study demonstrated that in the majority of isolates (78%), including two thirds of strains that harboured plasmids, resistance was due

to chromosomally encoded *tetO*. Six *C. jejuni* isolates were able to transfer a *tetO* harbouring plasmid to another *C. jejuni* strain. Plasmids were detected in approximately 74% of resistant strains, and ranged in size from small to larger plasmids (21 - 50kb). *Cla*I profiling of plasmids revealed genetic diversity and indicated that the *tetO* gene may be carried by a variety of plasmids.

High level trimethoprim resistance (MICs of 1000μg/ml and >1000μg/ml) was observed in all isolates (>100) examined from a second collection of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* spp. Just over half of isolates harboured plasmids indicating that plasmids may not be involved in trimethoprim resistance in campylobacters. Isolates were also examined for the presence of the previously identified *Campylobacter* associated trimethoprim resistance genes *dfr1* and *dfr9*. Although these genes play a significant role in this resistance, only approximately 16% of strains examined putatively harboured *dfr1*, and *dfr9* was not detected.

Integrons, antibiotic resistance gene acquisition and expression systems, play an important role in trimethoprim resistance due to carriage of *dfr* genes as inserted gene cassettes. Trimethoprim resistant *Campylobacter* isolates were examined for the presence of the *intI1* and *intI2* genes, encoding the class 1 and class 2 integrons. Only 5.56% of strains examined for *intI1* putatively carried this gene, and only 1.67% of isolates examined for *intI2* putatively carried *intI2*. Both putative *intI1* positive and negative isolates produced a variety of amplicons, ranging in size from ≈210bp to >1.5kb, when analysed for gene cassette sequences inserted into class 1 integrons.

This study has contributed to the knowledge of tetracycline and trimethoprim resistance, including the presence of resistance genes and associated genetic elements, in Australian isolates of *C. jejuni* and *C. coli*.

STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signed,

Alisa Pratt

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LIST OF ABBREVIATIONS & SYMBOLS

λ . Lambda bacteriophage

≈. Approximately

μg. Micrograms

μl. Microlitres

μm. Micrometers

μM. Micromolar

^{0}C. Degrees Celsius

aa. Amino acid

ATCC. American Type Culture Collection

B. subtilus. *Bacillus subtilus*

bp. Base pair/s

bv. biovar

C. coli. *Campylobacter coli*

C. fetus. *Campylobacter fetus* subsp. *fetus*

C. hyointestinalis. *Campylobacter hyotintestinalis*

C. jejuni. *Campylobacter jejuni* subsp. *jejuni*

C. lari. *Campylobacter lari*

C. sputorum. *Campylobacter sputorum* bv. *Sputorum*

C. upsaliensis. *Campylobacter upsaliensis*

CFU. Colony Forming Units

CJIE. *Campylobacter jejuni* Integrated Element

CO_2. Carbon dioxide gas

DHFR. Dihydrofolate reductase

DMSO. Dimethyl-Sulfoxide

DNA. Deoxyribonucleic Acid

dNTP. Deoxynucleic acid triphosphates

E. coli. *Escherichia coli*

EDTA. Ethylene diamine tetra-acetic acid

F. Fahrenheit

g. Grams

G+C. Guanine and cytosine nucleic acid percentage of DNA sequence

GBS. Guillain-Barré Syndrome

-GS. Genome sequenced variant of strain *C. jejuni* NCTC 11168

GTP. Guanidine Tri-phosphate

H. pylori. *Helicobacter pylori*

HBA. Horse Blood Agar

HCl. Hydrochloric acid

IS. Insertion Sequence

kb. Kilobase/s

kDa. KiloDaltons

L. Litre

LBA. Luria-Bertani Agar

Ltd. Limited

M. elsdenii. *Megasphaera elsdenii*.

M. Molar

Mb. Megabase/s

mg. Milligrams

$MgCl_2$. Magnesium chloride

MHA. Mueller-Hinton Agar

MIC. Minimum Inhibitory Concentration

ml. Millilitres

mm. Millimetres

mM. Millimolar

N/A. Not Applicable

N_2. Nitrogen gas

NaCl. Sodium chloride

NaOH. Sodium hydroxide

NCCLS. National Committee for Clinical Laboratory Standards

NCTC. National Collection of Type Cultures

ng. Nanograms

nm. Nanometres

nov. novel

O_2. Oxygen gas

ORF. Open Reading Frame

p. Plasmid

PBS. Phosphate Buffered Saline

PCR. Polymerase Chain reaction

pmoles. Picomoles

Pty. Proprietary

Qld. Queensland

R. Type. Resistance type

RE. Restriction Endonuclease

R-factor. Resistance factor

RFLP. Restriction Fragment Length Polymorphism

RNA. Ribonucleic Acid

rRNA. Ribosomal RNA

SDS. Sodium Doecyl Sulphate

sp. species

spp. species plural

SSC. Standard Sodium Citrate

subsp. subspecies

TAE. Tris Acetate EDTA

TBE. Tris Borate EDTA

Tn. Transposon

tRNA. Transfer RNA

TSA. Tryptone-Soya Agar

U. Units

UK. United Kingdom

US. United States of America

UV. Ultra Violet

V. cholerae. *Vibrio cholerae*

W. succinogenes. *Wolinella succinogenes*

w/v. Weight per volume

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Foodborne disease is an important issue among consumers, government agencies and industry alike. Foodborne disease is a public health concern in Australia and throughout the World where it causes significant morbidity and mortality (Kirk 2004 & Hall *et al*, 2005). In 2004, approximately 24,000 cases of eight potentially foodborne diseases were reported in Australia. It is estimated that foodborne disease may cost Australia as much as $1.2 billion annually (The OzFoodNet Working Group, 2005). Of great concern is the potential impact of industrial, societal and environmental changes on foodborne disease. Hall *et al*, 2002 proposed that factors such as climate change and the way we produce, distribute and consume food have the potential to affect foodborne disease in the coming century.

In 2004, campylobacteriosis caused by *Campylobacter* spp., was the most commonly reported sporadic foodborne disease in Australia with 15,640 cases (excluding figures for New South Wales) (The OzFoodNet Working Group, 2005). The genus *Campylobacter* comprises a group of closely related gram-negative bacteria which primarily colonise the gastrointestinal tract of a wide variety of host species (Wassenaar and Newell, 2000). *Campylobacter jejuni* and related organisms are important human pathogens, causing acute human entercolitis and they are the most common cause of food-borne diarrhoea in many industrialised countries (van Vliet and Ketley, 2001).

Campylobacteriosis is a zoonosis, an infectious disease transmissible under normal conditions from vertebrate animals to man (Aarestrup & Wegener, 1999). Foodborne infections caused by bacteria enzootic to food animals such as *Campylobacter*, *Salmonella* and entero-haemorrhagic *E. coli* are the most frequent zoonoses in industrial countries. The

main reservoir of zoonotic agents is the gastrointestinal tract of healthy food animals, and most foodborne infections originate from faecal contamination during slaughter or cross-contamination during subsequent processing (Aarestrup & Wegener, 1999).

In the context of foodborne disease, the transfer of antibiotic resistant bacterial pathogens to humans is of great concern. The development of resistance in zoonotic bacteria constitutes a public health risk, primarily through the increased risk of treatment failures (Aarestrup & Wegener, 1999 & Holmberg *et al*, 1984). In addition, development of resistance, notably by acquisition of transmissible genetic elements, may affect other aspects such as the ability of a pathogen to colonise an animal host or persist in the farm or food processing environment (Aarestrup & Wegener, 1999).

1.1 CAMPYLOBACTER TAXONOMY

The bacteria now recognised as members of the genus *Campylobacter* were first described at the beginning of the 20th century (Ketley, 1995). *Campylobacter* species belong to a phylogenetically distinct group referred to as either ribosomal RNA (rRNA) superfamily VI or the epsilon division of the class *Proteobacteria*. This group contains the genera *Campylobacter*, *Helicobacter*, *Arcobacter*, *Wolinella* and a number of other genera and species (On, 2001). The family Campylobacteraceae consists of *Campylobacter*, *Arcobacter* and *Bacteroides ureolyticus* (Snelling *et al*, 2005 & Vandamme, 2000). According to On, 2001 the genus *Campylobacter* contains 16 species and six subspecies, however, in 2005 Snelling *et al*, listed 14 species of *Campylobacter*.

C. jejuni subspecies *jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* are the most commonly isolated from human and animal diarrhoea. *Campylobacters* colonise moist body surfaces, including the human gingival cavity (e.g. *C. concisus*), the small intestinal mucosa, and the vaginal musoca of bovines (On, 2001 & Trust *et al*, 1994). In 2004, Foster *et al*, proposed a new species, *Campylobacter insulaenigrae* sp. nov., isolated from common seals and a carcass of a harbour porpoise. Phylogenetically, the novel species shows close affinity with *C. jejuni*, *C. lari* and *C. coli*. (Foster *et al*, 2004).

Although *C. jejuni* and *C. coli* derived their names from an initial association with enteric disease in animals, they are the most important human pathogens in the genus (Cover & Blaser, 1989 & Doyle, 1948 reference in Ketley, 1995). Within the species *C. jejuni* there are two subspecies, ssp. *jejuni* and ssp. *doylei*. The pathogenic role of *C. jejuni* ssp. *doylei* is unknown (Snelling *et al*, 2005 & Vandamme, 2000) and this subspecies is not commonly isolated from human and animal diarrhoea (On, 2001). All references to *C. jejuni* in the text from now on are to *C. jejuni* ssp. *jejuni*. *C. coli* is distinguished from *C. jejuni* by phenotypic tests, including the hippurate hydrolysis test (for which *C. coli* is negative) and genotypic tests (Steinbrueckner *et al*, 1999).

Other *Campylobacter* species of interest include *C. lari*, *C. fetus*, *C. hyointestinalis* and *C. sputorum*. *C. lari* is genetically close to *C. jejuni* and *C. coli* and is most commonly isolated from human and animal diarrhoea (Fouts *et al*, 2005 & On, 2001). This species is also prevalent in birds (seagulls in particular) but has also been isolated from dogs and swine (Fouts *et al*, 2005 & Hald *et al*, 2004). *C. fetus*, an opportunistic pathogen, is rarely found as a cause of enteritis in humans but is quite often isolated in systemic infections (Moore *et*

al, 2005 & Tu *et al*, 2004). *C. fetus* has been isolated from swine, humans, birds and reptiles, and is also found in bovine reproductive disorders (Tu *et al*, 2004 & On, 2001).

C. hyointestinalis can cause gastroenteritis in humans and infect animals (including bovine and reindeer sources), and there is evidence of transmission from swine to humans (Moore *et al*, 2005, Inglis *et al*, 2005, Laatu *et al*, 2005 & Gorkiewicz *et al*, 2002). *C. sputorum*, a hydrogen requiring *Campylobacter* species is found in the oral cavity and in enteric and reproductive tracts of various production animals (On, 2001). This species is also considered to be a commensal of the animals in which it can be found, and, has been associated with several diseases in humans (Duim *et al*, 2001).

1.2 BIOLOGY, PHYSIOLOGY & ECOLOGY OF *CAMPYLOBACTER*

Campylobacters are gram-negative, non-spore forming, curved, S-shaped, or spiral rods that are 0.2 to 0.9μm wide and 0.5 to 5μm long. Cells may form spherical or coccoid bodies under certain environmental conditions, such as in stationary phase, on exposure to atmospheric oxygen (Vandamme & De Lev, 1991 & Ketley, 1995) or temperature down shifts (Takkinen & Ammon, 2003). Organisms are motile by means of a single polar unsheathed flagellum at one or both ends (Vandamme & De Lev, 1991 & Ketley, 1995). The polar flagellum and the spiral shape endow the cells with a characteristic rapid darting corkscrew-like motility, enabling campylobacters to remain motile in mucous, a highly viscous environment which rapidly paralyses other motile rod-shaped bacteria (Park, 2002 & Shigematsu *et al*, 1998).

Campylobacter are microaerophilic and require reduced oxygen for best growth (as low as 3%) and a carbon dioxide concentration of 3-10%. The optimal growth atmosphere consists of 5% O_2, 10% CO_2 and 85% N_2 (Andrews, 1998), however, some species require hydrogen for growth (Blaser & Wang, 1987). Campylobacters can be divided into two groups, thermophilic and non-thermophilic. The temperature range for growth of the thermophilic *Campylobacter* species such as human pathogens *C. jejuni* and *C. coli* is 34-44^{o}C, with an optimal temperature of 42^{o}C (mirroring that of the avian gut), which probably reflects an adaptation to the intestines of warm-blooded birds (Park, 2002 & Blaser & Wang, 1987). Non-thermophilic species grow optimally at 25^{o}C and include species such as *C. fetus* and *C. hyointestinalis*.

Campylobacter species are fastidious organisms that require complex growth media and are unable to ferment or oxidise carbohydrates, instead obtaining energy from amino acids, or tricarboxylic acid cycle intermediates (Snelling *et al*, 200, van Vliet & Ketley, 2001 & Vandamme, 2000). *Campylobacter* spp. failure to ferment carbohydrates or degrade complex substances and requirement for supplemented media for growth is consistent with a small genome size (Taylor, 1992).

C. jejuni isolates are catalase, oxidase and hippurate hydrolysis positive (Snelling *et al*, 2005, Ottosson & Stenstrom & 2003 & Vandamme, 2000). The hippurate hydrolysis test differentiates most *C. jejuni* strains from other *Campylobacter* species (Butzler, 2004). The hippuricase gene is only found in *C. jejuni* (Rautelin *et al*, 1999) although some *C. jejuni* isolates are hippuricase-negative (Snelling *et al*, 2005). *C. coli* is also catalase and oxidase positive, but exhibits a number of specific growth characteristics that differentiates it from

C. jejuni (Snelling *et al*, 2005). As previously stated, *C. coli* is hippurate hydolysis negative.

Campylobacter organisms are sensitive to freezing, drying; acidic conditions (pH ≤5.0) and salinity (Altekruse *et al*, 1999). Campylobacters are more sensitive to osmotic stress than other bacterial foodborne pathogens and will not grow in concentrations of sodium chloride of 2% (Park, 2002 & Doyle & Roman, 1982). Park, 2002 proposed that campylobacters do not obey many of the physiological paradigms established for model foodborne bacterial pathogens such as *E. coli*, due to their uniquely fastidious growth requirements and the lack of many adaptive responses correlated with resistance to stress in other such pathogens. Unlike most other bacterial foodborne pathogens camplyobacters are not normally capable of multiplication in food during either processing or storage. Atmospheric and temperature requirements probably impair the ability to multiply outside an animal host (Park, 2002).

Campylobacters are highly variable organisms adapted to a wide range of ecological niches. The distribution of these organisms in the environment varies by species, and differing prevalences of *C. jejuni* and *C. coli* have been found in food, water and different animal species (Tam *et al*, 2003, Kramer *et al*, 2000, Chuma *et al*, 1997 & Cabrita *et al*, 1992b). Although birds (both wild and domestic) are the natural host for *Campylobacter*, campylobacters can be found in a wide variety of wild, farm and domestic animals such as cattle and rodents, and may even be carried on the exoskeleton of insects (Frost, 2001, Altekruse *et al*, 1999, , Jacobs-Reitsma *et al*, 1995 & Cabrita *et al*, 1992).

1.3 *CAMPYLOBACTER* GENETICS

In 2000, Parkhill *et al* published the complete genome sequence of an extensively studied human isolate of *C. jejuni*, NCTC 11168. This *C. jejuni* strain has a circular chromosome of 1.64 Mb with a G+C content of 30.6%. The genome is predicted to encode 1,654 proteins and 54 stable RNA species and 94.3% of the genome codes for proteins. Parkhill *et al* reported that strong similarities between *C. jejuni* and closely related *Helicobacter pylori* are mainly confined to housekeeping functions with only 55.4% of *C. jejuni* genes with orthologues in *H. pylori*. 28% of genes show closest similarity to *Escherichia coli* and 27% to *Bacillus subtilis*.

Parkhill *et al* reported the presence of hypervariable sequences and the lack of classical operon structure, except for genes involved in lipooligosaccharide biosynthesis, EP biosyntheis and flagellar modification. The genome of *C. jejuni* NCTC 11168 is unusual in that there was no evidence of any functional inserted sequence elements, transposons, retrons or prophages and very few repeat sequences, when compared to other genomes. Minimal similarity of one gene to part of the insertion sequence, IS605 *tnpB* from *H. pylori* was reported (Parkhill *et al*, 2000).

In addition to the sequencing of *C. jejuni* NCTC 11168, Fouts *et al*, in 2005 published the complete sequence of another *C. jejuni* strain, RM1221 and incomplete (though sequenced to at least 8 fold coverage) sequences of a strain each of *C. coli*, *C. lari* and *C. upsaliensis*. The RM1221 genome is a circular chromosome, 1.77Mb in length with a G+C content of 30.31%. There are a total of 1884 predicted coding regions and 94% of the genome represents coding sequence (Fouts *et al*, 2005). The findings of Fouts *et al* included major

structural differences between the four isolates, and these differences were associated with the insertion of phage and plasmid like genomic islands; and major variations in the lipooligosaccharide complex.

The four integrated elements in the RM1221 chromosome included a *Campylobacter* Mu-like phage; two integrated elements encoding phage related enzymes and possibly representing novel or nonfunctional prophages or genomic islands; and a genomic island or integrated plasmid. (Fouts *et al*, 2005). The sequence of the *C. coli* strain contained five copies of an insertion sequence, IS*Cco1*, located on both the chromosome and megaplasmid, hinting at recent acquisition and transposition competence (Fouts *et al*, 2005). The megaplasmid contained antibiotic resistance genes flanked by putative mobile genetic elements. Megaplasmids harboured by sequenced *C. lari* and *C. upsaliensis* strains, possessed a Type IV secretion system, probably involved in conjugation (Fouts *et al*, 2005).

A gene encoding a protein with identity to TraG, a protein involved in the formation of conjugative DNA transfer systems and an essential component of a Type IV secretion apparatus in several pathogens, including *H. pylori* was identified in two strains of *C. jejuni* (Poly *et al*, 2005, Poly *et al*, 2004, Lawley *et al*, 2003 & Frost *et al*, 1994). Poly *et al* speculated that the identification of a TraG like protein and other proteins involved in Type IV secretion suggests that *C. jejuni* might be able to produce a Type IV secretion system.

Horizontal gene transfer has been implicated in the acquisition of a number of genes and/or genetic elements harboured by campylobacters (O'Halloran *et al*, 2004 & Poly *et al*, 2004).

Mobile genetic elements or homologues to such elements, including conjugative and non-conjugative plasmids, bacteriophages, insertion sequences, transposons and integrons have been reported in *Campylobacter* (Nirdnoy *et al*, 2005, Schmidt-Ott *et al*, 2005, Fouts *et al*, 2005, Gibreel *et al*, 2004a, Gibreel *et al*, 2004b, Poly *et al*, 2004, Lee *et al* 2002, Bacon *et al*, 2002, Gibreel & Skold, 1998, Taylor *et al*, 1988 & Taylor *et al*, 1983). Such mobile genetic elements involved in horizontal gene transfer have also been implicated in the spread of antimicrobial resistance (Henriques Normark & Normack, 2002).

Intaspecies and interspecies horizontal genetic exchange for *C. jejuni* is common (Dingle *et al*, 2001 & Dorrell *et al*, 2001). de Boer *et al*, 2002 presented evidence that bidirectional inter-strain genetic exchange, such as horizontal transfer, as well as intragenomic alterations, such as rearrangements, point mutations, deletions, duplications and inversions, occurs between homologous and heterologous strains *in vivo* during *C. jejuni* infection of chickens and in an *in vitro* setting. Meinersmann *et al*, 2003 also reported evidence of regular interspecies, (e.g. between *C. coli* and *C. jejun*), recombination and horizontal genetic exchange. Gene transfer between *Campylobacter* and other bacterial species and genera has been implicated by a number of studies through G + C content and mechanisms involved in such transfer (Poly *et al*, 2004).

Natural transformation is one potential mechanism for horizontal gene transfer leading to genetic diversity among a population (Wiesner *et al*, 2003). *C. jejuni* is naturally competent for DNA uptake and transformation (Larsen *et al*, 2004, Pearson *et al*, 2003, Wiesner *et al*, 2003 & Wang & Taylor, 1990). Studies of bacteria, including *Campylobacter*, have implicated natural transformation as a means for spreading genetic determinants that

increase antibiotic resistance and virulence (Wilson *et al*, 2003 & Nuijten *et al*, 2000). Wilson *et al* reported that ciprofloxacin resistance in *C. jejuni* can be acquired through natural transformation.

Plasmids in *Campylobacter* species have been under study since the 1980's. A number of studies, analysing *Campylobacter* isolates from different geographical locations have reported plasmid carriage and also antibiotic resistance associated with the presence of plasmids. Several other studies have reported the involvement of the plasmid, pVir, in the virulence of some strains of *C. jejuni*, (Tracz *et al*, 2005, Bacon *et al*, 2002 & Bacon *et al*, 2000).

Plasmids have been detected in strains from Canada (Taylor *et al*, 1981 & Gibreel *et al*, 2004a), the USA (Boosinger *et al*, 1990 & Tenover *et al*, 1985), Brazil (Aquino *et al*, 2002), Poland (Lekowska-Kochaniak *et al*, 1996), the UK (Batchelor *et al*, 2004), Portugal (Cabrita *et al*, 1992b), Spain (Velazquez *et al*, 1995), Israel (Schwartz *et al*, 1993), Taiwan (Lee *et al*, 1994), Japan (Sagara *et al*, 1987), India (Prasad *et al*, 1994), Thailand (Nirdnoy *et al*, 2005), Malaysia (Ansary & Radu, 1992) and Australia (Pratt & Korolik, 2005 & Alfredson & Korolik, 2003).

Resistance to tetracycline, kanamycin and chloramphenicol, and recently reported streptomycin and streptothricin, may be associated with plasmids in *Campylobacter* (Nirdnoy *et al*, 2005, Gibreel *et al*, 2004a; Gibreel *et al*, 2004b & Sagara *et al*, 1987). Many reports examining antibiotic resistant isolates of *Campylobacter* have reported higher incidences of plasmid carriage with 70% to 100% of isolates harbouring plasmids (Lee *et*

al, 1994, Ansary & Radu, 1992, Sagara *et* al, 1987, Taylor *et al*, 1986, Tenover *et al*, 1985 & Taylor *et al*, 1981).

Plasmid carriage rates and the number of plasmids harboured by strains varies between reports. Plasmids have been found in between 13 to 53% of *C. jejuni*, with the majority being resistance plasmids (Gibreel *et al*, 2004a & Bacon *et al*, 2000). Plasmid incidence in *C. jejuni* varies from one geographical area to another and from source to source (Lekowska-Kochaniak *et al*, 1996). A number of studies have reported a higher plasmid carriage in *C. coli* strains than in *C. jejuni* strains (Cabrita *et al*, 1992a, Cabrita *et al* 1992b, Sagara *et al*, 1987, Tenover *et al*, 1985 & Bradbury *et al*, 1983).

Many of the plasmids isolated from strains have been reported to be conjugative and are transmissible among *Campylobacter* spp. but not to *E. coli* (Bacon *et al*, 2000). Schmidt-Ott *et al*, 2005 identified a distinct subgroup of *C. jejuni* plasmids harbouring ORFs with significant homology to genes of transfer systems from conjugative plasmids and genes of putative Type IV secretion systems of other bacteria such as *Brucella suis*, *E. coli*, *A. actinomycetemcomitans* and *H. pylori*.

1.4 PATHOGENICITY OF *CAMPYLOBACTER*

Infection with *C. jejuni* or *C. coli* can induce a spectrum of disease symptoms and *Campylobacter* pathogenesis reflects both the susceptibility of the host and the virulence of the infecting strain (Park, 2002). Host and pathogen specific factors such as the health and age of the host (Tauxe, 1992) and *C. jejuni*-specific humoral immunity from previous exposure may influence clinical outcome after infection (Altekruse *et al*, 1999).

The infective dose of *C. jejuni*, although influenced by factors such as host immunocompetency and strain virulence is generally low (Skirrow & Blaser, 2000 & Walker *et al*, 1986). Studies, including experimental human infection, have shown that the infective dose that results in symptoms can be as low as 500-800 organisms (van Vliet & Ketley, 2001, Skirrow & Blaser, 2000, Ketley, 1995).

The mechanisms by which campylobacters induce disease are not clearly understood, but on the basis of experimental evidence, at least two mechanisms for gastrointestinal illness have been postulated: (i) intestinal adherence and toxin production and (ii) bacterial invasion and proliferation within the intestinal mucosa (Park, 2002). Many pathogen-specific virulence determinants may contribute to the pathogenesis of *C. jejuni* infection, but none has a proven role (Ketley, 1997). Suspected determinants include chemotaxis, motility, and flagella, which are required for attachment and colonisation of the gut epithelium. Once colonisation occurs, other possible virulence determinants include iron acquisition, host cell invasion, toxin production, inflammation and active secretion, and epithelial disruption with leakage of serosal fluid (Altekruse *et al*, 1999 & Ketley, 1997).

Campylobacters are genotypically and phenotypically very diverse and the creation of this diversity may enable survival during transmission from host to host. This diversity may reflect differences in virulence at a strain specific level. For example, the efficiency with which campylobacters invade cultured human cells varies greatly and differences in toxigenic activities between strains have been observed (Park, 2002, Harvey *et al*, 1999 & Everest *et al*, 1992). Differences in virulence mechanisms may be linked to distinct clinical presentations of campylobacter enteritis (Bacon *et al*, 2000).

In 2000, Bacon *et al* identified the involvement of a plasmid in the virulence of a well studied *C. jejuni* strain, 81-176. The plasmid, pVir, approximately 37.5kb in size and encoding components of a Type IV secretion system (which is important for virulence in major bacterial pathogens) (Christie, 2001), was found to be involved in invasion (Tracz *et al*, 2005 & Bacon *et al*, 2002). pVir is not universally carried by *C. jejuni* strains and it was reported that pVir was absent from strains other than 81-176, including genome sequenced strain NCTC 11168, possibly indicating differences in pathogenic mechanisms among strains of *C. jejuni* (Bacon *et al*, 2002). Also, Tracz *et al*, 2005 reported the presence of pVir in only 17% of Canadian clinical isolates of *C. jejuni* examined.

In contrast, Louwen *et al*, 2006 reported the absence of an association between pVir and bloody diarrhoea, which is indicative of invasive capabilities of *Campylobacter* isolates. Only 1 patient out of 48 with bloody diarrhea was infected with a *C. jejuni* strain harbouring pVir. Louwen *et al* proposed that the absence of an association between pVir and bloody diarrhea suggests that other virulence factors may be involved.

There also may be differences in virulence between isolates of different origins, such as poultry, meat and humans. Takkinen & Ammon, 2003 reported that there is evidence that human isolates are more virulent that poultry isolates. Conversely, it is possible that certain strains of *C. jejuni* and *C. coli* may not actually be capable of causing disease in humans since the distribution of genotypes from poultry and humans are not necessarily the same (Clow *et al*, 1998 & Korolik *et al*, 1995).

1.5 EPIDEMIOLOGY OF *CAMPYLOBACTER* INFECTION

Campylobacteriosis is the most commonly reported bacterial cause of gastrointestinal infection in the United States, England and Wales, and Australia (The OzFoodNet Working Group, 2005, Frost, 2001, Altekruse *et al*, 1999, Stafford *et al*, 1996). The number of infections in developed countries often exceeds the combined total for diarrhoeal illness caused by *Salmonella*, *Shigella* and *E. coli* species (Blaser, 1997*).* The vast majority of *Campylobacter* infections are reported as sporadic (Frost, 2001).

Prevalence of campylobacteriosis in the United States has been estimated to be between 2.1 to 2.4 million cases per year (Altekruse *et al*, 1999), 55 000 cases were reported in England and Wales in 1999 (Frost, 2001) and 15 640 cases were reported in Australia in 2004 (The OzFoodNet Working Group, 2005). It was suggested by Stafford *et al*, 1996 that the actual incidence of C*ampylobacter* infection in Australia, as in other countries, is probably much greater than the currently reported rates. Hall *et al*, 2005 estimated that between 67,000 and 350,000 (median – 208,000) cases of foodborne gastroenteritis in Australia each year are due to *Campylobacter* species, however, a much smaller figure was reported (excluding figures for New South Wales) in 2004 (The OzFoodNet Working Group, 2005). Differences in numbers of infections notified and actual numbers may be due to under-reporting (Ketley, 1995).

C. jejuni and *C. coli* are the main *Campylobacter* species involved in human disease. *C. jejuni* accounts for between 80-90% of cases of enteric *Campylobacter* infections in industrialised countries. *C. coli* is the second most frequently isolated species and accounts for between 5-15% of cases (Moore *et al*, 2005 & Tam *et al*, 2003, Gillespie *et al*, 2002,

Nachamkin *et al*, 2000 & Ketley, 1995). Other campyobacters such as *C. lari*, *C. upsaliensis*, and *C. fetus* are more seldomly found, but vary depending on different regions of the world (Moore *et al*, 2005).

The highest age specific rates of campylobacteriosis in Australia in 2004 were in young children (0-4 years) (The OzFoodNet Working Group, 2005). In 2003, in addition to the high rates for the 0-4 age group, there was a secondary peak in the 20-29 year age group (The OzFoodNet Working Group, 2004). These figures correspond to figures reported by Blaser, 1997 of highest rates in children <1 year old and between 15 and 30 years. Stafford *et al*, 1996 proposed that the reasons for the highest rates in children may partly be due to parents being more likely to seek medical care for these types of illnesses compared with adults.

A higher rate of travel in young adults has been suggested as a reason for an increased isolation rate for the 20-29 age groups (Stafford *et al*, 1996). The popularity of poultry and fast food may also play a role. The incidence of reported infections is slightly higher in males than females (Blaser, 1997). In Australia, indigenous peoples in the Northern Territory also have a higher rate of infection than the non-indigenous population (The OzFoodNet Working Group, 2005).

C. jejuni infection is much more common in developing countries than in developed countries, but many characteristics are different (Blaser, 1997). Disease is usually restricted to children with no apparent peak in adults, no strong pattern of seasonality, a higher incidence of infection complicated by a higher rate of asymptomatic carriage and a larger

proportion of infections result in milder disease with a watery, non-inflammatory diarrhoea (Ketley, 1995). The difference in epidemiological and clinical pattern of disease is probably due to much higher exposure and infection rate early in life resulting in a different pattern of immunity (protective immunity) (Ketley, 1995 & Blaser, 1997). *C. jejuni* is a cause of traveller's diarrhoea in visitors (from developed countries) to developing countries (Andrews, 1998, Blaser, 1997 & Ketely, 1995), and the spectrum of illness in travellers is similar to that described in developed nations (van Vliet & Ketley, 2001).

Campylobacter enteritis in temperate countries exhibits a distinctive seasonal pattern with a typical spring/summer peak in reported cases (Tam *et* al, 2005 & Frost, 2001). In contrast, notification rates in Australia do not show as pronounced seasonal trends as compared to distinct trends in the northern hemisphere, suggesting factors other than climate in Australia may be involved (Stafford *et al*, 1996). In a study of pathogens causing community gastroenteritis in Melbourne, Victoria between 1997 and 1999, Sinclair *et al*, 2005 reported no clear seasonal trend for *Campylobacter* infections. Although there is not as distinct seasonal trends, the highest number of notifications in Australia occurs in the warmest months (January and February; October to December) (Communicable Diseases Network Australia, 1999).

Milder winter temperatures may favour some transmission routes, as well as enhance the survival and multiplication of the bacteria in the environment (Kovats *et al*, 2004). In this context, Stafford *et al*, 1996 reported that notifications of *Campylobacter* infections in Queensland (an Australian state with a tropical and subtropical climate) have a less marked seasonal trend, which may be related to the state's mild winters. Kovats *et al* reported a less

pronounced seasonality in Australian cites than in New Zealand, a country with a colder climate than Australia.

Factors leading to greater exposure of humans in summer months to campylobacters such as increased consumption of poultry, increased travel summer and increased contract with recreational waters, which may influence seasonal changes in human infections (Tam *et al*, 2005, & Ketley, 1995), may be less pronounced in Australia (Stafford *et al*, 1996). Imported cases of *Campylobacter* infection by travelers may also be an important factor influencing seasonal trends in this country (Stafford *et al*, 1996).

Campylobacters are widespread throughout animal, food and water environments (Frost, 2001). Campylobacteriosis is a zoonosis, and farm and companion animals are significant reservoirs of the organism with the potential for transmission to humans (Moore *et al*, 2005 & Tauxe, 2002). Prevalence studies among animals show different percentages for *Campylobacter* species and different types of animals. In animals, however, many species and serotypes are found at the same time. The most prevalent *Campylobacter* species in poultry and cattle has been shown to be *C. jejuni*, in swine, *C. coli*, and in dogs, *C. upsaliensis*. Broiler flocks are often contaminated with *C. jejuni* and *C. coli* (Takkinen & Ammon, 2003).

Transmission to humans occurs by ingestion of contaminated foods of animal origin, contaminated untreated water and by direct contact with infected animals, especially pets (Blaser, 1997). The presence of campylobacters in the environment is a sign of recent contamination with animal and avian faeces, agricultural run-off and sewage effluent

(Jones, 2001). Surveys of raw agricultural products and overlap in serotypes of *C. jejuni* found in humans, poultry and cattle, support epidemiologic evidence implicating poultry, meat, and raw milk as sources of human infection (Altekruse *et al*, 1999 & Nielsen *et al*, 1997).

Poultry, in particular, has been identified as a significant source of *Campylobacter* due to infection and contamination at pre-harvest and harvest levels (Moore *et al*, 2005 & Pezzotti *et al*, 2003). There is strong evidence for frequent cross contamination during slaughtering and product processing, although some studies have indicated that slaughter processes have a minor influence on the risk for human campylobacteriosis (Takkinen & Ammon, 2003). In total >95% of campylobacteriosis cases are recognised as being endemic and 50-70% of all endemic cases are due to ingestion of undercooked contaminated poultry (Blaser, 1997).

The Food Safety & Inspection Service suggested in 1996 that the high rate of infections due to the consumption of chicken should not be so surprising in light of the frequency with which poultry products are consumed and the nearly universal contamination of chicken carcasses with *Campylobacter* (Allos, 2001). Consumption of other foods cross contaminated with drippings from raw poultry is also a leading risk factor for human campylobacteriosis (Altekruse *et al*, 1999 & Park, 2002). Therefore, careful food preparation and cooking practices, including thorough cooking of chicken, as heat kills viable *Campylobacter* cells, should be emphasised as important food-safety and infection prevention measures (Allos, 2001).

1.6 CLINICAL FEATURES OF *C. JEJUNI* & *C. COLI* INFECTION

The mean incubation period of *Campylobacter* enteritis is 3.2 days with a range of 18 hours to eight days (Skirrow and Blaser, 2000). A spectrum of illness is seen during *C. jejuni* / *C. coli* infection, where patients range from asymptomatic to severely ill. The main symptom observed is diarrhea, which can vary from limited to voluminous stools which may be watery or bloody. Fever and abdominal cramping may also be present, but vomiting is uncommon (Moore et al, 2005 & Blaser, 1990). Symptoms and signs may last several days to more than one week. Symptomatic infections are self-limited, but relapses may occur in 5–10% of untreated patients (Blaser, 1990). *Campylobacter* infections are usually less acute than *Salmonella* or *Shigella* infections, with less fever and general symptoms (Moore *et al*, 2005).

Extraintestinal infection occurs and may affect contiguous organs, causing cholecystitis, pancreatitis, cystitis, septic abortion (Blaser, 1997), reactive arthritis, bursitis, meningitis, endocarditis, peritonitis and erythema nodosum (Blaser, 1990). Bacteriemia may occur, especially in immunocompromised hosts (Blaser, 1997). Death following *C. jejuni* infection is rare but does occur (Nachamkin *et al*, 2000, Font *et al*, 1997 & Tauxe, 1992). Life threatening systemic diseases caused by *Campylobacter* infections are diagnosed more and more readily. These include Guillain Barre Syndrome, the most severe post infectious consequence, making *Campylobacter* infection a major public health issue (Moore *et al*, 2005).

Guillain-Barré Syndrome (GBS), a demyelating disorder resulting in acute neuromuscular paralysis. An estimated one case of GBS occurs for every 1 000 cases of

campylobacteriosis and up to 40% of patients with the syndrome have evidence of recent *Campylobacter* infection (Altekruse *et al*, 1999 &Allos, 1997). An estimated 120 of the approximately 650 cases of GBS in Australia each year is likely to be foodborne and thus due to *Campylobacter* (Hall & Kirk, 2005). Symptoms of GBS usually occur one to three weeks after the onset of *Campylobacter* enteritis and cases associated with *Campylobacter* are usually more severe requiring intensive hospital treatment and leading to possible long term disability (Butzler, 2004). The pathogenic mechanism relies on antigen mimicry between oligosaccharides from *C. jejuni* lippopolysaccharides and the GM_1 ganglioside of the peripheral neuron membrane (Moore *et al*, 2005 & Yuki *et al*, 1997).

Maintenance of hydration and electrolyte balance, not antibiotic treatment, is the cornerstone of treatment of *Campylobacter* enteritis (Allos, 2001). Although *Campylobacter* infections are usually self limiting, antibiotic therapy may be prudent for patients who have high fever, bloody diarrhea, or more than eight stools in 24 hours; immunosupressed patients, patients with bloodstream infections, and those whose symptoms worsen or persist for more than one week from time of diagnosis (Altekruse *et al*, 1999). Patients who live in an institution or closed group where the risks of spread are high may also benefit from antibiotic therapy (Skirrow & Blaser, 2000). When indicated, antimicrobial therapy soon after the onset of symptoms can reduce the median duration of illness from approximately 10 days to five days (Altekruse *et al*, 1999).

Macrolides such as erythromycin and azithromycin are currently the treatment of choice, due to a high degree of efficacy and lack of serious toxicity. Fluoroquinolones including ciprofloaxcin are also used (Allos, 2001, Nachamkin et al, 2000, Altekruse *et al*, 1999).

Serious systemic infection should be treated with an aminoglycoside, such as gentamicin, or impenem (Skirrow & Blaser, 2000). Tetracyclines and chloramphenicol are alternative antibiotics when resistance or some other reason excludes fluoroquinolones and erythromycin, but up to 60% of strains may be resistant to tetracyclines. However, there is also increasing rates of resistance to the agents of choice used in the treatment of clinical enteric infection (Aarestrup *et al*, 1997).

1.7 ANTIMICROBIAL RESISTANCE IN *C. JEJUNI* AND *C. COLI*

The increasing rate of human infections caused by antimicrobial resistant strains of *C. jejuni* makes clinical management of cases of campylobacteriosis more difficult (Altekruse *et al*, 1999, Murphy *et al*, 1996 & Piddock, 1995). Antimicrobial resistance can prolong illness and compromise treatment of patients with bacteremia (Altekruse *et al*, 1999). Travers and Bazra, 2002 estimated an excess of days of diarrheoa due to domestically acquired fluoroquinolone resistant *C. jejuni* in the United States. Infection with either quinolone or marcolide resistant *Campylobacter* strains is also associated with an increased risk of invasive illness or death, compared to infections with drug susceptible strains (Helms *et al*, 2005).

Moore *et al*, 2006 stated that it is not clear whether additional problems (i.e. prolonged and/or more severe illness) arise because a strain is more resistant or whether strains are more virulent. Resistant strains may possess additional virulence factors that are not directly related to their reduced susceptibility to antimicrobials (Moore *et al*, 2006). Underlying mechanisms could be co-selection of virulence traits, up-regulation of virulence or improved fitness of the resistant isolates (Gibreel & Taylor, 2006, Molbak, 2005 &

Travers & Barza, 2002). Recently Zhang *et al*, 2006, noted that many fluoroquinolone resistant mutants of *Campylobacter* are as competent as or even more fit than sensitive strains, and this may also partly explain the increasing prevalence of fluoroquinolone resistant *Campylobacter* on a global scale

Several studies have linked the use of antimicrobial agents in veterinary medicine or as feed additives (as prophylactics and growth promoters) with the emergence and spread of resistance among *Campylobacter* strains, with potentially serious effects on food safety, and in both veterinary and human health (Chee-Sanford *et al*, 2001 & Saenz *et al*, 2000). Antibiotic use in human medicine has also been proposed to have had effect on the prevalence of antibiotic resistance. In both populations antibiotics are used for therapy and prophylaxis (van den Bogaard & Stobberingh, 2000).

Antimicrobial drug resistance arises in populations because of a combination of selective pressure from the use of antimicrobial agents and the emergence, import and dissemination of resistant bacteria and resistance genes (van den Bogaard & Stobberingh, 2000 & McGreer, 1998). Antibiotic use and resistance in food animals could be a selective force responsible for the accumulation of resistance genes in integrons, their movement to plasmids and other accessory elements, and then the movement of these genes from their ancestral bacteria into the commensal and pathogenic bacteria of mammals (Lipsitch *et al*, 2002).

The emergence of fluoroquinolone resistant isolates of *Campylobacter* is a clear example of the possible association between antibiotic use and the emergence of resistant isolates

implicated in human disease. The emergence of fluoroquinolone resistant *Campylobacter* infections in Europe and the United States was temporally associated with the approval of fluoroquinolone use in veterinary medicine (Altekruse *et al*, 1999). Resistance has been reported to develop among patients after treatment with fluoroquinolones (Bacon *et al*, 2000) and during treatment of *Campylobacter* colonised broiler chickens under experimental conditions (Moore *et al*, 2005 & Jacobs-Reitsma *et al*, 1994). Molecular epidemiological studies provide further support for the causal link between chicken consumption and fluoroquinolone-resistant *Campylobacter* infections. Strains of *Campylobacter* found in the meat of chickens seem to be identical to those responsible for human infections (Lipsitch *et al*, 2002 & Smith *et al*, 1999).

Campylobacter species are intrinsically resistant to a number of antibiotics, including cefoperazone, cephalothin, polymixin B, bacitracin, vancomycin, rifampin and trimethoprim (Allos, 2001, Gibreel & Skold, 1998, Andrews, 1998 & Koneman *et al*, 1992), some of which are utilised in selective media for isolation. The selective and isolation media developed by Skirrow contains the antibiotics trimethoprim, polymixin B and vancomycin (Oxoid, 1998 & Fricker, 1987). Most *Campylobacter* species also show varying levels of resistance to penicillin-like antibiotics (Alfredson & Korolik, 2005 & Lachance *et al*, 1991).

Resistance to a variety of antibiotics have been reported for *C. jejuni* and *C. coli*, including resistance to chloramphenicol, erythromycin, kanamycin, aminoglycosides, β-Lactams and ciprofloxacin (Moore *et al*, 2005, O'Halloran *et al*, 2004, Alfredson & Korolik, 2005, Beckmann *et al*, 2004, Trieber & Taylor, 2000, Acar & Goldstein, 1997, Taylor, 1992, Adler-Mosca *et al*, 1991, Wang & Taylor, 1990, Sagara *et al*, 1987). Resistance to other

antibiotics such as streptomycin (Ansary and Radu, 1992), cefalexin (Moore *et al*, 2001), gentamicin, nalidixic acid (Saenz *et al*, 2000) and sulphonamides (Lipscomb and Evers, 2000) has also been reported. Mechanisms involve plasmid, chromosome or integron mediated resistance genes or chromosomal mutations. Genetic and molecular mechanisms of tetracycline and trimethoprim resistance in *Campylobacter* will be addressed later in this report.

C. lari, *C. fetus* and *C. hyointestinalis* are all inherently resistant to nalidixic acid (Laatu *et al*, 2005 & Edmonds et al, 1987). Aarestrup *et al*, 1997 reported that isolates of *C. lari* from chickens and cattle were resistant to ampicillin. Resistance of *C. fetus* to tetracycline, cephalothin, and at low percentages to ciprofloxacin, cefotaxime and erythromycin has been reported in human and/or animal isolates of this species (including subspecies *fetus*) (Vandenberg *et al*, 2006, Inglis *et al*, 2006, Tremblay *et al*, 2003 & Kwon et al, 1994). Resistance to sulphonamide, streptomycin, tetracycline and erythromycin, and at very low percentages to ampicillin and ciprofloxacin, has been reported in bovine and/or reindeer isolates of *C. hyointestinalis* (Laatu *et al*, 2005 & Inglis *et al*, 2005). Published antibiotic susceptibility data for *C. sputorum* isolates seems very limited and perhaps non-existent.

Multidrug resistance in *Campylobacter* species appears to be occurring more frequently and poses the risk that an effective antimicrobial regimen to treat infection may be lacking (Nachamkin *et al*, 2000). Tee *et al*, 1995 reported the emergence of multi-drug resistance in *C. jejuni* from patients with HIV in Australia. *C. jejuni* strains progressively acquired resistance to antibiotics (erythromycin, ciprofloxacin and doxycyline) used during

treatment. Multi-drug efflux pumps have been found to to contribute to multi-drug resistance in *C. jejuni* and *C. coli* (Lin *et al*, 2002 & Pumbwe & Piddock, 2002).

The CmeABC efflux system, belonging to the Resistance and Nodulation Division family of efflux transporters was found to contribute intrinsic resistance of *Campylobacter* spp. to a broad range of unrelated antibiotics, including fluoroquinolones (including ciprofloxacin), erythromycin, ampicillin, tetracycline and chloramphenicol; detergents (bile salts & sodium dodecyl sulfate) and dyes (ethidium bromide & acridine orange) (Payot *et al*, 2006, Lin *et al*, 2002 & Pumbwe & Piddock, 2002). The pump is widely distributed in *Campylobacter*, including *C. jejuni* and *C. coli* and it is constitutively expressed (Cagliero *et al*, 2006). A second efflux pump, CmeDEF, was identified by Pumbwe *et al*, 2005 and substrates for this pump include ampicillin, ethidium bromide, acridine, sodium dodecyl sulphate, deoxycholate, triclosan and cetrimide, but not ciprofloxacin or erythromycin unlike CmeABC (Pumbwe *et al*, 2005). There may also be another efflux pump involved in multi-drug resistance in *Campylobacter* (Payot *et al*, 2006 & Pumbwe *et al*, 2005).

Modification of the outer membrane permeability as a mechanism of resistance to antibiotics has not yet been described in *Campylobacter* (Payot *et al*, 2006). No modification in expression nor porin sequence has been found to be associated with resistance (Payot *et al*, 2006, Pumbwe *et al*, 2004 & Luo *et al*, 2003). Page *et al*, 1989 characterised porins from *C. jejuni* and *C. coli*, extensively reviewed literature and concluded that pores in the outer membrane (due to their small size) are likely to contribute to resistance to many hydrophilic antibiotics, except those with small molecular weights.

Numerous studies have examined resistance of isolates from humans (Wagner *et al*, 2003, Isenbarger *et al*, 2002 & Fernandez *et al*, 2000) animals, including poultry, pigs and wild birds (Waldenstrom *et al*, 2005, Hart *et al*, 2004 & Fallon *et al*, 2003); food, such as raw chicken and beef meat (Taremi *et al*, 2006); the environment (Piddock *et al*, 2000) and a combination of isolates (Cardinale *et al*, 2006, Randall *et al*, 2003; Saenz *et al*, 2000 & Aarestrup *et al*, 1997). Resistance rates and Minimum Inhibitory Concentrations (MICs) have been found to differ geographically, and between isolates from humans and other animals (poultry and pigs) (Saenz *et al*, 2000), food (Moore *et al*, 2001) and environmental (sewage and abattoir drain water etc) isolates (Koenraad *et al*, 1995).

A small number of studies have examined antibiotic resistance in Australian isolates of *Campylobacter*. Alfredson *et al*, 2003 examined the resistance of clinical isolates of *Campylobacter* from the Gold Coast, Queensland and reported resistance to tetracycline, ampicilin and erythromycin, but only a small number of isolates were resistant to tetracycline and erythromycin. Also, 11% of isolates showed resistance to more than one antibiotic (Alfredson *et al*, 2003). Sharma *et al*, 2003 reported resistance of *C. jejuni* from the Hunter Region in New South Wales to ampicilin, erythromycin, nalidixic acid and tetracycline. Unicomb *et al*, 2006 assessed *C. jejuni* obtained from five Australian states and reported resistance to sulfisoxazole, ampicilin, roxithromycin, tetracycline, nalidixic acid, chloramphenicol, erythromycin, gentamicin and kanamycin.

Susceptibility tests for *Campylobacter* spp. are not standardised and consequently, the literature contains some variability in the susceptibility data reported (Tenover *et al*, 1992 and King, 2001). Recommendations for the agar dilution method (incorporation of various

concentrations of antibiotic in culture media prior to dispensing into petri dishes) include using Mueller-Hinton agar supplemented with 5% horse or sheep blood and incubated for 16 to 18 hours under microaerophilic conditions (Tenover *et al*, 1992). No internationally accepted criteria for susceptibility testing of *Campylobacter* spp. are available and breakpoints do not exist. Recently however the National Committee for Clinical Laboratory Standards (NCCLS) Sub Committee on Veterinary Antimicrobial Susceptibility Testing approved an agar dilution protocol as a valid method (Moore *et al*, 2005).

1.8 TETRACYCLINE RESISTANCE

Numerous studies have reported tetracycline resistance in *Campylobacter* species, including isolates from clinical settings, animals (poultry, pigs, cattle) and food (poultry meat, pork, beef and seafood). Tetracycline resistance in *Campylobacter* spp. has been reported in many countries around the world, including Ireland, Denmark, Spain, Iran, Canada, Brazil and Australia (McGill *et al*, 2006; Taremi *et al*, 2006; Unicomb *et al*, 2006; Gaudreau & Gilbert, 2003; Aquino *et al*, 2002; Saenz *et al*, 2000 & Aarestrup *et al*, 1997). Tetracycline resistance in *Campylobacter* has been reported to be mediated by plasmids (Taylor *et al*, 1983 & Taylor *et al*, 1981) and investigators in Canada, USA, Japan, India, Taiwan, Israel, Malaysia, Spain and Germany have isolated plasmids from tetracycline resistant *C. jejuni* and *C. coli* strains (Schmidt-Ott *et al*, 2005, Velazquez *et al*, 1995, Prasad *et al*, 1994, Lee *et al*, 1994, Schwartz *et al*, 1993, Ansary & Radu, 1992, Sagara *et al*, 1987; Tenover *et al*, 1985 & Taylor *et al*, 1981).

Tetracycline susceptibility may differ between *Campylobacter* species from different sources (such as humans, chicken and pigs) and between *C. jejuni* and *C. coli* isolates

(Lucey *et al*, 2000b, Saenz *et al*, 2000 & Aarestrup *et al*, 1997). Geographical differences in the susceptibility of *C. jejuni* and *C. coli* to tetracycline have been reported (Unicomb *et al*, 2006 & McGill *et al*, 2006). The percentage of isolates that are resistant to tetracycline may vary extensively, for example, one study may report that all isolates are susceptible to tetracycline and thus 0% are resistant, and another study may report that 78% and 100% of isolates are resistant (Aquino *et al*, 2002 & Lee *et al*, 1994). Rates of tetracycline resistance have been reported to vary from year to year (Andersen *et al*, 2005 & Gaudreau and Gilbert, 1998).

Tetracycline resistance in Australian *Campylobacter* isolates has been reported by a few studies. Alfredson *et al*, 2003 stated that 16% of clinical isolates from the Gold Coast, Queensland examined were resistant to tetracycline and MICs ranged from 16->256mg/L. Sharma *et al*, 2003 also reported a low rate of tetracycline resistance (11%) of *C. jejuni* isolates from humans in the Hunter Region of New South Wales. And, 7% of clinical *C. jejuni* isolates obtained from five Australian states were resistant to tetracycline (Unicomb *et al*, 2006). Resistance to tetracycline of *Campylobacter* isolates from animals (pigs) has also been reported (Hart *et al*, 2004).

Tetracyclines are broad-spectrum antimicrobial agents with activity against a wide range of bacterial pathogens, including both Gram-positive and Gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas and rickettsia, and protozoan parasites (Connell *et al*, 2003, Chopra & Roberts, 2001, Roberts, 1997, Roberts, 1996 & Chopra *et al*, 1992).Tetracyclines have been widely used throughout the world in both human and

animal medicine, however their use and effectiveness has declined as bacterial resistance has become more widespread (Chopra & Roberts, 2001 & Roberts, 1997).

Tetracyclines can be divided into two groups, typical tetracyclines (such as tetracycline, chlortetracycline and minocylcine) and atypical tetracyclines (such as anhydrotetracycline and 6-thiatetracycline) (Chopra & Roberts, 2001, Chopra, 1994 & Oliva & Chopra, 1992). Typical tetracyclines inhibit growth by reversibly binding to the ribosome, preventing the elongation phase of protein synthesis and thus inhibiting protein synthesis (Connell *et al*, 2003, Schnappinger and Hillen, 1996 & Roberts, 1997).

Tetracyclines bind to the bacterial 30S ribosomal subunit of the 70S ribosome and prevent the attachment of aminoacyl-tRNA to the ribosomal receptor (A) site (Chopra *et al*, 1992; Roberts, 1996; Roberts, 1997 and Manavathu *et al*, 1990) and therefore prevent the addition of new amino acids to the growing polypeptide (Connell *et al*, 2003). Binding of tetracycline to the ribosome weakens the ribosome-tRNA interaction and this has been thought to be the cause of inhibition of protein synthesis (Schnappinger and Hillen, 1996). The effect of tetracycline may be a direct blockage of aminoacyl-tRNA binding or may reflect a conformational change of the ribosome upon tetracycline binding which alters codon-anticodon interactions and thus prevents aminoacyl-tRNA binding (Treiber *et al*, 1998).

Tetracycline resistance may be mediated by one of three different mechanisms: (i) an energy-dependent efflux of the antibiotic, (ii) ribosomal protection by a soluble protein or (iii) enzymatic inactivation of the antibiotic (Taylor and Chau, 1996). Tetracycline

resistance in most bacteria is due to the acquisition of new genes and many of the ribosomal protection protein determinants are located on mobile genetic elements, such as plasmids and/or transposons, which may have facilitated their spread throughout the eubacteria via lateral gene transfer events (Roberts, 2005, Connell *et al*, 2003 & Chopra & Roberts, 2001).

Ribosomal protection genes are generally thought to be of Gram-positive origin but are now often found in a variety of aerobic and anaerobic Gram-negative species (Roberts, 1996). Their presence in natural Gram-negative isolates indicates that gene exchange between Gram-positive and Gram-negative bacteria is possible and does occur in nature (Roberts, 1997).

Ribosomal protection is a common mechanism of resistance to tetracycline, with 11 *tet* genes coding for ribosomal protection proteins (Roberts, 2005 & Taylor *et al*, 1998). Several related classes of ribosomal protection determinants have been identified and characterised at the DNA and protein levels and all appear to have similar function (Roberts, 1997 & Roberts, 1996). TetO and Tet(M) are the most extensively studied of the ribosomal protection proteins and TetO shares 78% homology with Tet(M) (Roberts *et al*, 1993).

Ribosomal protection proteins, such as TetO and Tet(M), are soluble cytoplasmic proteins (~72 kDa) that mediate tetracycline resistance by releasing tetracycline from the ribosome and thereby freeing the ribosome from the inhibitory effects of the drug, such that aa-tRNA can bind to the A site and protein synthesis can continue (Connell *et al*, 2003 & Taylor &

Chau, 1996). Manavathu *et al*, 1990 reported that the presence of TetO markedly diminished the inhibitory effect of tetracycline on protein synthesis.

Ribosomal protection proteins display sequence similarity to ribosomal elongation factors and are grouped into the translation factor superfamily of GTPases (Connell *et al*, 2003 & Leipe *et al*, 2002). Similarity between TetO and GTPases participating in protein synthesis, namely EF-Tu and EF-G was reported by Manavathu *et al*, 1988. TetO has been shown to bind and hydrolyse GTP, and with GTP TetO continually interacts with the ribosome displacing tetracycline, hydrolysing the GTP and dissociating from the ribosome (Taylor *et al*, 1998 & Taylor & Chau, 1996). It has been hypothesised that like EF-G-GTP, TetO would have a high affinity for the pre-translocational state and could bind to the ribosome, displace the tetracycline and induce a conformational shift back to a post-translational state, allowing tRNA to bind (Trieber *et al*, 1998).

The *tetO* gene encoding the TetO protein was first identified on a transferable plasmid from *Campylobacter jejuni* (Connell *et al*, 2003 & Taylor & Chau, 1996). The *tetO* gene, as of 2005, was found in eleven different genera, including the gram negative genera *Campylobacter*, *Butyrivibrio*, *Neisseria* and *Megasphaera* and the gram positive genera, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Aerococcus*, *Lactobacillus* and *Mobiluncus* (Roberts, 2005 & Chopra & Roberts, 2001).

The 40% G+C content of the protein coding regions of *tet*O is close to that of *tet*M (37%) but higher than the G+C value obtained for *C. jejuni* and *C. coli* chromosomal (32.5%) and plasmid (31 to 33%) DNAs suggesting that *tetO* and thus tetracycline resistance was

acquired from a Gram-positive bacterium, in particular a Gram-positive coccus. In addition to the higher G+C content of *tetO*, 75% homology of *tetO* with *tetM* of *Streptococcus pneumoniae* and the presence of *tetO* in Gram-positve cocci species also points to such an origin (Moore *et al*, 2005, Taylor and Courvalin, 1988, Manavathu *et al*, 1988, Sougakoff *et al*, 1987 & Taylor *et al*, 1983). Interestingly, all ribosomal protection protein genes, except the *tet*(W) gene and the *Streptomyces* genes, have G+C% contents compatible with a Gram-positive origin (<50% G+C) (Roberts, 2005). Taylor *et al*, 1983 reported no homology between the *Campylobacter* tetracycline resistance determinant (TetO) and enterobacterial resistance determinants.

The presence of the *tetO* gene encoding the TetO protein has been detected in *Campylobacter* strains throughout the world including, Canada (Tracz *et al*, 2005; Gibreel *et al*, 2004a; Gibreel *et al*, 2004b & Manavathu *et al*, 1988), USA (Fairchild *et al*, 2005 & Bacon *et al*, 2000;), UK (Pumbwe *et al*, 2004; Batchelor *et al*, 2004 & Randall *et al*, 2003;), Holland (Louwen *et al*, 2006), France (Avrain *et al*, 2004 & Sougakoff *et al*, 1987), Switzerland, Algeria and Vietnam (Sougakoff *et al*, 1987); Taiwan (Lee *et al*, 1994), and Thailand (Nirdnoy *et al*, 2005 & Sougakoff *et al*, 1987), indicating a wide distribution. In 2005 as part of this current project, we were the first to publish a study indicating the presence of the *tet*O gene in Australian clinical and chicken isolates of *C. jejuni* and *C. coli* (Pratt & Korolik, 2005).

The *tet*O gene has been detected in both *C. jejuni* and *C. coli* isolates from a variety of sources, including cases of gastroenteritis, Guillain-Barré and Miller-Fisher Syndromes,

chickens, chickens in slaughterhouses and chicken meat; and porcine origin (Louwen *et al*, 2006, Fairchild *et al*, 2005, Avrain *et al*, 2004, Lee *et al*, 1994 & Sougakoff *et al*, 1987).

*tet*O has been detected in tetracycline resistant *Campylobacter* strains with a variety of tetracycline MICs. MICs of *tet*O harbouring *C. jejuni* and *C. coli* isolates of diverse origins (clinical, chicken and porcine) range from lower values such as 8 or 16μg/ml to higher values such as ≤32 μg/ml, 64μg/ml, 128 μg/ml and 512μg/ml and in one study ≤1024μg/ml (Fairchild *et al*, 2005, Pumbwe *et al*, 2004; Gibreel *et al*, 2004a, Lee *et al*, 1994, Sougakoff *et al*, 1987 & T aylor *et al*, 1986). Gibreel *et al* reported an association between extremely high level tetracycline MICs (512μg/ml) of *C. jejuni* isolates and a mutated version of the *tet*O gene. It was proposed that these mutations could potentially allow for stronger binding to the ribosome, due to their exposure on the surface of the protein, and allow TetO to out-compete high levels of tetracycline thus mediating high level tetracycline resistance (Gibreel *et al*, 2004a).

Tet O appears most often to be plasmid-mediated in *C. jejuni* (Taylor *et al*, 1987) and *C. coli* (Sougahoff *et al*, 1987), but it has been identified in several gram-positive organisms where it is probably chromosomal (Roberts, 1993 and Taylor and Chau, 1996). A number of studies have reported plasmid mediated *tet*O carriage in *C. jejuni* and *C. coli* (Tracz *et al*, 2005, Gibreel *et al*, 2004a; Gibreel *et al*, 2004b, Bacon *et al*, 2000, Lee *et al*, 1994, Taylor *et al*, 1988 & Ng *et al*, 1987). *tet*O harbouring plasmids have been identified in both *C. jejuni* and *C. coli* isolates and from both clinical and chicken sources (Gibreel *et al*, 2004a, Lee *et al*, 1994 & Taylor *et al*, 1988). It has been reported that most tetracycline resistant strains of *Campylobacter* studied harboured plasmids carrying *tet*O (Gibreel *et al*, 2004a &

Ng *et al*, 1987). In semi contrast, Lee *et al*, 1994 reported that 87% of chicken isolates but only 47% of clinical isolates of *C. jejuni* from Taiwan carried *tet*O on a plasmid (Lee *et al*, 1994).

Although tetracycline resistance in *Campylobacter* is mostly plasmid mediated, a few researchers have reported resistance in isolates that lacked plasmid DNA and/or the presence of *tet*O on the chromosome. Plasmid free tetracycline resistant *Campylobacter* isolates have been reported by a number of investigators (Lee *et al*, 1994, Schwartz *et al*, 1993, Cabrita *et al*, 1992b; Bossinger *et al*, 1990 & Sagara *et al*, 1987). Tenover *et al*, 1985 reported that a tetracycline resistance plasmid showed homology to *C. jejuni* DNA from tetracycline-sensitive, plasmidless isolates. Gibreel *et al*, 2004a, Lee *et al*, 1994 and Ng *et al*, 1987 described chromosomally mediated tetracycline resistance (*tetO*) in Campylobacter isolates. Lee *et al* also reported the presence of *tet*O on the chromosome of Taiwanese *C. jejuni* isolates that harboured plasmids that did not carry *tet*O.

The *tet*O gene is usually associated with conjugative plasmids in *Campylobacter* spp. and it has been found to be transferable by conjugation among *C. jejuni* isolates *in vivo* and *in vitro* (Fairchild *et al*, 2005, Avrain *et al*, 2004 & Taylor *et al*, 1983). Conjugative plasmids from tetracycline resistant strains have been found to range in size mostly from 42 to 135kb (Batchelor *et al*, 2004, Gibreel *et al*, 2004a, Tenover *et al*, 1985 & Taylor *et al*, 1981). However smaller plasmids, such 23kb plasmids isolated in India (Prasad *et al*, 1994) and 3.3 and 12.6kb plasmids from Malyasia (Ansary and Radu, 1992) were conjugative and did transfer tetracycline resistance.

Bacon *et al*, 2000 detected the *tet*O gene on the pTet plasmid harboured by the well-characterised *C. jejuni* strain 81-176. Batchelor *et al*, 2004 subsequently completely sequenced this plasmid and another large conjugative *tet*O harbouring tetracycline resistance plasmid from a strain of *C. coli* from a human case of severe gastroenteritis. Both large plasmids, 44.7kb and 45.2kb in size respectively, showed striking similarities in DNA sequence and overall gene organization, although the two strains were isolated almost twenty years apart and on different continents (US and UK) (Batchelor *et al*, 2004). The *tet*O gene in both plasmids had a G+C content (40.4% G+C) substantially higher than that of the rest of the plasmid sequence, suggesting that this gene was horizontally transferred from another species and may have a different origin to the rest of the plasmid DNA (Batchelor *et al*, 2004).

Many genes present in the two plasmids were predicted to be involved in plasmid replication and conjugative transfer (Batchelor *et al*, 2004). Sequence analysis of the two plasmids revealed genes encoding a putative Type IV secretion system that was shown to be involved in conjugation and distinct from the Type IV secretion system found on the *C. jejuni* virulence plasmid pVir. Both plasmids were shown to be conjugative and transferred to recipient *C. jejuni* strains. The authors also experimentally showed that the Type IV secretion system carried by both plasmids is required for conjugation.

Analysis of conjugative tetracycline resistant plasmids from *Campylobacter* species was continued with the work of Nirdnoy *et al*, 2005. Nirdnoy *et al* reported several transposon related genes on a multiple drug resistant *tet*O harbouring conjugative plasmid from a *C. jejuni* strain. This plasmids was related to other *tetO* plasmids including pTet from *C. jejuni*

81-176, and, in addition to tetracycline resistance, conferred resistance to multiple antibiotics, including kanamycin, gentamicin, streptothricin, streptomycin and spectinomycin. Homologues to genes from the plasticity zone of *H. pylori* and multiple unknown genes from a variety of pathogenic and commensal bacteria were also identified on this plasmid (Nirdnoy *et al*, 2005).

In relation to the *tetO* gene, Schmidt-Ott *et al*, 2005 reported the presence of *tetO* on *mob* plasmids, but also on an unrelated plasmid and the authors suggested that this R-factor may eventually have been acquired with a transposon. This hypothesis was supported by the alignment of the *tetO* gene from the tetracycline resistance plasmid and its flanking sequences, with nucleotides for the transposon Tn*916* from *Enterococci* (Schmidt-Ott *et al*, 2005).

The presence of insertion sequences was reported on *tetO* harbouring plasmids of *C. jejuni* by Gibreel *et al*, 2004b. This insertion sequence, IS*607**, showed considerable similarity to IS*607* from the chromosome of some *H. pylori* strains worldwide. In addition to an association with plasmids carrying a kanamycin resistance gene, IS*607** was found on *tetO* harbouring tetracycline resistance plasmids from kanamycin susceptible clinical isolates of *C. jejuni*.

Avrain *et al*, 2004 demonstrated spontaneous *in vivo* conjugation between *C. jejuni* strains in the digestive tract of chickens inoculated with a pair of donor and recipient *C. jejuni* strains. The authors reported that the transfer of the *tetO* gene occurs rapidly and without antimicrobial selective pressure between *C. jejuni* strains in the digestive tract. It was also

proposed that the rapid and spontaneous transfer of the *tetO* gene may partly explain the high prevalence of tetracycline resistance in chicken *Campylobacter* strains and the high percentage of these resistant strains in an avian population (Avrain *et al*, 2004 & Avrain *et al*, 2001).

Avrain *et al*, 2004 also found that although the *tetO* gene was naturally present in the intestinal flora of specific pathogen free chickens, in the absence of tetracycline selective pressure, a recipient *Campylobacter* strain did not acquire the tetracycline resistance gene. Fairchild *et al*, 2005 were unable to experimentally detect *in vivo* transfer of resistance genes to *C. jejuni* even after finding commensal bacteria harbouring tetracycline resistance determinants and after the selective pressure of oral oxytetracycline administration.

1.9 TRIMETHOPRIM RESISTANCE

As previously stated, all *C. jejuni* and *C. coli* isolates are intrinsically resistant to trimethoprim (Gibreel and Skold, 2000, Gibreel and Skold, 1998, Taylor & Courvalin, 1988 & Karmali *et al*, 1981) and MICs range from 256μg/ml to ≥512μg/ml (Taylor & Courvalin, 1988). Due to this endogenous resistance, trimethoprim is regarded as an inefficient antimicrobial agent for the treatment of *Campylobacter* infections (Gibreel & Skold, 1998 & Karmali *et al*, 1981). Interestingly according to Huovinen, 1987, bacterial pathogens known to be intrinsically resistant to trimethoprim are fewer than susceptible ones.

Trimethoprim has a wide antibacterial spectrum, and is active *in vitro* against many bacteria (most aerobic gram-negative and gram-positive bacteria) including common urinary tract

pathogens, respiratory tract pathogens, skin pathogens as well as certain enteric pathogens such as *E. coli* and *Shigella* spp. (Huovinen *et al*, 1995 & Huovinen, 1987).

Trimethoprim is a synthetic antibacterial agent and is an antifolate, a structural analog of folic acid (Skold, 2001 & Huovinen, 1987). Through its structural analogy, trimethoprim competitively inhibits the reduction of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR), in microbial and eukaryotic cells (Skold, 2001, Huovenin *et al*, 1995 & Huovinen, 1987). This reduction in bacteria is necessary for the biosynthesis of several amino acids and nucleotides (Skold, 2001, Gibreel and Skold, 2000 & Huovinen, 1987). Trimethoprim exerts its anitbacterial action causing cell death, through the powerful selective inhibition of bacterial DHFRs, an essential enzyme in all living cells (Gibreel & Skold, 2000, Gibreel & Skold, 1998 & Huovinen *et al*, 1995). Trimethoprim is more active against microbial DHFRs than mammalian DHFRs (Huovinen, 1987).

Following extensive use in both human and veterinary medicine, bacterial strains resistant to trimethoprim developed rapidly after its introduction, although there were no naturally occurring enzymes to inactivate the antibiotic (Navia *et al*, 2003 & Huovinen *et al*, 1995). The use of trimethoprim, either alone or in combination, appears to be an important factor in the development and spread of resistance to this antibiotic.

A variety of resistance mechanisms to trimethoprim have been identified, including cell wall impermeability, alternative metabolic pathways, production of a resistant chromosomal dihydrofolate reductase enzyme, over production of a chromosomal enzyme and production of a plasmid mediated trimethoprim resistant dihydrofolate reductase

enzyme (Huovinen, 1987). The most frequently encountered mechanism of resistance is the acquisition by horizontal gene transfer, of a foreign *dfr* gene coding for an additional DHFR enzyme, which is less susceptible to trimethoprim than the chromosomal enzyme (Gibreel & Skold, 2000 & Gibreel & Skold, 1998).

As of 2003, Navia *et al* stated that there are at least twenty *dfr* genes, encoding different DHFR enzymes, and the corresponding enzymes can be distinguished by biochemical and biophysical properties. The transferable *dfr* genes can be divided into two subgroups/families and phylogeny analysis has revealed that the two groups are related (Huovinen *et al*, 1995).

White and Rawlinson, 2001 listed 21 transferable trimethoprim resistance genes (including two genes which have yet to be characterised) and their proteins in Gram-negative bacteria. These genes encode two distinct types of enzyme and are divided into two families. Most genes (19) belong to the family A, and only a small number (three) belong to family B. The type A gene family encodes DHFRs of between 157 and 187 residues. Alignments of 17 of these enzymes reveal high conservation within the N termini of these proteins, with 12 residues strictly conserved across all enzymes (White & Rawlinson, 2001). All proteins of family 1(A) mediate resistance to very high levels of trimethoprim (1g/L) (Huovinen *et al*, 1995).

The second family of DHFR enzymes are completely unrelated to other DHFRs in procaryotes and eucaryotes but are closely related to one another. These enzymes exhibit different characteristics to the other DHFRs, encode polypepetides of a shorter length and

are extremely resistant to trimethoprim (Skold, 2001, White and Rawlinson, 2001 & Huovinen *et al*, 1995).

Horizontal gene transfer has been implicated in the acquisition of trimethoprim resistance (Huovinen *et al*, 1995). It has been proposed that the genes for the drug insensitive DHFR enzymes moved horizontally into pathogenic bacteria from other microorganisms, but their exact origin is unknown. Skold, 2001 speculated that these genes originated from a wide variety of organisms. There is evidence that some of the DHFR enzymes are distantly related to bacterial chromosomal DHFR enzymes and that all these enzymes have a common ancestral DHFR gene (Huovinen, 1987). The wide-spread occurrence of trimethoprim resistance genes among pathogenic bacteria could be explained by the fact that most of them are located in gene cassettes, which via the integron mechanism are very horizontally mobile (Skold, 2001). White & Rawlinson, 2001 reported that in Gram-negative bacteria, 15 of the 19 known transferable DHFRs are encoded by genes that form part of a gene cassette (White *et al*, 2000).

*dfr*1 (*dfrA1*) was the first of the genes encoding transferable DHFR enzymes to be observed and characterised (Skold, 2001), and it encodes a DHFRI enzyme. *dfr*1 is located in a gene cassette which occurs in both class 1 and class 2 integrons and this cassette can be exchanged between the different integrons (Skold, 2001 & Huovinen *et al*, 1995). This gene is also the most prevalent resistance gene among Gram-negative bacteria and this is commonly attributed to the successful spread of its carrier transposon, Tn*7* (Huovinen *et al*, 1995 & Skold, 2001). Among clinically isolated trimethoprim-resistant bacteria, Tn*7* is usually located on the chromosome and less frequently on plasmids (Skold, 2001). Because

of the mobility of the cassette, *dfr*1 is also found in integrons similar to that borne by the transposon Tn*21*, a mercuric resistance transposon known to carry a class 1 integron (Hansson *et al*, 2002 & Huovinen *et al*, 1995).

Another transferable *dfr* gene is *dfr*9, a gene of unknown origin that was originally identified in *E. coli* isolates from swine (Skold, 2001). The gene occurs more frequently in trimethoprim resistant veterinary isolates than human isolates. The frequent veterinary prescription of trimethoprim in swine rearing may have influenced its spread among swine bacteria and a subsequent spread into commensal bacteria in human may have taken place (Skold, 2001). *dfr*9 in the swine *E. coli* isolates was found to be carried on large transferable plasmids, and in many of these plasmids the gene was borne on a disabled transposon, Tn*5393*, which was earlier found on a plasmid in the plant pathogen *Erwinia amylovora* (Skold, 2001).

A number of studies have reported trimethoprim resistance in *Campylobacter* spp. following the results of Karmali *et al*, 1981, *C. jejuni* was regarded as endogenously resistant to trimethoprim. Karmali *et al* reported that all Canadian clinical isolates of *C. jejuni* examined were highly resistant to trimethoprim, with only 3.6% of strains inhibited at a concentration of 256μg/ml and 96.4% of strains inhibited at a concentration of ≥512μg/ml. The susceptibility of strains to trimethoprim was not influenced by the inoculum size.

Winstanley *et al*, 1993 reported that a multiple antibiotic resistant isolate of *C. jejuni* acquired in Jordan was resistant to trimethoprim in addition to nine other antibiotics. Many

(77%) of the human and animal isolates of *Campylobacter* spp. examined by Lucey *et al*, 2000a were resistant to three or more agents with part of the R-type including resistance to trimethoprim. Lucey *et al*, 2000b also reported that all isolatesof Irish *Campylobacter* spp. from a variety of sources were resistant to trimethoprim in accordance with the findings of Karmali *et al*.

Gibreel and Skold, 2000 and Gibreel and Skold, 1998 reported high level trimethoprim resistance (500 to 1000μg/ml) in Swedish clinical isolates of *C. jejuni*. Foreign *dfr* genes coding for resistant variants of DHFR enzymes were found to mediate the high level resistance in the majority of strains examined by Gibreel and Skold, 1998. The most commonly found gene was *dfr1* (DHFR gene of type 1), but another gene, *dfr9* (DHFR gene of type 9), was also detected in about one-third of isolates. In about 10% of the strains, the *dfr1* and *dfr9* genes occurred simultaneously.

Both *dfr1* and *dfr9* were observed to be chromosomally located in the context of remnants of an integron and transposon respectively. The *dfr1* gene was found to be inserted in a cassette-like manner, with repeat sequences similar to those of the Tn*7 dfr1* cassette. The surroundings of the *dfr9* gene were identical to the right hand end of the transposon Tn*5393* (Gibreel and Skold, 1998). The authors proposed that the insertion of the *dfr9* gene into the *C. jejuni* chromosome could have been mediated by this transposon. The G+C content (about 40%) of both *dfr1* and *dfr9* was reported by Gibreel and Skold to be higher than that of the genome of *C. jejuni*, suggesting that the genes originated in another organism.

1.10 INTEGRONS

Integrons are horizontal gene transfer systems containing elements necessary for acquisition, site-specific recombination and expression of foreign DNA (Nemergut *et al*, 2004, Rowe-Magnus *et al*, 2001 & Hall & Collis, 1998). Foreign DNAs captured from the environment and incorporated into the integron by an integrase enzyme are referred to as gene cassettes. Many gene cassettes encode antibiotic resistance and in Gram-negative bacteria, integrons play a major role in the dissemination of antibiotic resistance genes (Barlow *et al*, 2004 & Hansson *et al*, 2002).

Integron platforms are defective for self-transposition, however, integrons harbouring antibiotic resistance cassettes are often found associated with transposons and/or conjugative plasmids which serve as transmission vehicles (Nemergut *et al*, 2004 & Rowe-Magnus & Mazel, 2001). Due to this location, integrons have been found among a large number of phylogenetically diverse Gram-negative and Gram-positive isolates (Rowe-Magnus & Mazel, 2001).

Integrons are divided into two major groups: the resistance integrons and the super-integrons (Fluit & Schmitz, 2004). The chromosomally located super integrons, which are probably not exchanged between different strains or species, harbour hundreds of gene cassettes which encode adaptations that extend beyond antibiotic resistance and pathogenicity (Barlow *et al*, 2004, Fluit & Schmitz, 2004 & Rowe-Magnus *et al*, 1999). Super-integron integrase-like genes have been found in *Gamma*-, *Delta*- and *Beta*-proteobacteria and in the spirochete *Treponema denticola* (Nemergut *et al*, 2004 & Rowe-Magnus *et al*, 2001). Super integrons and their cassettes have been proposed to be the

source of resistance integron cassettes (Fluit & Schmitz, 2004 & Rowe-Magnus *et al*, 2001).

Three classes (1, 2 and 3) of resistant integrons, which contain antibiotic resistance gene cassettes, have been defined on the basis of the divergence among their integrase genes (Barlow *et al*, 2004, Rowe-Magnus & Mazel, 2001 & Hall & Collis, 1998). Homology between the three classes indicates that their evolutionary divergence has extended longer than the 50 years of the antibiotic era (Rowe-Magnus & Mazel, 1999). Each class appears to be able to acquire the same gene cassettes, and more than 70 different antibiotic resistance gene cassettes, covering most antimicrobials presently in use, have been detected in the three classes (Rowe-Magnus & Mazel, 2001, Mazel & Davies, 1999 & Hall & Collis, 1998). The selective pressure of antibiotic therapy regimes has been proposed to be a biased force driving the specific capture of resistance gene cassettes (Rowe-Magnus *et al*, 2001). Some resistance integrons harbouring up to five different resistance cassettes have been described (Rowe-Magnus *et al*, 2001 & Poirel *et al*, 2000).

Class 1 integrons have been reported in many Gram negative genera including *Aeromonas*, *Burkholderia*, *Campylobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella* and *Vibrio*; and in other bacteria such as *Corynebacterium*, *Mycobacterium* and *Enterococcus faecalis*. Class 1 integrons have been detected in isolates from humans, cattle, swine, chickens, fish, pets and zoo animals and environmental samples (Fluit & Schmitz, 2004). Class 1 integrons code for an integrase, IntI1 (337 amino acids in length), and are generally borne on elements derived from Tn*5090*, such as that found in the central part of transposon Tn*21* (Hansson *et al*, 2002).

Class 1 integrons are associated with a variety of resistance gene cassettes, with commonly inserted cassette genes including *aadA*, encoding streptomycin-spectinomycin resistance, and *dfr* genes encoding trimethoprim resistance, such as *dfr1* (Blahna *et al*, 2006 & Fluit & Schmitz, 2004). Class 3 integrons have been described in a number of species, and although the structure is comparable to that of class 2 integrons, the properties of the class 3 integrase, IntI3 are similar to those of the class 1 integrase (Fluit & Schmitz, 2004, Senda *et al*, 1996 & Arakawa *et al*, 1995).

Class 2 integrons have been detected in *Acinetobacter*, *Shigella*, *Salmonella* and Enterobacteriaceae from urinary tract infections (Fluit & Schmitz, 2004 & White *et al*, 2001). Class 2 integrons encode for an integrase, IntI2* which is not functional due to the presence of an internal stop codon (Hansson *et al*, 2002 & Radstrom *et al*, 1994). Class 2 integrons therefore cannot acquire new gene cassettes and may be dependent on the class 1 integrase, which frequently occur in the same isolates as class 2 integrons, for integration and excision of gene cassettes (Blahna *et al*, 2006, Fluit & Schmitz, 2004, Hansson *et al*, 2002 & Bennett, 1999). Class 2 integrons are embedded in the Tn*7* family of transposons and has a similar organisation to that of class 1 (Hansson *et al*, 2002 & Radstrom *et al*, 1994). Class 2 integrons usually carry three resistance gene cassettes - *dfrA1*, *sat* and *aadA1*, encoding resistance to trimethoprim, streptothricin and spectinomycin respectively (Hansson *et al*, 2002 & Sundstrom & Skold, 1990).

Integrons consist of two major parts, the stationary integron platform and the mobile gene cassettes, which are promoterless open reading frames with a recombination site, *attC* / 59-base element (Nemergut *et al*, 2004). Integrons possess two conserved segments separated

by a variable region into which the gene cassettes are inserted (Levesque *et al*, 1995). One component of the integron platform is the 5'conserved segment which contains the integrase (*intI*) gene and a strong common promoter region on the opposite strand. The promoter is directed toward the site of integration and the gene cassettes are expressed from this region as a resistance operon (Nemergut *et al*, 2004 & Levesque *et al*, 1995).

Another important component of the integron platform is the recombination site, *attI*, where gene cassettes are integrated into the integron. In antibiotic resistance associated class 1 integrons, the second conserved region, known as the 3' conserved segment, contains *qacEΔ1* and *sul1* genes conferring resistance to ethidium bromide and quaternary ammonium compounds and sulfonamide respectively; and an open reading frame, *orf5* (Levesque *et al*, 1995, Paulsen *et al*, 1993, Stokes & Hall, 1989 & Sundstrom *et al*, 1988).

Nemergut *et al*, reported that in 2004, there were 32 unique integron integrase genes known and 22 of these genes were found exclusively in the *Gammaproteobacteria.* Integron integrases belong to the tyrosine family of recombinases and reversibly catalyse a site-specific recombination event between *attI* sites and *attC* sites of gene cassettes, and integrate or excise cassettes (Nemergut *et al*, 2004, Fluit & Schmitz, 2004 Holmes *et al*, 2003, Hansson *et al*, 2002 & Recchia & Hall, 1997).

Gene cassettes are not necessarily part of the integron, but they become an integral part when integrated (Fluit & Schmitz, 2004). Cassettes can exist in two forms: as free circular molecules that are unable to replicate, or integrated at the *attI* site. Most cassettes include very little non-coding sequence and do not contain a promoter, and are dependent on

integration in the correct orientation into an integron that supplies an upstream promoter (Recchia & Hall, 1997). Rowe-Magnus *et al*, 2001 stated that it is conceivable that any open reading frame can be structured as a gene cassette. Integrons may be important for gene transfer in response to selective pressures other than the presence of antibiotics (Nemergut *et al*, 2004).

Gene cassettes in resistance integrons consist of a resistance gene and a short imperfect inverted repeat element, the 59-base element / *attC* site. This element is located downstream of the resistance gene and is involved in recombination (Levesque *et al*, 1995 & Hall *et al*, 1991). Each inserted gene has its own version of this element and as of 2001; most of those identified are unique with varying lengths and sequences (Rowe-Magnus *et al*, 2001). 59-base element sites include a core site with the consensus GTTRRRY and for recombination events mediated by the class 1 integrase, the recombination crossover point is between the G and first T of this site (Holmes *et al*, 2003, Grainge & Jayaram, 1999 & Stokes *et al*, 1997).

It is clear from the studies of Gibreel and Skold, 1998 and 2000 that integrons play a role in trimethoprim resistance in *Campylobacter* spp., and these structures have also been found to mediate other forms of antibiotic resistance in campylobacters (O'Halloran *et al*, 2004 & Lee *et al*, 2002). An early study by Lucey *et al*, 2000a detected putative gene cassettes in *Campylobacter* strains of human, chicken and porcine origin and amplified a variety of different sized fragments. Only one incomplete ORF matched database sequences and this was found to match a glycyl-tRNA synthetase from *H. pylori*. The authors also identified

conserved features of class 1 integrons including the core site of the 59-base element, the integrase gene and the *qacEΔ1* and *sul1* genes.

Irish human and poultry isolates of *Campylobacter* analysed by O'Halloran *et al*, 2004 were found to harbour class 1 integrons with a recombined cassette containing a aminoglycoside resistance gene. Lee *et al*, 2002 reported that only a small number of US chicken *C. jejuni* isolates possessing the class 1 integrase gene harboured gene cassettes. These cassettes contained an aminoglycoside (tobramycin and gentamicin) resistance gene, *aacA4*, which had previously been found in both class 1 and class 3 integrons.

Microarray analysis by Poly *et al*, 2004 identified four ORFs with significant homology to integrases of the tyrosine recombinase family in *C. jejuni* strain 43431. Three ORFs were closely related to putative integrases from the epsilon bacterium, *Wolinella succinogenes*. *W. succinogenes* is a nonpathogenic, host-associated bacterium that was originally isolated from the rumen of cattle and is closely related to *H. pylori* and *C. jejuni* (Baar *et al*, 2003). The remaining ORF was closely related to an integrase from the cyannobacterium, *Nostoc punctiforme* (Poly *et al*, 2004).

1.11 AIMS & SIGNIFICANCE

1.11.1 Aims

1. To identify and characterise tetracycline resistant determinants and associated genetic elements present in tetracycline resistant Australian human and chicken isolates of *Campylobacter jejuni* and *Campylobacter coli.*

2. To identify and characterise the trimethoprim resistant determinants present in trimethoprim resistant Australian isolates of *C. jejuni* and *C. coli*.

3. To identify integron like structures and associated elements in Australian isolates of *C. jejuni* and *C. coli*.

1.11.2 Significance

Campylobacter jejuni and *Campylobacter coli* are the leading cause of food-borne diarrhoea in industrialised countries (van Vliet and Ketley, 2001). Severe cases of campylobacteriosis are treated with antibiotics, with erythromycin being the current choice of treatment (Blaser, 1997). Resistance of *Campylobacter* spp. to various antibiotics has been reported, and rates of resistance are increasing due to use both in human populations and in animals raised for food production (Blaser, 1997). As previously stated, antibiotic resistant campylobacter can influence both the extent and duration of Campylobacteriosis. Infection with antibiotic resistant isolates can make clinical management of the disease more difficult (Murphy *et* al, 1996). Antibiotics such as tetracycline are considered no longer effective against *Campylobacter* spp. and this may be the case for a number of other antibiotics in the future.

The emergence of fluoroquinolone resistance in *Campylobacter* isolates, which coincided with the introduction of these antibiotics (such as enrofloxacin) in food production animals (Snelling *et al*, 2005, White *et al*, 2002; Altekruse *et al*, 1998 & Allos, 2001), is a poignant example of the relevance of antibiotic resistance surveillance and research. Following a rapid rise in resistance levels in both poultry and human isolates of *Campylobacter* spp., the

United States withdrew approval for the use of fluoroquinolones in animals, including the use of enrofloxacin for prophylaxis and growth-promotion in poultry (Unicomb, *et al*, 2006 & Iovine & Blaser, 2004). Therefore, with data gained from research, practical strategies can be put into place to combat or prevent problems arising in the human population, animals and associated industries.

Only a small amount of data has been published on antibiotic resistance in Australian *Campylobacter* isolates. Taking into consideration that this pathogen is the leading cause of bacterial gastroenteritis in this country, and of which a proportion of cases are possibly due to infection with an antibiotic resistant strain, the issue of resistance is important indeed. Data on antibiotic resistance prevalence and mechanisms in Australian isolates of this organism could aid medical, health and associated agencies in monitoring, regulation and treatment strategies.

Emergence and dissemination of antibiotic resistance among *Campylobacter* spp., and genes encoding this resistance, has been linked to antibiotic use in veterinary medicine and as prophylactics and growth promoters in animal husbandry (Saenz *et al,* 2000). Antibiotic resistance genes in *Campylobacter* spp. are proposed to have been acquired from Gram positive and other Gram-negative bacteria. Acquisition of foreign antibiotic resistance genes and dissemination in the *Campylobacter* population could have serious effects on human and animal health.

The prevalence and mechanisms mediating tetracycline and trimethoprim resistance in Australian strains of *Campylobacter* have not previously been reported. The incidence of

*tet*O, the characterised *Campylobacter* tetracycline resistance determinant identified in various countries throughout the world, and/or the presence of other tetracycline resistance determinants in Australia, has yet to be determined at the time this project was undertaken.

Although most campylobacters are intrinsically resistant to trimethoprim, the only reports on resistance mechanisms concern *C. jejuni* and *C. coli* isolates from Sweden. Mechanisms and genes harboured by isolates from other countries have yet to be determined. The presence of *dfr* genes encoding trimethoprim resistance and other mechanisms involved in Australian strains have yet to be reported. Also, nothing is known of the prevalence, distribution and contribution to antibiotic resistance in Australian *Campylobacter* spp. of integrons, genetic elements implicated in the acquisition and phenotypic expression of antibiotic resistance genes.

Although antibiotics such as tetracycline and trimethoprim are no longer used in the clinical management of Campylobacteriosis, determination of the mechanisms by which *Campylobacter* spp. are resistant to various antibiotics as well as how they acquire these mechanisms will aid in understanding an extensive problem throughout the world. This is of great importance since *Campylobacter* spp. are naturally conjugative and transformable and the mechanisms of antibiotic resistance in these organisms are poorly understood. Knowledge of these mechanisms could lead to the development of compounds to counteract the effect of antibiotics and could be used to predict and control the rate of emergence of antibiotic resistance. And, through the accumulation of knowledge, we can achieve a better understanding of the biology and ecology of this unique, yet important, pathogen.

CHAPTER 2

MATERIALS & METHODS

2. MATERIALS & METHODS

2.1 GENERAL PROCEDURES

Media was sterilised at 121^0C for 15-20 minutes, except where specified, and allowed to cool to 50^0C – 55^0C prior to addition of horse blood, antibiotics or supplements. Media was then mixed and manually dispensed into 90mm gamma irradiated petri dishes (Sarstedt) in a laminar flow cabinet and stored at 4^0C. Solutions were sterilised at 121^0C for 15-20 minutes, cooled and stored at room temperature or 4^0C. Preparation of media, use of sterile solutions and bacterial work was carried on in a laminar flow hood.

Media inoculated with *Campylobacter* strains was incubated in Anaerobic jars (BQ BDL or Oxoid) at 37^0C in a Sanyo incubator or warm room; or at 42^0C in a Contherm Scientific incubator. *E. coli* inoculated solid media was incubated in either at 37^0C in a Sanyo incubator or warm room.

Agarose gels containing electrophoresed DNA were viewed using a UV-transilluminator (UVP) at 302nm and the Grab-It computer program.

2.1.1 General Chemicals

- Streptomycin, Chloramphenicol, Agarose and Boric Acid were obtained from Progen Industries Limited (Archerfield, Qld)
- Tetracycline, Erythromycin, Trimethoprim, Ampicillin, Rifampicin, Isoamyl Alcohol, SDS, Polyvinylpyrrolidone, Bromophenol Blue, Sodium Phosphate di-basic, Ficoll and Bovine serum albumin were obtained from Sigma

- Potassium Chloride, Ethanol (100% Analytical Grade), Chloroform and Sodium Chloride were obtained from BDH AnalaR
- Sodium Chloride, Sodium Hydroxide and Potassium Acetate were obtained from ChemSupply Pty Ltd
- Isoprophanol, Hydrochloric Acid, Potassium di-hydrogen orthophosphate, Glucose and Sodium Acetate were obtained from Univar
- Sodium Chloride and SSC were obtained from ICN Biomedicals
- Phenol and Tris Base/Tris were obtained from MP Biomedicals
- Urea and Sucrose were obtained from Spectrum
- EDTA and Ethidium Brommide were obtained from Ameresco Industries Limited
- Glycerol was obtained from USB
- Glacial Acetic Acid was obtained from Lab Scan Analytical Sciences
- Kanamycin was obtained from Roche
- Sephadex G50 - Fine was obtained from Pharmacia Biotech

2.1.2 Media

- Horse Blood Agar (HBA)

  HBA was prepared from dehydrated media (Columbia Agar Base (Oxoid)) suspended in distilled water and following sterilisation, supplemented with 5% defibrinated horse blood (bioMérieux Australia & Institute of Medical and Veterinary Science, South Australia). 2%HBA was prepared as above with the addition of 1% w/v bacteriological agar (Oxoid & Spectrum Quality Products Inc.) prior to sterilisation.

- Skirrow Media

  Skirrow medium was prepared as for HBA (or 2%HBA), but with the addition of Skirrow Supplement (*Campylobacter* Selective Supplement) (Oxoid) to cooled sterile media prior to dispensing into Petri dishes.

- Mueller-Hinton Agar (MHA)

  MHA was prepared from dehydrated media (Oxoid) suspended in distilled water and supplemented with 5% defibrinated horse blood (bioMérieux Australia & Institute of Medical and Veterinary Science, South Australia) prior to dispensing. 2%MHA was prepared as above with the addition of 1% w/v bacteriological agar (Oxoid & Spectrum Quality Products Inc.).

- Tryptone-Soya Agar (TSA)

  TSA was prepared from dehydrated media (Oxoid) with the addition of 0.5% yeast extract (Oxoid) prior to sterilisation, and 5% defibrinated horse blood (bioMérieux Australia & Institute of Medical and Veterinary Science, South Australia) before dispensing into Petri dishes.

- Luria-Bertani Agar (LBA)

  LBA was prepared from dehydrated Luria-Bertani Base (Millers) media (Oxoid) with the addition of 1.5% bacteriological agar (Oxoid & Spectrum Quality Products Inc.) prior to sterilisation.

- Brucella Broth

  Brucella Broth was prepared according to the manufacturer's instructions. Dehydrated media (Difco) was suspended in distilled water, mixed thoroughly and boiled for 1 minute. The broth was then sterilized, cooled and stored at 4^0C.

- Heart Infusion Broth

  Heart Infusion Broth was prepared according to the manufacturer's instructions. Dehydrated media (Difco) was suspended in distilled water, sterilised, cooled and stored at 4^0C.

- Storage Medium

  Storage medium was prepared by suspending 10% w/v Skim Milk powder (Dutch Jug) in distilled water with 1% w/v tryptone and 10mM Tris-Cl pH 7.4 – 7.5. Following thorough mixing, the medium was sterilised at 109^0C for 30 minutes, allowed to cool and stored at 4^0C.

2.1.3 Enzymes

- Pancreatic RNase A, Pronase and Lysozyme were obtained from Roche
- DNase 1U/µl was obtained from MBI Fermentas
- Taq Polymerase was obtained from Eppendorf, Invitrogen, New England Biolabs and Biotech
- Ampli-Taq Gold was obtained from Perkin-Elmer
- Pfu Turbo polymerase was obtained from Stratagene

2.1.4 Restriction Endonucleases

- *Cla*I was obtained from Promega & New England BioLabs
- *Hind*III and *Pst*I were obtained from New England Biolabs
- *Dde*I was obtained from Promega & Invitrogen
- *Alu*I and *Hinf*I were obtained from Promega

2.1.5 Molecular Weight Markers

- 100bp ladder and 1kb ladder were obtained from New England Biolabs
- λ*Hind*III marker consisted of 20μg of λ DNA, obtained from MBI Fermentas, digested with 30U of *Hind*III at 37^0C for 5 hours. The reaction was stopped by the addition of stop solution or storage at -20^0C.

2.1.6 Stock Solutions

- 0.5M EDTA pH 8.0
- 1M HCl
- 5M NaCl
- 1M Tris-Cl pH 7.4 - 7.5
- 10% SDS (w/v) in sterile distilled water
- 20 X SSC
- 10N NaOH
- 10 X TE Buffer pH 7.4 – 100mM Tris-Cl pH 7.4 – 7.5, 10mM EDTA pH 8.0
- Ethidium Bromide Solution – 10mg/ml in distilled water
- 50 X TAE Buffer
- 5 X TBE Buffer

- 50 X Denhardt's solution – 1% (w/v) ficoll 400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (fraction V), sterilized by filtration, stored at -20^{0}C.
- 10 X Loading Buffer – 50% (v/v) glycerol, 0.25% bromophenol blue, 0.25 Xylene cyanole FF in 1 X TAE buffer, diluted 1 in 4 and stored at room temperature.
- Stop Solution pH 7.0 – 7M Urea, 50% (w/v) sucrose, 50mM EDTA, 0.025% bromophenol blue
- Pronase – Powdered enzyme at 10mg/ml or 20mg/ml was added to sterile distilled water and stored at -20^{0}C. Working solutions of 1mg/ml of enzyme were prepared in 0.1M Tris-Cl pH 7.5, 0.5% SDS in sterile distilled water. Solutions were heated to 35 – 40^{0}C, cooled and stored at -20^{0}C
- RNase – Pancreatic RNase A was dissolved at a concentration of 10mg/ml in 0.01M sodium acetate (pH 5.2) in distilled water. The solution was heated to 100^{0}C for 15 minutes and cooled slowly to room temperature. The pH was adjusted by adding 0.1 volume of 1M Tris-Cl pH 7.4–7.5. Solutions were dispensed into 100µl aliquots and stored at -20^{0}C. Working solutions of 1mg/ml were diluted from stock solutions in sterile distilled water.

2.1.7 Antibiotic Stock Solutions

- Erythromycin – 2mg/ml in 95% analytical ethanol, stored at 4^{0}C
- Kanamycin – 10mg/ml in distilled water, sterilised by filtration and stored at -20^{0}C.
- Streptomycin – 100mg/ml in water, sterilised by filtration, stored at -20^{0}C
- Chloramphenicol – 10mg/ml in 100% analytical ethanol, stored at -20^{0}C
- Tetracycline – 5mg/ml in 100% analytical ethanol, vortexed to dissolve, stored away from light at -20^{0}C

- Trimethoprim – 10mg/ml dissolved in ½ volume 0.1M HCl in distilled water, then made up to appropriate volume with distilled water according to the method of EUCAST, 2000 or 10mg/ml in Dimethyl-Sulfoxide (DMSO), stored at 4^0C or 10mg/ml suspended in a solution of 0.05M HCl in distilled water (ensuring HCl is less than 10% of total volume), dissolved by shaking at 37^0C, sterilized by filtration and stored at -20^0C according to National Committee for Clinical Laboratory Standards, 1997 recommendations
- Rifampicin – 10mg/ml in distilled water, stored at -20^0C or 50mg/ml in methanol, stored at -20^0C or 10mg/ml in methanol, stored away from light at -20^0C

2.1.8 General Solutions

- Phosphate Buffered Saline (PBS) – 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM $KHPO_4$ adjusted to pH 7.4 with HCl and sterilised by autoclaving at 121^0C for 15 minutes.
- Alkaline Lysis Solution I / Glucose Buffer – 50mM Glucose, 25mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0 in distilled water, sterilized at 121^0C for 15 minutes and stored at 4^0C.
- Alkaline Lysis Solution II / 0.2N NaOH, 1% SDS
- Alkaline Lysis Solution III / Potassium acetate pH 4.8 – 5M Potassium acetate, glacial acetic acid in distilled water (3M with respect to potassium and 5M to acetate)
- Phenol-Chloroform-Isoamyl alcohol – 25:24:1
- Chloroform-Isoamyl alcohol – 24:1
- Depurination Solution – 0.125M - 0.2M HCl in distilled water
- Denaturation Solution – 1.5M NaCl, 0.5N NaOH in distilled water

- Neutralisation Solution – 1.5M NaCl, 0.5M Tris-Cl ph 7.4 – 7.5 in distilled water
- Hybridisation Solution – 6 X SSC, 0.5% SDS, 5 X Denhardts solution, 100μg/ml Herring sperm DNA in distilled water and stored at -20^{0}C.

2.2 BACTERIAL METHODS

2.2.1 Bacterial Strains

For analysis of tetracycline resistance in Australian strains of *C. jejuni* and *C. coli*, species and source data for each strain under study is displayed in **Table 1**. Strains under study were previously identified as tetracycline resistant by a disk diffusion test. The majority of strains were obtained from humans with gastroenteritis; however, isolates from healthy chickens were also included. Previously characterised tetracycline resistant and *tet*O carrying strains JC8 and JC6 isolated in Japan were used as positive controls. Tetracycline sensitive strain 331, isolated from a Victorian chicken flock, was used as a negative control. Six *Campylobacter* isolates were chosen for use as recipient strains in conjugation studies. Two strains employed as recipients using one method (Method A), and, four strains were employed as recipients using the second method (Method B). Strains are shown in **Table 2**.

For analysis of trimethoprim resistance in *Campylobacter* spp., a selection of *C. jejuni* and *C. coli* isolated in Australia and overseas were chosen for study and are displayed in **Table 3**, **Table 4**, **Table 5** and **Table 6**. 82 Australian strains of *C. jejuni* and *C. coli* isolated from humans and healthy chickens were selected. The 31 strains of *C. jejuni* and *C. coli* from Japan, USA, UK and Canada selected for study were mainly isolated from humans and chickens. A small selection of non-*C. jejuni/C. coli* isolates were also chosen for analysis and these strains are displayed in **Table 5**. These strains, including isolates of *C. lari* and *C.*

fetus, were isolated in a number of countries including Australia. An *E. coli* strain, ColE1:Tn*7*, obtained from Ruth Hall of the CSIRO was employed as a positive control in trimethoprim resistance studies.

2.2.2 Storage of strains

All bacterial strains were stored at -80^{0}C in storage medium.

2.2.3 Revival of strains

Strains for which only a few colonies were recovered from frozen stocks during culturing were 'revived' through culture methods. 1-3ml of Heart-Infusion broth in bijou bottles were inoculated with either a loopful of frozen stock or a volume (e.g. 60µl - 150µl) of thawed frozen stock and incubated in a microaerophilic atmosphere for 24 hours at 37^{0}C or 42^{0}C. 1-1.5ml of culture was transferred to a 1.5ml microcentrifuge tube and centrifuged for 2 minutes at 3000xg. The supernatant was removed and the cell pellet was resuspended in 200-500µl of PBS. The cell suspension was then plated on Skirrow medium and incubated in a microaerophilic atmosphere at 37^{0}C or 42^{0}C for 48 hours. Strains were subsequently cultured on HBA plates and harvested into storage medium for storage.

2.2.4 Growth Conditions

Campylobacter strains were cultured in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) at either 37^{0}C or 42^{0}C for 48 hours, except where specified. *Campylobacter* strains were routinely cultured on HBA plates. Skirrow medium was also occasionally employed in the culturing of strains. Prior to antibiotic susceptibility testing, strains were cultured on HBA at 37^{0}C or occasionally at 42^{0}C. *E. coli* strain ColE1:Tn*7* was either

Table 1. *Campylobacter* strains, source and speciation data for tetracycline resistance analysis

Strain	Species	Source
JC8 (Japan)	*C. jejuni*	Human ◇#
JC6 (Japan)	*C. jejuni*	Human ◇#
331	*C. jejuni*	Chicken #
FF7	*C. jejuni*	Human * #
FF12	*C. jejuni*	Human ❖ #
293	*C. coli*	Human ⌘ #
GC012	*C. jejuni*	Human
QH104	*C. jejuni*	Human
QH120	*C. jejuni*	Human
QH127	*C. jejuni*	Human
QH222	*C. coli*	Human
QH230	*C. jejuni*	Human
QH304	*C. jejuni*	Human
QH428	*C. jejuni*	Human
QH509	*C. jejuni*	Human
QH514	*C. coli*	Human
GC1874	*C. jejuni*	Human
GC2732	*C. jejuni*	Human
GC3346	*C. jejuni*	Human
GC5252	*C. jejuni*	Human
GC6108	*C. jejuni*	Human
GC10019	*C. jejuni*	Human
GC15913	*C. jejuni*	Human
GC16723	*C. jejuni*	Human
GC18943	*C. jejuni*	Human
GC21098	*C. jejuni*	Human
GC21172	*C. jejuni*	Human
GC22479	*C. jejuni*	Human
GC23330	*C. jejuni*	Human
GC23503	*C. jejuni*	Human
GC23730	*C. jejuni*	Human
GC27179	*C. jejuni*	Human
GC28719	*C. jejuni*	Human
GC29395	*C. jejuni*	Human
GC31677	*C. jejuni*	Human
GC32304	*C. jejuni*	Human
GC32626	*C. jejuni*	Human
GC35618	*C. jejuni*	Human
GC36409	*C. jejuni*	Human
GC42121	*C. jejuni*	Human
GC470308	*C. coli*	Human
C1	*C. coli*	Chicken #
115	*C. jejuni*	Chicken #
231	*C. jejuni*	Chicken #
801	*C. jejuni*	Chicken #
802	*C. jejuni*	Chicken #
824	*C. jejuni*	Chicken #
835	*C. jejuni*	Chicken #
3017	*C. jejuni*	Chicken #

Table 2. Name, species and source data for recipient strains in conjugation experiments

Method A		
Strain	**Species**	**Source**
81116	*C. jejuni*	Human♣
CSIRO40	*C. coli*	Human* #
Method B		
Strain	**Species**	**Source**
RM10	*C. jejuni*	Human #
887	*C. jejuni*	Human❖ #
9126	*C. jejuni*	Human #
NCTC 11366	*C. coli*	Pig +❖ #

Strain 81116 was obtained from Veterinary Laboratories Agency (Weybridge), UK

The majority of human isolates used for tetracycline resistance analysis were obtained from the Pathology Department, Gold Coast Hospital, Southport, Queensland, 4212. Remaining strains of *C. jejuni* and *C. coli* were from the Royal Melbourne Institute of Technology culture collection (#)

Table 3. Strain, species and source data for Australian isolates of *Campylobacter* selected for analysis for trimethoprim resistance.

Strain	Species	Source	Strain	Species	Source
004	*C. jejuni*	Chicken #	351	*C. jejuni*	Human❖ #
007	*C. jejuni*	Chicken❖ #	354	*C. jejuni*	Human❖ #
8	*C. jejuni*	Human❖ #	375	*C. jejuni*	Human❖ #
008	*C. jejuni*	Chicken #	388	*C. coli*	Human❖ #
011	*C. lari*	Chicken #	405	*C. jejuni*	Chicken #
013	*C. jejuni*	Chicken #	410	*C. jejuni*	Human❖ #
017	*C. jejuni*	Chicken #	413	*C. jejuni*	Chicken #
018	*C. coli*	Chicken #	415	*C. jejuni*	Chicken #
019	*C. jejuni*	Chicken #	430	*C. jejuni*	Human❖ #
32	*C. jejuni*	Human❖ #	435	*C. jejuni*	Human❖ #
93	*C. jejuni*	Human #	439	*C. jejuni*	Human❖ #
108	*C. jejuni*	Human❖ #	440	*C. jejuni*	Human❖ #
131	*C. jejuni*	Chicken #	458	*C. jejuni*	Human❖ #
133	*C. jejuni*	Chicken #	506	*C. jejuni*	Chicken #
134	*C. jejuni*	Chicken #	515	*C. jejuni*	Human #
141	*C. jejuni/coli* *	Chicken #	520	*C. jejuni*	Human⌘ #
142	*C. jejuni/coli* *	Chicken #	605	*C. jejuni*	Chicken #
173	*C. jejuni/coli* *	Human #	657	*C. coli*	Human❖ #
231	*C. jejuni*	Chicken #	691	*C. coli*	Human❖ #
235	*C. jejuni*	Chicken #	705	*C. jejuni*	Human❖ #
239	*C. jejuni*	Human❖ #	801	*C. jejuni*	Chicken #
293	*C. coli*	Human⌘ #	886	*C. jejuni*	Human❖ #
303	*C. jejuni*	Chicken #	887	*C. jejuni*	Human❖ #
336	*C. jejuni*	Chicken #	957	*C. jejuni*	Human❖ #

* - Strains not speciated further

Table 4. Strain, species and source data for Australian isolates of *Campylobacter* for analysed for trimethoprim resistance.

Strain	Species	Source	Strain	Species	Source
961	*C. coli*	Chicken [#]	CSIRO40	*C. coli*	Human [*#]
4547	*C. jejuni*	Human [#]	FF1	*C. jejuni*	Human* [#]
3015	*C. jejuni*	Chicken [#]	FF3	*C. jejuni*	Human* [#]
4002	*C. jejuni*	Chicken [#]	FF5	*C. jejuni*	Human❖ [*#]
5001	*C. jejuni*	Chicken [#]	FF18	*C. jejuni*	Human❖ [#]
7005	*C. jejuni*	Chicken [#]	FF34	*C. jejuni*	Human [#]
7006	*C. coli*	Chicken [#]	FF45	*C. jejuni*	Human❖ [#]
8004	*C. coli*	Chicken [#]	RM2	*C. jejuni*	Human [#]
9014	*C. jejuni*	Chicken [#]	RM3	*C. coli*	Human [#]
9126	*C. jejuni*	Human [#]	RM6	*C. jejuni*	Human [#]
0519/010	*C. coli*	Human [#]	RM7	*C. jejuni*	Human [#]
0520/072	*C. jejuni*	Human [#]	RM10	*C. jejuni*	Human [#]
C50	*C. jejuni*	Chicken▼	RM16	*C. jejuni*	Human [#]
C163	*C. jejuni*	Chicken▼	RM17	*C. jejuni*	Human [#]
C170	*C. jejuni*	Chicken▼	RM19	*C. jejuni*	Human [#]
C491	*C. jejuni*	Chicken▼	RM20	*C. jejuni*	Human [#]
C560	*C. jejuni*	Chicken▼	RM103	*C. jejuni*	Human [#]

Table 5. Strain, speciation and source data of *Campylobacter* isolates from overseas selected for trimethoprim resistance analysis.

Strain	Species	Source	Country
108	*C. jejuni*	Chicken	Netherlands [#]
JC1	*C. jejuni*	Human	Japan◇ [#]
JC2	*C. jejuni*	Human	Japan◇ [#]
JC3	*C. jejuni*	Human	Japan◇ [#]
JC5	*C. jejuni*	Human	Japan◇ [#]
JC6	*C. jejuni*	Human	Japan◇ [#]
JC8	*C. jejuni*	Human	Japan◇ [#]

Table 6. Strain, speciation and source data of *Campylobacter* isolates from overseas selected for trimethoprim resistance analysis.

Strain	Species	Source	Country
JC10	*C. jejuni*	Chicken	Japan◇#
JC11	*C. jejuni*	Chicken	Japan #
JC13	*C. jejuni*	Cat	Japan #
JC14	*C. coli*	Dog	Japan #
5565	*C. coli*	Human	Japan ∩#
D123	*C. jejuni*	Human	Canada ⊗#
0-10	*C. jejuni*	Chicken #	USA #
3-9	*C. jejuni*	Chicken	USA #
B5Ft8	*C. jejuni*	Chicken	USA #
CE01	*C. jejuni*	Chicken	USA #
CE04	*C. coli*	Chicken	USA #
CE05	*C. jejuni*	Chicken	USA #
CE06	*C. jejuni*	Chicken	USA #
CE07	*C. jejuni*	Chicken	USA #
CE7-8	*C. jejuni*	Chicken	USA #
CE09	*C. coli*	Chicken	USA #
D117	*C. jejuni*	Human	USA ⊗#
PRC13	*C. jejuni*	Chicken	USA #
PRC67	*C. jejuni*	Chicken	USA #
TC19	*C. jejuni*	Chicken	USA #
RM1221	*C. jejuni*	Chicken	USA♥
NCTC 11168-GS•	*C. jejuni*	Human	UK♥
NCTC 11351	*C. jejuni*	Bovine	USA+#
NCTC 11366	*C. coli*	Pig	UK+❖#
81116	*C. jejuni*	Human	UK♣

• Designated by Gaynor *et al*, 2004

Table 7. Strain, species, source and country data for non-*C.jejuni*/*C. coli* isolates analysed for trimethoprim resistance analysis

Strain	Species	Source	Country
011	*C. lari*	Chicken	Australia⌘ #
85-239	*C. lari*	Human	USA ∪ #
85-241	*C. lari*	Human	USA ∪ #
85-244	*C. lari*	Human	USA ∪ #
ATCC 35223	*C. lari*	Human	UK+ #
CSIRO11	*C. fetus* subsp. *fetus*	Human	Australia * #
85-256	*C. fetus* subsp. *fetus*	Human	USA∪ #
D2189	*C. hyointestinalis*	Human	USA⊗ #
ATCC 33562	*C. sputorum* bv. *sputorum*	Bovine	Belgium + #

Strains from the current collection (including those obtained from the Royal Melbourne Institute of Technology culture collection (#)) were obtained from the following -

* P. Coloe Strain Collection, Department of Applied Biology, Royal Melbourne Institute of Technology, Melbourne, Victoria, 3000

❖ J. W. Tee Strain Collection, Department of Clinical Pathology, Fairfield Infectious Diseases Hospital, Fairfield, Victoria, 3078

⌘ T. W. Steele, Institute of Medical and Veterinary Science, Adelaide, South Australia, 5000

◇ K. Muramatsu, Nagano Research Institute for Health and Pollution, 1978 Komamure, Amori, Nagano, Japan

\+ National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom

□ K. Pinkard Strain Collection, Department of Microbiology, Monash Medical School, Prince Henry's Hospital, Melbourne, Victoria

▼ Department of Primary Industries, Queensland Government, Brisbane, Queensland

⊗ C. M. Patton, Hospital Infectious Disease Program, Centres for Disease Control, Atlanta, Georgia, USA

◠ T. Itoh, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan

◡ M. J. Blaser, Infectious Disease Section, Veterans Administration, Denver, Colorado, USA

♣ Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey, United Kingdom

♥ The Institute for Genomic Research, Rockville, Maryland, USA

cultured on HBA or LBA media at 37^0C in an aerobic or microaerophilic atmosphere (when included as a control strain in trimethoprim susceptibility testing) for 24 – 48 hours.

Prior to plasmid DNA extractions, *Campylobacter* strains were cultured on HBA, TSA or media containing antibiotics at 37^0C. Strains from the tetracycline resistance study were cultured on TSA with tetracycline (1μg/ml) to maintain resistance or on HBA at 37^oC. Strains from the trimethoprim resistance study were cultured on HBA, TSA or occasionally on 2%HBA with trimethoprim (20μg/ml) to maintain resistance.

Transconjugants obtained from conjugations using Method A were cultured on HBA with tetracycline (1μg/ml), HBA with a combination of tetracycline (16μg/ml) and rifampicin (50μg/ml) or TSA, at 37^0C. Transconjugants obtained from conjugations using Method B

were cultured on HBA with combinations of tetracycline (16μg/ml) and selecting antibiotics - erythromycin (20μg/ml), chloramphenicol (5μg/ml) or streptomycin (10μg/ml) at 37^0C or 42^0C.

Campylobacter strains, prior to genomic DNA extractions, were routinely cultured on HBA and occasionally on TSA containing tetracycline (1μg/ml) at 37^0C for 48 hours. Transconjugants, prior to genomic DNA extractions, were cultured on HBA containing tetracycline (16μg/ml) and erythromycin (20μg/ml) at 37^0C or 42^0C for 48 hours.

Strains used as donors or recipients in conjugation studies were grown on HBA or media containing antibiotics. Donor strains used in Method A conjugation experiments were cultured on HBA, or HBA containing tetracycline (1μg/ml) to ensure tetracycline resistance, at 42^0C. Recipient strains in Method A conjugations were cultured on HBA, or, HBA or MHA containing rifampicin (50μg/ml or 100μg/ml) for rifampicin resistant strains or variants of strains, at 42^0C. Both donor and recipient strains used in Method B conjugation experiments were cultured on HBA at 42^0C. Transconjugants were cultured on HBA containing the selecting antibiotics used in matings and occasionally on 1-2%HBA with tetracycline (1μg/ml or 16μg/ml).

2.3 ANTIBIOTIC SUSCEPTIBILITY DETERMINATION

Minimum Inhibitory Concentrations (MICs) and susceptibility of strains to various antibiotics were determined using the agar dilution method based on methods recommended by the NCCLS, 1997.

2.3.1 Tetracycline MIC determination

Tetracycline MICs for strains under study were determined using agar dilution complying with methods recommended by NCCLS. MHA plates with tetracycline concentrations of 8, 16, 32, 64, 128 and 256μg/ml were inoculated with 1μl of a spectophotometrically determined 1-2 x 10^7 CFU/ml suspension in brucella broth, and incubated at 37^{o}C under microaerophilic conditions for 40–48 hours. Control strains consisted of two *C. jejuni* strains known to be tetracycline resistant and a *C. jejuni* strain considered tetracycline sensitive (MIC 8μg/ml). Control 2% MHA plates were also inoculated with bacterial suspensions. MICs were defined as the lowest concentration of antimicrobial agent producing no visible growth in accordance with Tee *et al*, 1995. No internationally accepted criteria for breakpoints are available for *Campylobacter* spp. (Aarestrup, 1997). Therefore we used 8μg/ml as a breakpoint concentration, where isolates with a tetracycline MIC greater this were considered resistant.

2.3.2 Rifampicin, Kanamycin, Erythromycin, Chloramphenicol & Streptomycin Susceptibility

Susceptibility of potential recipient and donor strains in conjugation studies, to a variety of potential selective antibiotics, was determined using modified agar dilution methods. Potential recipient strains were also tested for susceptibility to tetracycline at concentrations of 1μg/ml, 16μg/ml or 8-256μg/ml by agar dilution following the methods described below.

Susceptibility to rifampicin, kanamycin and erythromycin was determined by inoculating MHA or 1-2%HBA plates supplemented with various concentrations of antibiotics, and

control 2%HBA plates, with 1μl of a 10^6 – 1-2 x 10^7 cell suspension in PBS or brucella broth. Inocula were taken from either a 1:10 or 1:100 dilution of the original cell suspension. Plates were incubated at 37^0C for 48 hours. Concentrations tested included – 50μg/ml and 100μg/ml of rifampicin, 5, 10, 20 and 50μg/ml of kanamycin, and, 5, 10 or 15μg/ml of erythromycin. Due to the exploratory nature of the testing, neither positive nor negative control strains could be used; however, we did examine strains that had previously been found to be resistant to some of these antibiotics. MICs were determined as above and we utilised the lowest concentration to which strains were tested as a crude breakpoint.

Susceptibility to chloramphenicol, streptomycin and erythromycin, of a different selection of potential recipient and donor strains, was determined by inoculating 2%HBA plates containing varying concentrations of antibiotics, and control 2%HBA plates, with 1μl of a 1- 4 x 10^7 cell suspension in brucella broth. Inocula were taken from 1:10, 1:20 or 1:100 dilutions of the original suspension. Plates were incubated as above. Concentrations of antibiotics were 10μg/ml of streptomycin and chloramphenicol, and 10, 20, 50 and 100μg/ml of erythromycin. Again, due to the nature of the work we could not employ control strains. The lowest concentrations of antibiotics tested were also employed as crude breakpoints.

2.3.3 Trimethoprim MIC determination

In initial investigations of trimethoprim resistance, trimethoprim MICs were determined using agar dilution according to the NCCLS method, with some modifications. HBA or 2%HBA plates with trimethoprim concentrations of 10, 20, 50 and 100μg/ml were inoculated with 1μl of a spectophotometrically determined, approximately 10^7 CFU/ml cell

suspension in brucella broth. Inocula were taken from either the original cell suspension or a 1:20 or 1:100 dilution. Antibiotic containing plates, along with 2%HBA control plates that were also inoculated, were incubated at 37^0C for 48 hours. *E. coli* strain ColE1:Tn7 which was known to be trimethoprim resistant was employed as a positive control strain. No negative control strain was employed, as, due to the exploratory nature of the work, a trimethoprim sensitive Campylobacter strain had yet to be identified. MICs were determined as for tetracycline MICs.

Subsequent investigations of trimethoprim resistance also used agar dilution, but complied with NCCLS recommended methods, with the exception of the use of HBA. Also, different concentrations of trimethoprim to those used initially were employed. 2%HBA plates with trimethoprim concentrations of 25, 50, 100, 500 and 1000μg/ml, and control 2% HBA plates, were inoculated with 1μl of a spectophotometrically determined 1-2 x 10^7 CFU/ml suspension in brucella broth, and incubated at 37^oC for 48 hours. *E. coli* strain ColE1:Tn7 was employed as a control strain. MICs were determined as for above.

2.4 CONJUGATION OF TETRACYCLINE RESISTANT PLASMIDS

Two methods, Method A and Method B, were employed to determine the conjugative ability of selected tetracycline resistant strains. Recipient strains are shown in **Table 2,** and resistance phenotypes and plasmid carriage of these strains are described in **Table 8**.

2.4.1 Method A

One *C. jejuni* and one *C. coli* strain were chosen as recipient strains in intraspecies and interspecies transfer studies. Resistance determinants and localisation to plasmid or

chromosome was unknown for recipient strains. Four tetracycline resistant, plasmid harbouring *C. jejuni* donor strains, and tetracycline sensitive control strain 331 (which did not harbour detectable plasmid DNA), were mated (one strain twice) with plasmid harbouring *C. coli* recipient strain, CSIRO40. One plasmid harbouring *C. jejuni* donor strain was also mated with a rifampicin resistant variant of the *C. jejuni* strain, 81116.

Table 8: Name, species, antibiotic resistance and plasmid carriage data for recipient strains

Method A			
Strain	**Species**	**Antibiotic resistance and MIC**	**Plasmid carriage**
CSIRO40	*C. coli*	Kanamycin resistant – MIC > 10μg/ml Rifampicin resistant – MIC 100μg/ml	Harbouring multiple plasmids
81116	*C. jejuni*	Rifampicin resistant – MIC > 100μg/ml[↓]	Harbouring no detectable plasmids
Method B			
Strain	**Species**	**Antibiotic resistance and MIC**	**Plasmid carriage**
RM10	*C. jejuni*	Streptomycin resistant - MIC >10μg/ml,	Harbouring no detectable plasmids
887	*C. jejuni*	Erythromycin resistant - MIC >100μg/ml	Harbouring a plasmid
9126	*C. jejuni*	Streptomycin resistant - MIC >10μg/ml	Harbouring a plasmid
NCTC 11366	*C. coli*	Chloramphenicol resistant - MIC >10μg/ml	Harbouring multiple plasmids

[↓] Reisistance and MIC refer to those for rifampicin resistant variants of recipient strains

To maintain resistance, recipient strain CSIRO40 was routinely cultured on HBA containing rifampicin (50μg/ml). CSIRO40 cultured on media containing rifampicin was also harvested and stored for future use. If CSIRO40 was observed to not grow fastidiously

on rifampicin (50μg/ml) containing media, the strain was lawn cultured on antibiotic containing media and harvested as described above.

A rifampicin resistant variant of strain 81116 was generated for use as a recipient strain. Three volumes (300μl, 500μl and 700μl) of a 10^8 cell suspension of 81116 were spread on MHA plates containing rifampicin (100μg/ml) and incubated at 37^0C for 48 hours. Following growth, cells were harvested into storage medium and stored at -80^0C. Rifampicin resistant variants of 81116 were then routinely cultured on MHA or HBA plates containing rifampicin (50μg/ml or 100μg/ml) to maintain resistance. Resistant variants, if observed to be not growing fastidiously on rifampicin containing media, were lawn cultured on plates containing rifampicin (50μg/ml and 100μg/ml) and harvested as above.

Conjugations were performed according to the plate-mating method of Taylor *et al*, 1981 with the following modifications. Strains were suspended in 1ml PBS or brucella broth to 10^7 – 10^8 cells per ml. 150μl - 200μl of donor and recipient strains (cell suspensions or dilutions) were mixed in a 1.5ml microcentrifuge tube (or on the surface of an HBA plate). Mixed cell suspensions were then spread on HBA plates and incubated for between 2 and 6 hours at 42^0C. Cells were washed off plates with PBS or brucella broth, diluted and spread onto transconjugant selecting media containing selective antibiotics. 100μl of harvested cells and or a 1:10, 1:100 and 1:1000 dilution (or additional dilutions of 1:10000 and 1:1000000 in one mating group) were spread onto antibiotic containing plates and for some mating groups also on 2%HBA plates as controls. Donors and recipients were mated for between three and five hours, matings were then spread onto antibiotic containing media and were incubated for 48-72 hours at 42^0C.

Transconjugants were selected on HBA, MHA or 2%HBA plates containing 16μg/ml or occasionally 8μg/ml of tetracycline and selecting antibiotics. These included rifampicin (50μg/ml) or kanamycin (5μg/ml). The combination of tetracycline and rifampicin was most frequently used along with the combination of tetracycline and kanamycin. Donor and recipient strains were also cultured on transconjugant selecting media as controls to monitor resistance to selecting antibiotics and detect resistance by mutation. 100μl of cell suspensions used in matings and/or 1:10 dilutions of suspensions were spread onto antibiotic containing media and onto 2%HBA. Control plates were incubated for similar periods as mating plates, but were occasionally examined for growth after shorter periods of incubation (e.g. approximately 40 hours).

2.4.2 Method B

Three *C. jejuni* strains and one *C. coli* strain were chosen as recipients in intraspecies and interspecies transfer studies. Resistance determinants and localisation to plasmid or chromosome was unknown for recipient strains. Ten *tetO*-plasmid harbouring, tetracycline resistant donor strains, including control strain JC6, were mated with the four recipient strains. The two remaining donor strains were only mated with recipients 887 and NCTC *C. coli* 11366 due to resistance patterns.

Conjugations were performed according to the plate-mating method of Taylor *et al*, 1981 and the first round of conjugation studies with the following modifications. Strains were suspended in 1ml pre-warmed brucella broth to 10^8 - 10^9 cells per ml. 200μl of donor and recipient strain cell suspensions were mixed in a 1.5ml microcentrifuge tube containing 10U of DNase (1U/μl), Buffer 1 and water. Mixed cell suspensions were then DNase

treated through incubation for 10 minutes at 37^{o}C. In two mating groups, 300µl of recipient cell suspensions were mixed with 200µl of donor cells and treated as above. In one mating group, cells were not treated with DNase. Instead, 200µl of donor and recipient cell suspensions were mixed on the surface of a HBA plate and incubated. These matings were repeated which included treatment with DNase as described above. For all other matings, DNase treated cells were then spread onto HBA plates and incubated for approximately 6 hours at 42^{o}C. Two mating groups were incubated for shorter (4 hours and 20 minutes) or longer (7 hours) periods of time.

Cells were washed off plates with 1ml of room temperature brucella broth. 100µl of harvested cell suspensions and 1:10, 1:100 and 1:1000 dilutions of harvested suspensions were spread onto transconjugant selecting media. Also, 100µl of harvested cell suspensions were spread on control 2% HBA or HBA plates for all mating groups except the non-DNase treated group. Plates for all matings were incubated at 42^{0}C for approximately 48 hours ± 4 - 6 hours. For two mating groups, plates were incubated for shorter (38 – 40 hours) or longer (58 hours) periods of time. Transconjugants were selected on 2%HBA plates containing 16µg/ml of tetracycline and selecting antibiotics. These included erythromycin (20µg/ml), streptomycin (10µg/ml) and chloramphenicol (5µg/ml). Combinations used were tetracycline and erythromycin, tetracycline and streptomycin, and, tetracycline and chloramphenicol.

Suspensions of donor and recipient strains were also spread on selective media to detect antibiotic resistance by mutation. 100 - 200µl of cell suspensions or harvested cell suspensions of recipient strains (from matings where recipient strains were also incubated

on HBA plates and subsequently harvested as described above) were spread on 2%HBA plates and antibiotic containing media. 1:10 dilutions of suspensions were also spread on antibiotic containing media or 2%HBA. 100µl of donor cell suspensions or harvested cell suspensions were plated on 2%HBA and antibiotic containing media. 1:10 dilutions of suspensions were also plated on antibiotic media.

Transfer frequencies were calculated as the number of transconjugants per recipient. Rates were calculated for dilution plates from which transconjugants were recovered and then averaged to achieve the final transfer rate.

2.5 MOLECULAR METHODS

2.5.1 Preparation of DNA from cell lysates

Crude cell lysates were prepared by the boiling method as follows. A small loopful of cultured bacteria was washed with 500µl of sterile PBS and pelleted by centrifugation at 9,000 × g for 5 minutes at room temperature. The pellet was resuspended in 100µl of sterile water and boiled for 5 minutes to release the DNA. Final centrifugation at 9,000 × g for 5 minutes at room temperature pelleted cell debris and supernatant with bacterial DNA was collected. Crude lysate extracted DNAs were stored at -20^0C.

2.5.2 Plasmid isolation and analysis

Plasmid DNA was extracted using a modified alkaline lysis method of Sambrook and Russell, 2001 with the following modifications. Cells from one plate were harvested into 1ml PBS and centrifuged for 2 minutes at 18,000 × g at room temperature. Pellets were resuspended by vortexing in 200µl of ice-cold Solution I containing 4mg/ml of lysozyme

and stored for five minutes at room temperature. A 400µl volume of Solution II was added and the mixture was incubated on ice for 5 minutes. 300µl of ice-cold Solution III was added to the lysate, which was placed on ice for 5 minutes, then centrifuged for 7 minutes at 18,000 × g at room temperature. Lysates were divided between two tubes and treated with 8-10µl of 10mg of Pancreatic RNase A per ml for 30-45 minutes at 37^{o}C. Following phenol-chloroform extractions and ethanol precipitation, pellets from duplicate tubes were resuspended in 10µl of sterile water, combined, and stored at -20^{0}C. Plasmid DNA was isolated from transconjugants and strains from the trimethoprim resistance study according to the method described above with some modifications. Lysates were treated with 3-10µl of RNase A for 45 minutes – 1½ hours and pellets from duplicate tubes were resuspended in 10-15µl of sterile water, combined, and stored as above.

2.5.3 Small-scale genomic DNA extraction

Genomic DNA was prepared by a modified large-scale extraction method of Korolik *et al*, 1995. A lawn culture was flooded with 3ml PBS and 1.5ml of cells was harvested and centrifuged for 1.5 minutes at 18,000 × g at room temperature. The pellet was resuspended by vortexing in 150µl of glucose buffer and stored for 5 minutes at room temperature. 300µl of TE buffer pH 7.4 containing 0.5-1% SDS was added and the lysate was incubated for 15 minutes at 37^{o}C. Lysates were treated with 100µl of 1mg of Pancreatic RNase A per ml or, 10-30µl of 10mg of RNase per ml and incubated for 15 minutes at 37^{o}C. Following RNase treatment, 100µl of 1mg of Pronase per ml, or 20-40µl of 10mg Pronase per ml, or 20µl of 20mg Pronase per ml was added, and the mixture was incubated for 1½ - 2½ hours

at 37^{o}C. A 225µl volume of ice-cold potassium acetate, pH 4.8, was added and the mixture was gently vortexed and then centrifuged for 10 minutes at 18,000 × g at room temperature.

DNA was extracted three times (more if required) with phenol-chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol. DNA was precipitated with an equal volume of ice-cold isopropanol and pelleted by centrifugation for 10 minutes at 18,000 × g at room temperature. DNA pellets were washed with 1ml of isopropanol and resuspended in 30µl (or smaller volumes of 10µl or 20µl if required) of sterile water and stored at –20^{o}C.

2.5.4 PCR analysis

tetO amplification

The *tet*O gene was detected in isolates using primer sequences (listed in Table 7) and PCR parameters based on those of Bacon *et al*, 2000 with modification. Also included in the reaction were internal control primers (listed on Table 7) detecting the 23S rRNA of thermophillic *Campylobacter* spp. (Korolik *et al*, 2001). PCR components consisted of 15 pmoles of *tet*O primer and 8 pmoles of internal control primer, 200µM dNTPs, PCR buffer, 3.3mM $MgCl_2$ and 1U of Eppendorf Taq polymerase. 2µl of crude lysate extracted DNA was used as template in each 25µl PCR reaction. 0.5µl of genomic DNA was used if amplification from crude lysate extracted DNA was unsuccessful. 2µl of sterile water was employed as a water control.

Japanese isolates, JC6 and JC8, were employed as positive controls and tetracycline sensitive strain, 331 was used as a negative control. PCR parameters consisted of initial denaturation at 95^{0}C for 2 minutes, followed by 35 cycles of 95^{0}C for 2 minutes, 52^{0}C for

30 seconds and 72^{0}C for 1 minute. Final extension was at 72^{0}C for 2 minutes. A probe for Southern blot experiments was generated via a modified PCR protocol. 2µl of genomic DNA or crude lysate extracted DNA was used as template in 50µl reactions that lacked internal control primers.

flaA amplification

*fla*A PCR was performed with primer sequences (listed in Table 7) stated in Wassenaar and Newell, 2000. PCR components consisted of 200µM dNTPs, 2mM $MgCl_2$, 25 pmoles of primer, 5 µl of PCR buffer and ½U. Taq polymerase concentration was increased to 1U or 1.5U if amplification was unsuccessful. 2µl of crude lysate extracted DNA was generally used as template. 3-5µl of crude lysate extracted DNA or 2µl of genomic DNA was used if problems occurred with amplification. 2µl of sterile water was employed as a water control. PCR reactions were performed in volumes of 50µl. PCR parameters were initial denaturation at 94^{o}C for 2 minutes, followed by initially 36 cycles, then reduced to 30 cycles of 94^{o}C for 30 seconds, 60^{o}C for 1 minute and 72^{o}C for 2 minutes. Final extension was at 72^{o}C for 5 minutes.

dfr1 amplification

The *dfr1* gene was detected in isolates using primer sequences and PCR parameters based on the method of Gibreel & Skold, 1998 and 2000 with modification. Primer sequences are contained in Table 7. PCR components consisted of 200µM dNTPs, 1.5mM $MgCl_2$, 10 pmoles of primer, 1 X PCR buffer and ½U Taq polymerase. 2µl of crude lysate extracted DNA was used as template and PCRs were carried out in 25µl volumes. PCR parameters

were initial denaturation at 94^0C for 1 minute, followed by 30 cycles of 94^oC for 1 minute, 53^oC for 1 minute and 72^oC for 2 minutes, and final extension at 72^oC for 5 minutes. *E. coli* strain ColE1:Tn7, known to carry the *dfr1* gene, was used as a positive control. 2µl of sterile water was employed as a water control.

dfr9 amplification

dfr9 was detected in isolates using primer sequences and PCR parameters based on the method of Gibreel and Skold, 1998 with modification. Primer sequences are listed in Table 7. PCR components were the same as for the *dfr1* PCR. PCRs were carried out in 25µl volumes with 2µl of crude lysate extracted DNA as template in the majority of reactions. Volumes of crude lysate extracted DNA was decreased to 0.5µl or 1µl for some isolates and template volumes were made up to 2µl with sterile water. PCR parameters were the same as those employed for the *dfr1* PCR with the exception of annealing temperature (50^0C). A positive control strain was not employed due to the lack of an appropriate *dfr9* carrying strain. 2µl of sterile water was used as a water control.

intI1 amplification

The class I integrase gene, *intI1*, was detected in isolates using primer sequences and PCR parameters based on those of Lee *et al*, 2002 and Bass *et al*, 1999 with modification. Primer sequences are contained in Table 7. PCR components consisted of 200µM dNTPs, 2mM $MgCl_2$, 50 pmoles of primer, 1 X PCR buffer and ½U – 1U of Taq polymerase. PCRs were carried out in 25µl volumes and 2µl of crude lysate extracted DNA was used as template. Crude lysate extracted DNA volumes were reduced to 1µl or increased to 3µl for some strains. If required, template volumes were made up with sterile water. PCR parameters

were initial denaturation at 94^0C for 2 minutes followed by 30 cycles of 94^oC for 1 minute, 55^oC for 1 minute and 72^oC for 1 minute. Final extension was at 72^oC for 5 minutes. *E. coli* strain ColE1:Tn*7* was employed as a putative positive control. 2µl of sterile water was used as a water control.

intI2 amplification

The class 2 integrase gene, *intI2*, was detected in isolates using primer sequences and PCR parameters based on those of Goldstein *et al*, 2001 with modification. Primer sequences are listed in Table 7. PCR components were 200µM dNTPs, 3mM $MgCl_2$, 50 pmoles of primer, 1 X PCR buffer and ½U – ¾U of Taq polymerase. PCRs were carried out in 25µl volumes with 2µl, increased to 3µl for some strains, of crude lysate extracted DNA as template. PCR parameters were the same as those for *intI1* PCRs with the exception of annealing temperature (54^0C). *E. coli* strain ColE1:Tn*7* was used as a positive control as it was known to harbour the Tn*7* transposon which carries an *intI2* gene. 2µl of sterile water was employed as a water control.

Gene cassette amplification

We amplified the variable regions of class I integrons - gene cassettes, using primers targeting 5' and 3' conserved regions of integron sequences, according to the methods of O'Halloran *et al*, 2004 and Levesque *et al*, 1995. Primer sequences are listed in Table 7. PCR components consisted of 200µM-300µM dNTPs, 1.5mM-2mM $MgCl_2$, 25-30 pmoles of primer, 1 X PCR buffer and ½U – ¾U of Taq polymerase. Taq polymerase concentration was occasionally increased to 1U. PCRs were performed in 25µl volumes and 2µl of crude lysate extracted DNA was used as template. PCR parameters consisted of initial

Table 9. Primer sequences and product size for each PCR experiment

Primer Sequence	Product size
tetO	
*tet*OF: 5'-GGCGTTTTGTT TATGTGCG-3'	559bp
*tet*OR: 5'-ATGGACAACCCGACAGAAGC-3'	
Therm 1.1: 5'-TATTCCAATACCAACATTAGT-3'	306-309bp
Therm 2.1: 5'-GAAGATACGGTGCTATTTTG-3'	
flaA	
*flaA*F: 5'-ATGGGATTTCGTATTAACAC-3'	1731bp
*flaA*R: 5'-CTGTAGTAATCTTAAAACATTTTG-3'	
dfr1	
P3: 5’-ACGGATCCTGGCTGTTGGTTGGACGC-3’	254bp
P4: 5’-CGGAATTCACCTTCCGGCTCGATGTC-3’	
dfr9	
P1: 5’-ATGAATTCCCGTGGCATGAACCAGAAGAT-3’	398bp
P2: 5’-ATGGATCCTTCAGTAATGGTCGGGACCTC-3’	
intI1	
*intI1*F: 5’-CCTCCCGCACGATGATC-3’	280bp
*intI1*R: 5’-TCCACGCATCGTCAGGC-3’	
intI2	
*intI2*F: 5’-TTATTGCTGGGATTAGGC-3’	233bp
*intI2*R: 5’-ACGGCTACCCTCTGTTATC-3’	
Gene cassettes	Variable base pair product because cassettes are unknown
5-CS: 5’-GGCATCCAAGCAGCAAG-3’	
3-CS: 5’-AAGCAGACTTGACCTGA-3’	

denaturation at 94^0C for 2 minutes followed by 35 cycles of 94^oC for 1 minute, 55^oC for 1 minute and 72^oC for 5 minutes. Final extension was at 72^oC for 5 minutes. *E. coli* strain ColE1:Tn7 which produced an *intI1* PCR product was used as a positive control strain.

2.5.5 flaA-Restriction Fragment Length Polymorphism Analysis

flaA-Restriction Fragment Length Polymorphism (RFLP) analysis was employed to determine whether transconjugants from conjugation studies were true transconjugants or resistant variants of donor or recipient strains. 10µl (occasionally 8µl or 12µl) of *flaA* PCR product was digested with 2U of *Dde*I for 1½ (occasionally 1¾) hours at 37^oC. If *Dde*I failed to distinguish between donors, recipients and transconjugants, 10µl of PCR product was digested with 2U of *Hinf*I, *Alu*I or *Pst*I for 1½ hours at 37^oC.

2.5.6 Restriction Endonuclease Digestion with ClaI

1µg of genomic DNA was digested with 30-40U of *Cla*I for 2¼ (occasionally 2½) hours at 37^oC. 300-500ng of plasmid DNA or all of the remaining DNA preparation (12.5µl, 15µl or 17µl) was digested with 20-30U of *Cla*I for 2¼ hours at 37^oC.

2.5.7 Verification of transconjugants

Transconjugant colonies from successful matings were verified by plasmid analysis, *tet*O amplification, *fla*A-RFLP analysis and restriction endonuclease (RE) profiling. Two or four transconjugants from each successful mating were verified. In some cases only one transconjugant could be analysed. If *fla*A-RFLP analysis failed to discriminate between transconjugants and donor and recipient strains, RE profiling was used. Genomic DNA

from undistinguished isolates was cleaved with *Cla*I as described above, resolved by agarose gel electrophoresis and profiles were compared.

2.5.8 Agarose Gel Electrophoresis

All agarose gels, unless specified, contained ethidium bromide at a concentration of 1 μg / 10 ml. *tet*O, *flaA* and *W. succinogenes* integrase PCR products were resolved on 1, 1.5 or 2% TAE gels at between 20 and 100 volts. *dfr1, dfr9, intI1, intI2*, and gene cassette PCR products were resolved in 1.5% or 2.5% TAE gels either at high voltages (100 –130 volts) or low voltages (20-60 volts). Plasmid DNA was resolved in 0.7-0.8% TBE gels at 50 volts. *Cla*I digested genomic DNA was electrophoresed in 0.8% or 2% TAE gels at 30-50 volts. Plasmid DNA, *Cla*I digested plasmid DNA and *Cla*I digested genomic DNA for Southern blot experiments was resolved in 0.8% TBE gels at 20-50 volts. Restriction fragments in *flaA*-RFLP analysis were resolved using 3% TAE (occasionally TBE) gels at 80-100 volts, and following electrophoresis, were stained in an ethidium bromide bath for 15-20 minutes, de-stained for 5 minutes in distilled water and viewed.

2.5.9 Southern blot analysis

Southern Blotting

Plasmid DNA, *Cla*I digested plasmid DNA and *Cla*I digested genomic DNA was transferred to Hybond-N+ nitrocellulose membranes (Amersham Pharmacia Biotech) by the neutral transfer method of Sambrook and Russell, 2001 and Southern, 1975 with modification. Gels were subjected to depurination in 0.125-0.2M HCl for 10-15 minutes prior to denaturation for 30-40 minutes and neutralisation for 30 minutes. DNA was transferred by downward transfer overnight (up to 24 hours) at room temperature.

Following transfer and fixation, DNA was cross-linked to membranes by UV-irradiation (2 minutes on a UV-transilluminator (UVP) at 302nm) Membranes were air-dried at room temperature and stored in foil at 4^0C.

Radio-labelling of probe

The *tet*O PCR product amplified from positive control JC8 was purified using a Wizard PCR Preps DNA Purification System kit (Promega). The probe was radiolabelled using a Nick Translation kit (Promega or Roche) with [^{32}P]dCTP (Amersham Pharmacia Biotech). The radio-labelled probe was purified through columns using 1 X TE buffer or through spun column chromatography according to the method of Sambrook and Russell, using Sephadex G50.

Hybridisation conditions

Solutions and conditions were used according to Sambrook and Russell with modification. Hybridisation solution contained 100μg/ml of herring sperm DNA. Membranes were pre-hybridised for 3-3½ hours in hybridisation solution at 65^0C. Hybridisations were performed at 65^0C overnight (14-19 hours) in fresh hybridisation solution with radio-labelled probe. Following hybridisation, membranes were washed twice in 2 X SSC, 0.1% SDS at 65^0C for 7 or 10 minutes, and once in 1 X SSC, 0.1% SDS at 65^0C for 10 or 15 minutes. If membranes were ‘dirty’, membranes were washed in 0.5 X SSC for 20 minutes at 65^0C.

Autoradiography

Blots were exposed to phosphor screens (Bio Rad) for 1 - 24 hours or longer (3 - 4 days) if required, and analysed with a Bio Rad Personal Imager FX and the Quantity One program (Bio Rad).

Stripping of membranes

Membranes were incubated in 0.4M NaOH at 43^0C for 1-1½ hours and then incubated in 0.1 X SSC, 0.1% SDS, 0.2M Tris-Cl pH 7.4 - 7.5 for 1 hour at 43^0C. Membranes were then checked for radioactivity as described above. The process was repeated if the membranes were not thoroughly stripped. Membranes were then re-probed as described above.

CHAPTER 3

TETRACYCLINE RESISTANCE

The work presented in this chapter was previously published as - **Pratt, A. & Korolik, V.** (2005) Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates, *Journal of Antimicrobial Chemother*apy **55:**452-460. Refer to **Appendix 4** for the published journal article.

3. TETRACYCLINE RESISTANCE

Tetracycline resistance in *Campylobacter* spp. is encoded by the *tetO* gene and is usually associated with conjugative plasmids, although occasionally *tetO* may be chromosomally located (Tracz *et al*, 2005, Batchelor *et al*, 2004, Gibreel *et al*, 2004a, Gibreel *et al*, 2004b, Bacon *et al*, 2000, Lee *et al*, 1994, Taylor & Courvalin, 1988, Taylor *et al*, 1988, Ng *et al*, 1987). Although, the presence of *tetO* has been detected in *Campylobacter* strains throughout the world, little is known about the genetics and mechanisms of tetracycline resistance in Australian *Campylobacter* species.

3.1 TETRACYCLINE SUSCEPTIBILITY

Tetracycline MICs were determined for 41 *C. jejuni* and 5 *C. coli* isolates firstly identified to be tetracycline resistant by a disk diffusion test (**Table 10** and **11**). Tetracycline MICs ranged from 32μg/ml to >256μg/ml. 23 strains had a MIC of 128μg/ml, 8 strains had a MIC of 256μg/ml and 8 strains had a MIC of 64μg/ml. MICs of *C. jejuni* isolates ranged from 32 to >256μg/ml and MICs of *C. coli* isolates ranged from 32 to 256μg/ml. MICs of clinical isolates ranged from 32μg/ml to >256μg/ml and of chicken isolates ranged from 32μg/ml to 128μg/ml.

3.2 PLASMID CARRIAGE

46 tetracycline resistant strains and control strains JC6, JC8 and 331 were examined for carriage of plasmid DNA. Plasmid carriage and size data is summarised in **Table 10** and **11**. Plasmid profiles of 6 representative strains are shown in **Figure 1**. 34 strains harboured plasmids. Of these, 30 strains harboured single plasmids and the remaining four strains

harboured multiple plasmids. All multiple plasmid harbouring strains were of human origin of which two isolates were of *C. jejuni* and two of *C. coli*. Control strain JC6 also harboured multiple plasmids. Two strains carried two small plasmids, and, two strains and control strain JC6 harboured small plasmids and a large plasmid. Thirty *C. jejuni* strains and four *C. coli* strains carried plasmids; of which, twenty-five *C. jejuni* and four *C. coli* strains were isolated from humans with gastroenteritis.

Plasmids ranged in size from small plasmids to larger plasmids of 21-50kb in size. Approximate sizes of large plasmids were determined by the addition of fragment sizes from *Cla*I plasmid digestions. Sizes of small plasmds could not be determined, as these plasmids appeared undigestable with *Cla*I. Sizes could not be determined using *Cla*I digestion for large plasmids from seven strains, as distinguishable fragments could not be obtained. Sizes for these plasmids were estimated by comparing these uncut plasmids with the uncut form of plasmids for which sizes were previously determined by the addition of *Cla*I fragments.

Plasmids between 31-40kb were the most frequently isolated, with 13 strains harbouring plasmids in this range. All strains harbouring plasmids of this size were isolates of *C. jejuni*, with the majority of human origin. Two additional chicken strains carried plasmids estimated to be 31-41kb in size. Seven strains and control strain JC6 harboured 20-30kb plasmids, and, four strains harboured plasmids from 41-50kb in size. Plasmids from four other strains were estimated to be 20-40kb in size and two strains harboured a plasmid that was >20kb in size. Two *C. coli* isolates harboured only small plasmids.

Table 10. Tetracycline MICs, *tetO* PCR, plasmid carriage, *tetO* hybridisation and conjugation results of tetracycline resistant strains under study

Strain	**Species**	**Source**	**MIC (μg/ml)**	**Plasmid Carriage & Size (kb)**	***tet*O PCR**	***tetO* Hybridisation**	**Conjugation[a]**
JC8 (Japan)	*C. jejuni*	Human	128	-	+	Chromosome	N/A
JC6 (Japan)	*C. jejuni*	Human	128	2 small plasmids & a 26kb plasmid	+	Plasmid	4.7×10^{-6}
331	*C. jejuni*	Chicken	8	-	-	-	N/A
FF7	*C. jejuni*	Human	128	44-50	+	Plasmid	No conjugation
FF12	*C. jejuni*	Human	128	-	+	Chromosome	N/A
293	*C. coli*	Human	64	21	+	Chromosome	N/A
GC012	*C. jejuni*	Human	64	33	+	Plasmid	No conjugation
QH104	*C. jejuni*	Human	256	-	+	Chromosome	N/A
QH120	*C. jejuni*	Human	>256	Small plasmids & a 32kb plasmid	+	Plasmid	No conjugation
QH127	*C. jejuni*	Human	256	-	+	Chromosome	N/A
QH222	*C. coli*	Human	265	2 small plasmids	+	Chromosome	N/A
QH230	*C. jejuni*	Human	64	-	+	Chromosome	N/A
QH304	*C. jejuni*	Human	32	-	+	Chromosome	N/A
QH428	*C. jejuni*	Human	256	-	+	Chromosome	N/A
QH509	*C. jejuni*	Human	128	-	+	Chromosome	N/A
QH514	*C. coli*	Human	256	2 small plasmids	+	Chromosome	N/A
GC1874	*C. jejuni*	Human	128	-	+	Chromosome	N/A
GC2732	*C. jejuni*	Human	128	37	+	Chromosome	No conjugation
GC3346	*C. jejuni*	Human	128	22	+	Chromosome	N/A
GC5252	*C. jejuni*	Human	128	31-34	+	Plasmid	2×10^{-6}
GC6108	*C. jejuni*	Human	128	30	+	Plasmid	0.8×10^{-8}
GC10019	*C. jejuni*	Human	64	31	+	Plasmid	No conjugation
GC15913	*C. jejuni*	Human	256	36	+	Chromosome	N/A

[a] The transfer frequency was calculated as number of transconjugants per recipient

NA: Not Applicable

Table 11. Tetracycline MICs, *tetO* PCR, plasmid carriage, *tetO* hybridisation and conjugation results of tetracycline resistant strains under study continued

Strain	Species	Source	MIC (μg/ml)	Plasmid Carriage & Size (kb)	*tetO* PCR	*tetO* Hybridisation	Conjugation[a]
GC16723	*C. jejuni*	Human	128	31	+	Chromosome	N/A
GC18943	*C. jejuni*	Human	128	25-33	+	Plasmid	2.3×10^{-7}
GC21098	*C. jejuni*	Human	>256	>20	+	Plasmid	2.3×10^{-7}
GC21172	*C. jejuni*	Human	128	22-30	+	Chromosome	N/A
GC22479*	*C. jejuni*	Human	64	32	+	Chromosome	N/A
GC23330	*C. jejuni*	Human	128	30-34	+	Chromosome	N/A
GC23503	*C. jejuni*	Human	128	32-33	+	Chromosome	N/A
GC23730	*C. jejuni*	Human	128	32-33	+	Chromosome	N/A
GC27179*	*C. jejuni*	Human	>256	22-36	+	Chromosome	N/A
GC28719	*C. jejuni*	Human	>256	50	+	Plasmid	Conjugation[b]
GC29395	*C. jejuni*	Human	128	42	+	Chromosome	N/A
GC31677	*C. jejuni*	Human	256	2 smaller plasmids & a 22kb plasmid	+	Plasmid	1.9 x 10-6
GC32304	*C. jejuni*	Human	128	29	+	Chromosome	N/A
GC32626	*C. jejuni*	Human	128	29	+	Chromosome	N/A
GC35618	*C. jejuni*	Human	128	37	-	Chromosome	N/A
GC36409	*C. jejuni*	Human	256	-	+	Chromosome	N/A
GC42121	*C. jejuni*	Human	128	41	+	Chromosome	N/A
GC470308	*C. coli*	Human	128	40	+	Chromosome	N/A
C1	*C. coli*	Chicken	32	-	+	Chromosome	N/A
115*	*C. jejuni*	Chicken	128	31-41	+	Chromosome	N/A
231	*C. jejuni*	Chicken	128	-	+	Chromosome	N/A
801*	*C. jejuni*	Chicken	64	21-37	+	Chromosome	N/A
802*	*C. jejuni*	Chicken	64	37	+	Chromosome	N/A
824*	*C. jejuni*	Chicken	64	>20	+	Chromosome	N/A
835*	*C. jejuni*	Chicken	32	31-41	+	Chromosome	N/A
3017	*C. jejuni*	Chicken	128	-	+	Chromosome	N/A

* Strains in which closed circular forms of plasmids were compared

b Transfer frequency was not calculated as the donor strain was found to be resistant to the selecting antibiotic. However, transconjugants were identified as true transconjugants (Section 3.4)

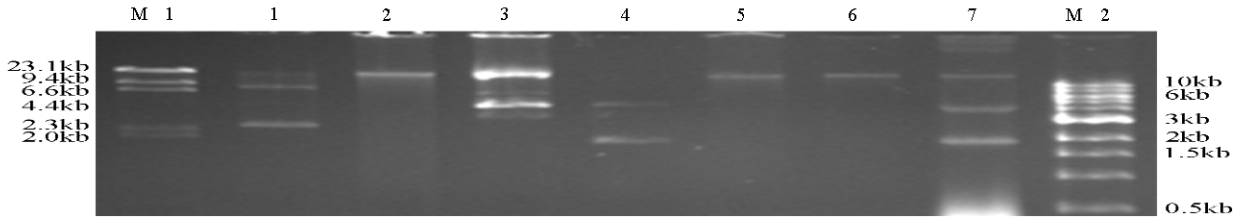

Figure 1. Plasmid profiles of tetracycline resistant isolates of *C. jejuni* and *C. coli*. Lanes: M1, molecular weight marker *Hin*dIII digested λ DNA; 1, positive control strain JC6; 2 – 7, FF7, QH120, QH222, GC21172, GC23330, GC31677 & M2 molecular weight marker 1kb ladder (New England Biolabs).

The similarity between plasmids harboured by resistant strains was analysed through digestion with *Cla*I. *Cla*I profiles of plasmids from 25 isolates, including number and size of fragments were analysed and then visibly compared. *Cla*I fragments of plasmids examined ranged in size between <2.0kb and ≈23.1kb. *Cla*I profiles of a selection of plasmids from tetracycline resistant isolates are displayed in **Figure 2** and **3**.

13 strains appeared to harbour similar plasmids, according to comparisons of *Cla*I profiles. Some smaller fragments were not detectable from some plasmids, but due to similarity in other fragments observed, these plasmids were considered similar to those in which more fragments were visible.

The plasmid from an additional strain appeared to have a *Cla*I profile with some similarities to those exhibited by the plasmids described above and was considered to be some what similar to these plasmids. *Cla*I profiles of plasmids from the remaining 11 strains examined were unique and differed from each other and the profiles of the plasmids described above. Such unique profiles indicated that these plasmids may possess differing *Cla*I restriction sites.

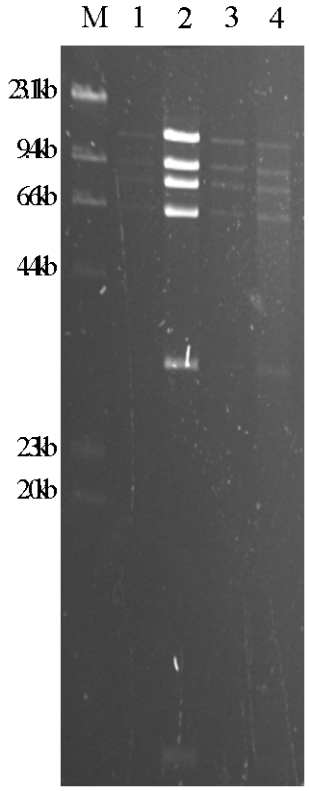

Figure 2. RFLP patterns of *Cla*I digested plasmids from selected tetracycline resistant *Campylobacter* isolates. Lanes: M, molecular weight marker - *Hin*dIII digested λ DNA; 1, strain GC2732; 2, strain GC29395; 3, GC35618 & 4, strain GC42121.

12 tetracycline resistant strains did not harbour detectable plasmid DNA. Also, control strains JC8 (tetracycline resistant) and 331 (tetracycline sensitive) did not harbour plasmid DNA. Of the 12 strains, 11 isolates were of *C. jejuni* and one of *C. coli*. Nine isolates were of human origin and three of chicken origin.

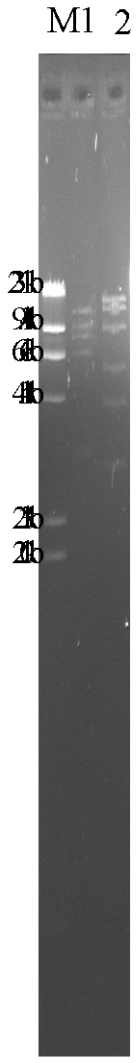

Figure 3. RFLP patterns of *Cla*I digested plasmids from selected tetracycline resistant *Campylobacter* isolates. Lanes: M, molecular weight marker - *Hin*dIII digested λ DNA; 1, strain GC470308 & 2, strain GC28719.

3.3 DETECTION & LOCALISATION OF *tetO* ON PLASMID & GENOMIC DNA

3.3.1 Detection of tetO by PCR

All tetracycline resistant isolates under study were examined for the *tetO* gene by PCR analysis, employing primers described in Bacon *et al*, 2000 and as described in **Materials and Methods**, section 2.5.4. Forty-five of the forty-six strains were positive for *tetO* when analysed by this PCR method. Results for all strains are displayed in **Table 10** and **Table 11**, and for a selection of strains in **Figure 4**.

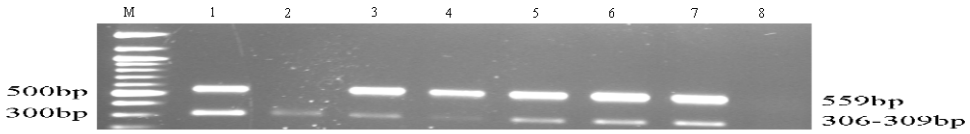

Figure 4. Agarose gel electrophoresis of *tetO* PCR products amplified from tetracycline resistant strains of *C. jejuni* and *C. coli*. The *tetO* band is 559bp and the internal control band is 306-309bp. Lanes: M, molecular weight marker (100bp ladder); 1, positive control strain JC8; 2, tetracycline sensitive strain 331; 3-7, QH104, QH120, GC012, QH222, QH514 and 8, water control.

3.3.2 Localisation of tetO

Southern blot hybridisation at medium stringency, using the *tetO* coding sequence as a probe was used to determine the localisation of *tetO* in the genome (chromosome, plasmid

or both) of tetracycline resistant isolates. The *tetO* probe was hybridised to all blots at 65^0C, and all strains were found to hybridise with the *tetO* probe. Complete data is listed in **Table 10** and **11**. The one strain for which *tetO* was not detected using PCR analysis was shown to carry the *tetO* gene by Southern blot analysis.

Plasmid DNA and *Cla*I digested plasmid and genomic DNA of plasmid harbouring strains was examined. Only plasmid DNA and *Cla*I digested genomic DNA was examined for seven strains and two control strains, JC8 and 331. Genomic DNA from positive control strain JC8 that did not harbour plasmid DNA was included on all blots. The plasmid DNA preparation from JC8 was only examined once. *Cla*I digested plasmid DNA from plasmid harbouring control strain JC6 was only included on one blot. However, *Cla*I digested genomic DNA of this strain was included on all blots, and, undigested plasmid DNA was included all blots except one. Hybridisation results of a selection of strains are presented in **Figure 5**.

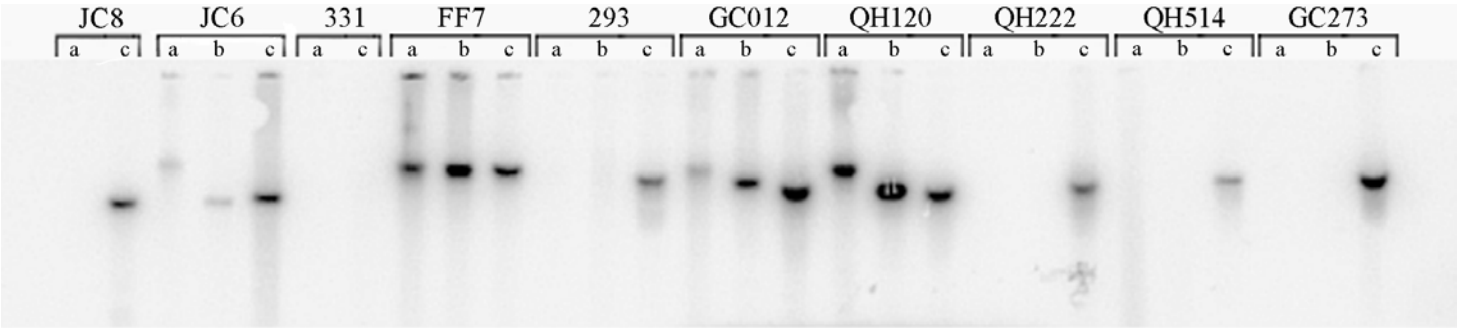

Figure 5. Southern hybridisation of *tetO* to plasmid DNA and *Cla*I digested plasmid and genomic DNA of tetracycline resistant *Campylobacter* isolates. a, b and c represent un-digested plasmid DNA, *Cla*I digested plasmid DNA and *Cla*I digested genomic DNA respectively. Only plasmid extractions and genomic DNA were probed with *tetO* for control strains JC8 and 331.

All 34 plasmid harbouring strains examined hybridised to *tetO*, including strain GC35618 that was negative for *tetO* by PCR. Of the 34 plasmid harbouring stains, plasmids from only 10 strains hybridised to *tetO*. Labelled *tetO* hybridised with chromosomal DNA from the remaining 24 plasmid harbouring strains (including GC35618). Genomic DNA from control strain JC8 hybridised to *tetO*. Genomic and plasmid DNA of control strain JC6 hybridised to *tetO* in all blots analysed. Genomic DNA and the plasmid DNA preparation from negative control strain 331 did not hybridise to *tetO*.

Plasmid and genomic DNA isolated from 10 plasmid harbouring strains hybridised to *tetO*. When compared visually, similar sized *tetO* hybridising *Cla*I digested plasmid and genomic DNA fragments were observed for eight strains and positive control strain JC6, indicating a plasmid location for *tetO*. For four strains, digested plasmid DNA fragments that hybridised to *tetO* appeared slightly larger than genomic DNA fragments that hybridised (data not shown). *Cla*I digested plasmid and genomic DNA fragments that hybridised to *tetO* differed considerably in size for strain GC31677 (data not shown), where the digested plasmid fragment that hybridised was smaller in size than the *Cla*I genomic DNA fragment. For strain GC28719, only undigested plasmid DNA and *Cla*I digested plasmid DNA hybridised to *tetO*. *Cla*I digested genomic DNA from this strain did not hybridise (data not shown).

As previously stated, *tetO* hybridised to chromosomal DNA from the remaining 24 plasmid harbouring strains. For five strains - 801, GC10019, GC15913, GC22479 and GC32626, *tetO* appeared to hybridise to two *Cla*I genomic DNA fragments. *Cla*I digested genomic DNA of 801, GC22479 and GC32626 was examined a second time and *tetO* hybridised to

two fragments for both 801 and GC22479, but to only one fragment for GC32626. Hyrbidisation results for the three strains re-examined are presented in **Figure 6**.

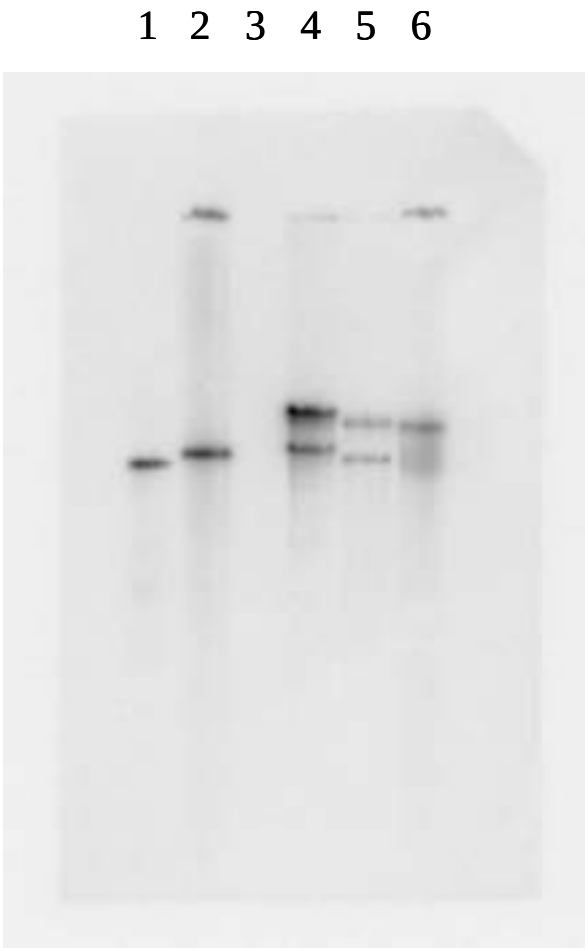

Figure 6. Southern hybridisation of *tetO* to *Cla*I digested genomic DNA of tetracycline resistant *Campylobacter* isolates from which two fragments previously hybridised. Lanes: 1, positive control strain JC8; 2, positive control strain JC6; 3, tetracycline sensitive negative control strain 331; 4-6, strains 801, GC22479 & GC32626.

Total genomic DNA digested with *Cla*I was examined from 12 tetracycline resistant isolates found not to harbour plasmid DNA. *tetO* hybridised to genomic DNA from all 12 strains. *Cla*I digested genomic DNA fragments of different sizes hybridised to the probe.

3.4 CONJUGATIVE TRANSFER OF TETRACYCLINE RESISTANCE

The ability of a selection of tetracycline resistant, plasmid harbouring strains to transfer tetracycline resistance through conjugation was examined using two methods employing different recipient strains. In conjugation experiments using Method A (refer to **Materials and Methods** section 2.4.1), four plasmid harbouring *C. jejuni* donor strains and tetracycline sensitive control strain 331 (lacking detectable plasmid DNA) were mated with two recipient strains. All tetracycline resistant donor strains and 331 were mated with a rifampicin resistant, plasmid harbouring *C. coli* recipient strain, CSIRO40, in interspecies experiments. One donor strain was mated twice with CSIRO40. This donor strain was also mated with a rifampicin resistant variant of *C. jejuni* strain 81116 in intraspecies experiments.

The four *C. jejuni* donor strains, GC012, GC5252, GC18943 and GC28719 were mated with *C. coli* CSIRO40 but did not conjugatively transfer tetracycline resistance. Tetracycline sensitive strain 331, which did not harbour plasmid DNA, did not conjugate with the recipient strains as was expected. Putative transconjugants were produced from the mating of GC012 with CSIRO40, but all four colonies analysed had the *flaA*-RFLP pattern of the donor strains and were therefore not true transconjugants. GC18943 was also mated once with 81116 (rifampicin resistant variant), but did not conjugatively transfer tetracycline resistance as no transconjugant colonies were recovered. The plasmids

harboured by the four tetracycline resistant donor strains were subsequently found by Southern blot to harbour *tetO*.

The 10 tetracycline resistant *C. jejuni* strains that harboured plasmids carrying the *tetO* gene as determined by Southern blot experiments (section 3.3.2), and positive control strain JC6 were analysed for the ability to transfer plasmid-encoded tetracycline resistance using Method B (refer to **Materials and Methods** section 2.4.2). Results of conjugation experiments and transfer frequencies are shown in **Table 10** and **11**. All 10 donor strains, and control strain JC6 were mated with recipient strains 887 and *C. coli* NCTC 11366. Eight donor strains and control strain JC6 were mated with recipient strains RM10 and 9126. Two donor strains, QH120 and GC28719, were only mated with two recipient strains – 887 and *C. coli* NCTC 11366 due to resistance to the selecting antibiotic (streptomycin).

Conjugative transfer of tetracycline resistance occurred between six of the 10 *C. jejuni* strains examined and one recipient strain. Control strain JC6, GC5252, GC6108, GC18943, GC21098, GC28719 and GC31677 transferred resistance to recipient *C. jejuni* strain 887. Transfer frequencies ranged from 0.8×10^{-8} to 1.9×10^{-6} transconjugants per recipient in six hour matings. Resulting transconjugants were both tetracycline resistant and erythromycin resistant.

Two transconjugants from each successful mating were verified using *tetO* PCR, *fla*A-RFLP analysis or RE profiling, and, plasmid carriage analysis. The *tetO* gene was amplified from transconjugants from all successful matings. Patterns of selected donor and recipient strains and transconjugants are displayed in **Figure 7**. *fla*A-RFLP typing verified

transconjugants from five of the successful matings and the mating of control strain JC6 with 887 (data not shown). *fla*A-RFLP profiles of transconjugants were that of recipient strain 887.

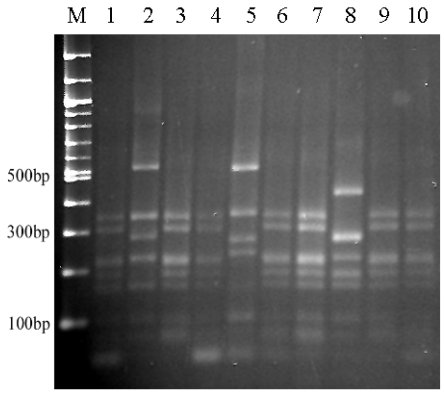

Figure 7. *fla*A-RFLP patterns of representative donor and recipient strains and transconjugants. Lanes: M, molecular weight marker - 100bp ladder (New England Biolabs); 1, recipient strain 887; 2, 5, 8, control strain JC6, GC5252 and GC6108; 3-4, 6-7 and 9-10, transconjugants.

Donor strain GC28719 was found to be resistant to the selecting antibiotic (erythromycin) in addition to tetracycline. Transconjugants however were verified as true transconjugants by *fla*A-RFLP analysis, as transconjugants had the same pattern as 887, and, by detection of the

tetO gene. Transconjugants from the mating of GC18943 with 887 were verified through RE profiling with *Cla*I. *Dde*I and three additional restriction enzymes, *Alu*I, *Hinf*I and *Pst*I,

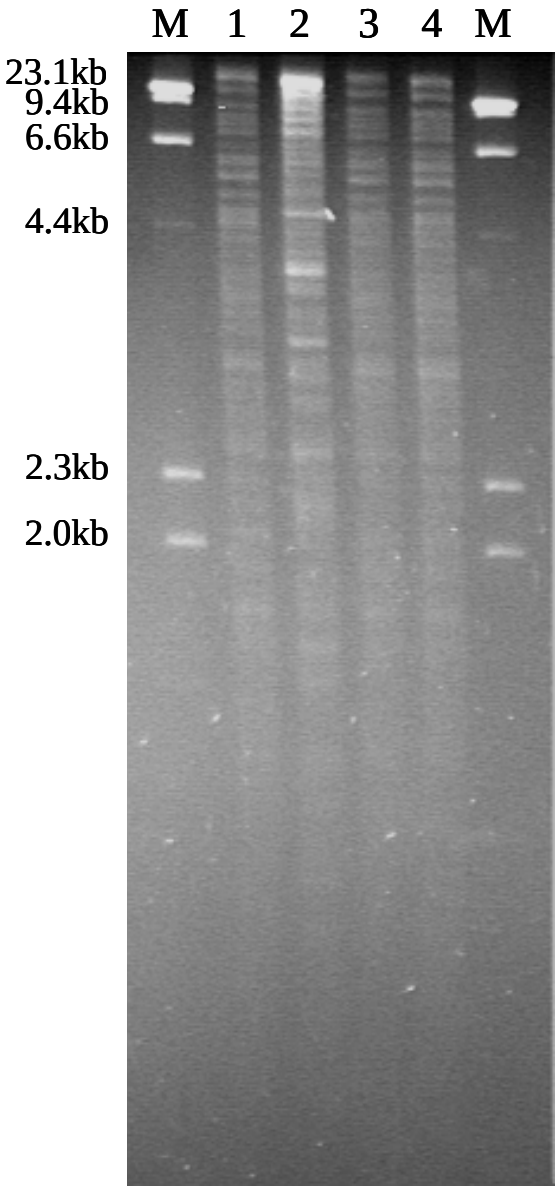

Figure 8. *Cla*I RE profiles of donor strain GC18943, recipient strain 887 and transconjugants. Lanes: M, molecular weight marker *Hin*dIII digested λ DNA; 1, 887; 2, GC18943; 3-4, transconjugants.

failed to distinguish between GC18943, transconjugants and the recipient strain. A restriction pattern similar to that of recipient strain 887 was observed for transconjugants and transconjugants were therefore considered true transconjugants. RE profiles of donor strain GC18943 and recipient strain 887 and transconjugants are displayed in **Figure 8**.

Strains FF7, GC012, GC10019 and QH120 did not conjugate with 887. GC012 was mated twice with 887 and one mating was DNase treated, however both matings did not produce transconjugants. Strain QH120 was mated twice with the recipient strain, but no transconjugants were produced.

All eight *tetO* plasmid carrying donor strains and control strain JC6 mated with recipients RM10 and 9126 failed to conjugatively transfer tetracycline resistance. Matings of strains FF7, GC012, GC5252, GC10019, GC18943, GC21098, GC31677, and JC6 with the two recipients did not produce transconjugants. Matings of GC6108 with RM10 and 9126 produced putative transconjugants, but *fla*A-RFLP analysis revealed that transconjugants had the same pattern as the donors and were therefore not true transconjugants, but were likely to be spontaneous.

All 10 *tetO* plasmid carrying *C. jejuni* donor strains and control strain JC6 were mated with *C. coli* NCTC 11366. Interspecies transfer of tetracycline resistance between eight donor strains, JC6 and the *C. coli* recipient strain did not occur. Matings of FF7, GC012, GC5252, GC6108, GC10019, GC18943, GC21098, GC31677 and JC6 with *C. coli* NCTC 11366 did not produce transconjugants.

Strains GC28719 and QH120 were mated twice with *C. coli* NCTC 11366, but conclusions could not be drawn from these matings. This was due to resistance of donor strains to chloramphenicol (the selecting antibiotic) and resistance of the recipient strain to tetracycline in one mating set. *flaA*-RFLP patterns of donors, recipient and putative transconjugants could not be compared due to the lack of amplification of a *flaA* fragment from crude lysate DNA preparations of *C. coli* recipient strain NCTC 11366.

When comparing plasmid carriage data of transconjugants with donor and recipient strains, we found that in one successful mating a small plasmid had also been transferred. Transconjugants of the mating between control strain JC6 and recipient strain 887 harboured a small plasmid of similar size to that of the donor strain, in addition to a larger plasmid. The donor strain harboured a small plasmid of this size. Transconjugants from the mating between strain GC31677 and recipient strain 887 did not harbour two small plasmids that were harboured by the donor strain. For other matings, comparisons of plasmid carriage could not be used as both donor strains and the recipient harboured plasmids of approximately the same size, when covalently closed circular plasmids were compared.

In Summary. Tetracycline resistance was examined in 46 Australian isolates of *C. jejuni* and *C. coli*. MICs ranged from 32 to >256μg/ml and 34 strains harboured single or multiple plasmids ranging in size from small plasmids to 21-50kb in size. All strains examined harboured the *tetO* gene but this gene was chromosomally located in 24 of the 34 plasmid harbouring strains and in strains that did not harbour visible plasmid DNA. Six *C. jejuni*

strains carrying plasmids that harboured *tetO* conjugatively transfered tetracycline resistance to a *C. jejuni* recipient strain.

3.6 DISCUSSION

Little was known about tetracycline resistance in Australian *Campylobacter* spp.; therefore we examined this resistance in Australian *C. jejuni* and *C. coli* isolates. High-level tetracycline resistance was observed in the 46 *C. jejuni* and *C. coli* isolates examined, with MICs ranging from 32 to >256μg/ml. This data concurs with similar ranges of MICs reported from a number of countries around the world (Lee *et al*, 1994, Schwartz *et al*, 1993 & Ansary & Radu, 1992). A similar range of tetracycline MICs was observed for both *C. jejuni* and *C. coli* isolates. Also, similar ranges were observed for chicken and human isolates, although ranges did not match as closely as did those for both species. Interestingly, half of the isolates examined were resistant to the high MIC of 128μg/ml.

The high tetracycline MICs of the isolates examined in this study may be explained by nucleotide changes within the DNA sequence of the *tetO* gene, as observed by Gibreel *et al*, 2004a. Such changes may have allowed for stronger binding of the TetO protein to the ribosome and thus influenced MICs (Gibreel *et al*, 2004a). The sequence of the *tetO* gene in highly resistant isolates from our collection could be assessed for nucleotide changes and other factors that may influence MICs according to the methods of Gibreel *et al*, 2004a.

All tetracycline resistant isolates examined in our study harboured the *tetO* gene, confirming that tetracycline resistance in Australian *Campylobacter* isolates was due to the Tet O determinant. Other investigators have also reported the presence of the *tetO* gene in

Campylobacter isolates from Canada, the USA, the United Kingdom, Germany, Poland, The Netherlands, France, Taiwan, Vietnam, Thailand, Switzerland and Algeria (Wardak *et al*, 2007, Dasti *et al*, 2007, Louwen *et al*, 2006, Nirdnoy *et al*, 2005, Tracz, *et al*, 2005, Fairchild *et al*, 2005, Avrain *et al*, 2004, Pumbwe *et al*, 2004, Bacon *et al*, 2000, Lee *et al*, 1994, Manavathu *et al*, 1988 & Sougakoff *et al*, 1987).

PCR amplification was successful in detecting *tetO* in 97.8% of strains under study. One isolate was consistently negative for *tetO* by PCR, but was subsequently shown to carry the *tetO* gene by Southern blot analysis. Heterogeneity between the *tetO* sequence in this strain and primers used in PCR detection may account for the lack of amplification. Sequencing of the PCR product from this strain may confirm this. The possibility of a hybrid tetracycline resistance gene in *C. jejuni* could also possibly account for the conflicting results between the *tetO* PCR and Southern blot results observed.

Stanton and Humphrey, 2003 reported a mosaic tetracycline determinant in the swine anaerobic bacterium *Megasphaera elsdenii*. The tetracycline resistance gene of *M. elsdenii* was initially identified as *tetO* by PCR; however subsequent sequence analysis revealed that the gene consisted of specific regions matching both *tetO* of *C. jejuni* and *tet*W of *Butyrivibrio fibrisolvens*.

Campylobacters may have acquired mosaic genes from other bacterial species that inhabit the same environments. Interestingly an element carrying *tetW* and a streptomycin resistance gene and upstream sequence 100% identical to a *C. jejuni tetO* carrying plasmid, was identified in a porcine tetracycline resistant isolate of *Arcanobacterium pyogenes*. It

would be of interest to examine tetracycline resistant campylobacters for the presence of hybrid tetracycline resistance genes and mobile genetic elements that contribute to acquisition and dissemination of such resistance genes.

It is particularly interesting to note that in the Australian tetracycline resistant isolates examined in this study, *tetO* and therefore tetracycline resistance was mostly (78.26%) chromosomally encoded. Notably, isolates in which *tetO* was chromosomally located included two thirds of strains that harboured plasmids. Dasti *et al*, 2007 similarly reported that *tetO* was not plasmid mediated in three plasmid harbouring *C. coli* isolates.

Chromosomally located *tetO* was also observed in tetracycline resistant *Campylobacter* spp. isolates that do not harbour detectable plasmid DNA. Similar percentages have been observed in Canada (21%) and Israel (30%) (Tracz *et al*, 2005 & Schwartz *et al*, 1993). Chromosomally mediated *tetO* strains that lack plasmids have also been reported by other investigators (Friis *et al*, 2006), but not at such a high rate as that observed in our study (Gibreel *et al*, 2004a & Lee *et al*, 1994).

Plasmid carriage of *tetO* in *C. jejuni* and *C. coli* has been reported by many studies (Ng *et al*, 1987, Taylor *et al*, 1988, Lee *et al*, 1994, Bacon *et al*, 2000, Gibreel *et al*, 2004a; Gibreel *et al*, 2004b & Tracz *et al*, 2005), and is thought to be the major factor in tetracycline resistance in campylobacters. Integration of *tetO* and surrounding sequences, or the entire plasmid into the chromosome of isolates from our collection, may have occurred explaining resistant isolates that do not harbour detectable plasmid DNA, as suggested by Taylor, 1992, Bossinger *et al*, 1990 and Walker *et al*, 1986.

Walker *et al* speculated that there may be a general class of conjugative plasmids in *C. jejuni* that are capable of recombining with a region(s) of the chromosome and mobilising these chromosomal sequences into other strains in a fashion analogous to F or R prime plasmids of *E. coli*. Also, it has been observed that even in the absence of functional homology, heterologous plasmid DNA introduced into *C. coli* by natural transformation could integrate into the genome at random sites by illegitimate recombination (Richardson and Park, 1997).

Putative integrated plasmids have been identified in the genome sequenced strain *C. jejuni* RM1221, *Campylobacter* isolates from various geographical locations and clinical and veterinary sources, and, *C. jejuni* strain 81-176 (Parker *et al*, 2006, Hofreuter *et al*, 2006, Fouts *et al*, 2005). Interestingly, one or more sequences matching sequenced tetracycline resistance plasmids have been observed in some *C. jejuni* isolates from humans, cattle and birds (Hepworth *et al*, 2007). Additionally, genes with similarity to the putative integrated plasmid from sequenced strain RM1221 have been identified on tetracycline resistance and *tetO* carrying plasmids (Parker *et al*, 2006, Nirdnoy *et al*, 2005 & Batchelor *et al*, 2004).

The location of *tetO* on the chromosome of tetracycline resistant isolates, even those harbouring plasmids, and, the recent identification of elements with similarity to transposable elements and IS elements on *tetO* carrying plasmids, implies mobile genetic elements other than transmissible plasmids may be involved in the acquisition and dissemination of *tetO* in campylobacters. Transposon related genes from a variety of bacterial species were identified on *tet*O harbouring plasmid from a *C. jejuni* strain (Nirdnoy *et al*, 2005), and similarly, plasmid encoded *tetO* and flanking sequences have

been found to align with nucleotides from the *Enterococci* Tn*916* transposon (Schmidt-Ott *et al*, 2005). Insertion Sequences have also been identified on *tetO* carrying plasmids, including elements with similarity to the chromosomally located IS607 of *H. pylori* and an apparent hybrid of two other *H. pylori* transposons IS*Hp608* and IS*606* (Nirdnoy *et al*, 2005 & Gibreel *et al*, 2004b).

Although approximately 74% of the Australian *Campylobacter* strains examined in this study harboured plasmids, less than a third of plasmid carrying isolates had tetracycline resistance mediated by a *tetO* harbouring plasmid. In contrast to the low rate of *tetO* carriage by plasmids observed in this study, 96% and 88% of plasmid harbouring tetracycline resistant chicken and clinical isolates of *C. jejuni* examined by Lee *et al*, 1994, carried *tetO* on a plasmid. Also, all plasmid harbouring, tetracycline resistant *Campylobacter* isolates examined by Dasti *et al*, 2007, Wardak *et al*, 2007 and Gibreel *et al*, 2004a carried *tetO* on a plasmid.

Plasmid carriage rates for isolates of both species and isolation sources were high. High plasmid carriage rates of between 80 and 100% for tetracycline resistant *C. jejuni* and *C. coli*, with sizes ranging from 1.7 to 133kb, have also been reported in other countries worldwide (Wardak *et al*, 2007, Lee *et al*, 1994 & Schwartz *et al*, 1993, Cabrita *et al*, 1992, Sagara *et al*, 1987, Tenover *et al*, 1985). Most plasmid harbouring strains examined in this study carried single plasmids, however, four strains, all of human origin, harboured multiple plasmids. Multiple plasmid combinations included small plasmids or small plasmids and a large plasmid. Large plasmids from ten strains carried *tetO*.

Plasmids ranged in size from small plasmids to larger plasmids (21 - 50kb in size). The most frequently isolated plasmids were between 31 – 40kb in size, with approximately one third of plasmid harbouring strains carrying plasmids of this size. Interestingly, all strains harbouring plasmids of this size were isolates of *C. jejuni*. Wardak *et al*, 2007 reported that Polish tetracycline resistant *Campylobacter* isolates harboured similarly sized plasmids between 34 and 45kb. Larger plasmids in the range of 41–50kb in size were carried by 11.8% of plasmid harbouring strains examined in our study, and have previously been isolated from tetracycline resistant *C. jejuni* and *C. coli* isolates from the USA and UK (Batchelor *et al*, 2004).

Similarities between plasmids carried by 25 isolates from our collection were determined through examination of *Cla*I profiles. 56% of plasmids examined had similar profiles, indicating that these strains harboured similar or the same plasmids. Interestingly, two plasmids carried *tetO* and conjugatively transferred to a recipient strain. Although profiles of plasmids from these strains were similar, sizes of plasmids ranged from approximately 22-30kb to 42kb.

The remaining 44% of plasmids subjected to *Cla*I profiling had unique profiles that were only harboured by that individual plasmid. Plasmids possessing unique profiles ranged in size from approximately 20kb to 50kb and interestingly, eight plasmids carried *tetO*, of which four conjugatively transfer resistance. The plasmids possessing unique profiles are likely to be unrelated indicating genetic diversity amongst plasmid harboured by our strains. Genetic heterogeneity amongst plasmids from tetracycline resistant *C. jejuni* and *C. coli* isolates was also noted by Wardak *et al*, 2007.

It is interesting to note that when analysed with *Cla*I, more than two thirds of *tetO* carrying plasmids isolated from resistant strains in this study had unique profiles. It is likely that the the *tetO* gene is not harboured by a commonly occurring plasmid, but instead may be carried by a variety of plasmids. This observation is in contrast to the proposal of Friis *et al*, 2006 that the large plasmids observed in our study (Pratt & Korolik, 2005) are likely to be related to or contain a backbone of the two sequenced *tetO* carrying plasmids. However, similar to our observations, *tetO* carriage has been observed in closely related plasmids and unrelated plasmids (Schmidt-Ott *et al*, 2005). This may be due to the previously proposed association between mobile genetic elements, such as transposons and IS elements, with *tetO*.

Campylobacter isolates in our study found to harbour *tetO* plasmids were examined for the ability to transfer this gene and thus tetracycline resistance by conjugation. Six of the 10 *C. jejuni* strains in which *tetO* was plasmid mediated were able to transfer tetracycline resistance to a recipient *C. jejuni* strain by intraspecies conjugation. Plasmid harbouring positive control strain JC6 also conjugatively transferred resistance to the same recipient strain. Transfer of tetracycline resistance by conjugation has previously been reported for *Campylobacter* species from many countries including Spain, India, Japan and Canada (Gibreel *et al*, 2004, Velazquez *et al*, 1995 Prasad *et al*, 1994, Sagara *et al*, 1987). Avrain *et al*, 2004 reported spontaneous *in vivo* transfer of *tetO* between *C. jejuni* strains in the chicken digestive tract.

Conjugative plasmids identified in our study may harbour type IV secretion system genes or homologues involved in the conjugation process as recently identified by Friis *et al*,

2006, Schmidt-Ott *et al*, 2005 & Batchelor *et al*, 2004. Type IV secretion system genes were detected on conjugative *tetO* carrying plasmids, such as those sequenced by Batchelor *et al* and also other campylobacter plasmids. As proposed by Friis *et al*, 2006 the presence of type IV secretion system genes implies that isolates carrying these plasmids are capable of conjugative transfer.

Donor strains examined for the ability to transfer tetracycline resistance were found to transfer resistance a recipient strain that harboured native plasmids. Plasmids harboured by recipient strains may have no effect on the uptake of donor plasmid DNA as observed in *H. pylori*. Ando *et al*, 2000 reported that the presence of competing endogenous plasmids in *H. pylori* strains was found to not constitute a substantial barrier to transformation by an *E. coli-H. pylori* shuttle vector.

Also, the presence of plasmids in recipient strains may have an advantageous effect on the uptake ability of recipient strains. Plasmids native to a recipient strain might act as a rescue plasmid by recombining with the incoming plasmid, as proposed by Wang and Taylor, 1990, who reported a 100-fold increase in natural transformation frequencies of plasmid DNA in *Campylobacter* isolates fold when the recipient strain harboured similar plasmids.

In our study a small plasmid was co-transferred with the larger conjugative, *tetO* carrying plasmid when positive control strain JC6 was mated with the recipient strain. Ansary and Radu, 1992 reported that Malaysian tetracycline resistant isolates also co-transferred small plasmids (3.3 and 12.6kb) with a larger plasmid (78kb) by conjugation. Miller *et al*, 2006 identified genes homologous to plasmid mobilisation genes on small cryptic plasmids from

a *C. coli* isolate, indicating that these plasmids are mobilisable. Identification of *mob* genes and an origin of transfer (*oriT*) in the small plasmid harboured by JC6 may confirm our observations.

Transfer of tetracycline resistance between *C. jejuni* strains under study, control strain JC6, and remaining *C. jejuni* and *C. coli* recipient strains could not be demonstrated. Conclusions from matings of two donor strains with one of the *C. coli* recipients could not be drawn due to cross resistance of donor and recipient strains, and the lack of *flaA* amplification from the recipient strain. This lack of amplification is unlike that observed for other *C. coli* strains examined in our laboratory, and may be due to an insufficient region of homology in the genomic DNA used for primer annealing in *C. coli* NCTC 11366.

Of the recipient strains that failed to transfer resistance, two strains were plasmid free and three strains harboured plasmids. Failure of tetracycline resistance plasmids to transfer resistance was similarly reported by Bacon *et al*, 2000 and Sagara *et al*, 1987. Plasmids harboured by some donor strains may be non-conjugative and non-mobilisable and therefore were unable to transfer. Taylor *et al*, 1988 reported that *tetO* in a Canadian tetracycline resistant *C. coli* isolate was located on a non-transmissible plasmid.

Barriers to conjugal transfer, such as, the host range of the plasmid, incompatibility between plasmids, the inability of plasmids to replicate in the recipient, strain specificity, and restriction modification systems may be responsible for lack of tetracycline resistance transfer in some strains. Incompatibility between plasmids may prevent transfer as many

conjugative plasmids exhibit surface exclusion against plasmids of the same incompatibility group (Van der Hoeven, 1985).

Additionally, differences in restriction modification systems between strains may determine whether conjugal transfer takes place, as these systems enable cells to selectively destroy unmethylated foreign DNAs (Edmonds *et al*, 1992). Differences in restriction modification systems between strains have previously been observed in both campylobacters and *H. pylori* (Ahmed *et al*, 2002, Dorrell *et al*, 2001, Ando *et al*, 2000 & Edmonds *et al*, 1992). Predicted DNA restriction and DNA modification systems in *C. jejuni* strain NCTC 11168 appeared to be particularly variable, with many of these systems absent or highly divergent in other strains examined by Dorrell *et al*, 2001.

Although conjugal transfer of tetracycline resistance between different species of *Campylobacter* has been reported by a number of investigators, with *C. jejuni* donor strains transferring tetracycline resistance plasmids to *C. fetus* subsp. *fetus*, *C. coli* and *C. lari* recipient strains, (Schwartz *et al*, 1993, Tenover *et al*, 1985, Taylor *et al*, 1983, Taylor *et al*, 1986), such interspecies transfer was not observed among our isolates. Four plasmid harbouring *C. jejuni* strains did not transfer tetracycline resistance to a plasmid harbouring *C. coli* recipient strain using Method A. Three of these four strains were found using Method B to transfer the *tetO*-harbouring plasmids to *C. jejuni* recipient strain 887. Plasmids from these three strains may have not been able to conjugate with the *C. coli* strain used in these conjugations due to barriers preventing transfer, such as those described above.

In conclusion, we found high-level tetracycline resistance in Australian *Campylobacter* spp. isolates under study. Although 74% of strains analysed harboured plasmids, the *tetO* gene was mostly chromosomally encoded. Six *C. jejuni* isolates transferred a tetracycline resistance plasmid to a *C. jejuni* recipient strain; however transfer to other *C. jejuni* and *C. coli* recipient strains did not occur. Genetic diversity amongst plasmids, including those that harboured *tetO* was observed, therefore *tetO* and tetracycline resistance may be carried on a variety of plasmids.

CHAPTER 4

TRIMETHOPRIM RESISTANCE

4. TRIMETHOPRIM RESISTANCE

Trimethoprim resistance is commonly elicited through the presence of drug insensitive DHFR enzymes. Generally, most *Campylobacter* spp. isolates are trimethoprim resistant. Despite this fact, little is known of the meachanisms of this resistance. Nothing was known of the prevalence of trimethoprim resistance in Australian *Campylobacter* spp. A selection of *C. jejuni* and *C. coli* isolates, along with additional isolates of other *Campylobacter* species were examined for trimethoprim susceptibility and the presence of plasmids, mobile genetic elements possibly involved in trimethoprim resistance.

4.1 TRIMETHOPRIM SUSCEPTIBILITY

A random selection of 83 *Campylobacter* spp. isolates from our culture collection was examined for trimethoprim MICs. Strain, species and source data for strains is displayed in **Table 3**, **4**, **5**, **6** and **7** in **Materials and Methods**. Isolates examined included strains of *C. jejuni* (62 isolates), *C. coli* (11 isolates) and one isolate only identified to *C. jejuni/coli* level, and a selection of isolates of other *Campylobacter* species including thermophilic species *C. lari* (five strains) and non-thermophilic species *C. fetus* subsp. *fetus* (two isolates) and single isolates of *C. hyointestinalis* and *C. sputorum* bv. *sputorum*.

Genome sequenced *C. jejuni* strains NCTC 11168 and RM1221 were included in the selection of strains. Isolates examined were from different sources, including human and chicken origin (which constituted the largest number of strains) and five isolates of other origins such as porcine, canine and bovine.

82 of the 83 isolates examined were found to have MICs of >1000μg/ml. The single remaining isolate, a strain of *C. hyointestinalis*, had an MIC of 1000μg/ml. MICs of all strains examined are displayed in **Tables 12** to **16**.

4.2 PLASMID CARRIAGE

121 trimethoprim resistant strains were analysed for native plasmid DNA. Strains under study included the 83 isolates for which trimethoprim MICs were determined described above, and, an additional 38 *C. jejuni* and *C. coli* strains which were previously found to have trimethoprim MICs of >100μg/ml when tested against concentrations up to 100μg/ml. Plasmid carriage and size data is summarised in **Tables 12** to **16**. Plasmid profiles of a selection of strains are presented in **Figure 9** and **10**.

As plasmids were not subjected to restriction endonuclease digestion, exact sizes could not be determined. Therefore we classified our plasmids into two size groups - 'small' and 'large'. 'Small plasmid(s)' referred to plasmids appearing smaller than the 23kb fragment of the λ*Hind*III marker when analysed by agarose gel electrophoresis. 'Large plasmid(s)' referred to plasmids which appeared larger than the 23kb fragment of the λ*Hind*III marker when analysed by agarose gel electrophoresis.

65 of the 121 strains examined were found to harbour plasmid DNA. Plasmid harbouring strains included 24 *C. jejuni* isolates of chicken origin, 21 *C. jejuni* strains of human/clinical origin, five human/clinical *C. coli* strains, five chicken *C. coli* isolates, one C. jejuni/coli isolate of chicken origin and one C. jejuni/coli isolate of human origin.

Table 12. Trimethoprim MICs and plasmid carriage data of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Trimethoprim MIC (μg/ml)	Plasmid carriage & size
0-10	*C. jejuni*	Chicken	USA	>100μg/ml*	2 large plasmids
004	*C. jejuni*	Chicken	Australia	>100μg/ml*	Large plasmid
007	*C. jejuni*	Chicken	Australia	>100μg/ml*	Large plasmid
8	*C. jejuni*	Human	Australia	>100μg/ml*	2 large plasmids
008	*C. jejuni*	Chicken	Australia	>1000	–
011	*C. lari*	Chicken	Australia	>1000	3 large plasmids
013	*C. jejuni*	Chicken	Australia	>100μg/ml*	Large plasmid
017	*C. jejuni*	Chicken	Australia	>100μg/ml*	–
018	*C. coli*	Chicken	Australia	>100μg/ml	2 small plasmids
019	*C. jejuni*	Chicken	Australia	>100μg/ml	Large plasmid
3-9	*C. jejuni*	Chicken	USA	>1000	Large plasmid
32	*C. jejuni*	Human	Australia	>1000	Large plasmid
93	*C. jejuni*	Human	Australia	>100μg/ml*	–
108	*C. jejuni*	Human	Australia	>100μg/ml*	–
108	*C. jejuni*	Chicken	Netherlands	>100μg/ml*	–
131	*C. jejuni*	Chicken	Australia	>100μg/ml*	2 large plasmids
133	*C. jejuni*	Chicken	Australia	>100μg/ml*	Large plasmid
134	*C. jejuni*	Chicken	Australia	>1000	Large plasmid
141	*C. jejuni/coli*	Chicken	Australia	>100μg/ml*	–
142	*C. jejuni/coli*	Chicken	Australia	>1000	Small plasmid
173	*C. jejuni/coli*	Human	Australia	>100μg/ml*	Large plasmid
231	*C. jejuni*	Chicken	Australia	>100μg/ml*	–
235	*C. jejuni*	Chicken	Australia	>1000	–
239	*C. jejuni*	Human	Australia	>100μg/ml*	Large plasmid
293	*C. coli*	Human	Australia	>100μg/ml*	Large plasmid (≈21kb)
303	*C. jejuni*	Chicken	Australia	>100μg/ml*	–
336	*C. jejuni*	Chicken	Australia	>1000	–

Table 13. Trimethoprim MICs and plasmid carriage data of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Trimethoprim MIC (μg/ml)	Plasmid carriage & size
351	*C. jejuni*	Human	Australia	>100μg/ml*	2 large plasmids
354	*C. jejuni*	Human	Australia	>100μg/ml*	–
375	*C. jejuni*	Human	Australia	>100μg/ml*	Small plasmid
388	*C. coli*	Human	Australia	>100μg/ml*	2 small plasmids & large plasmid
405	*C. jejuni*	Chicken	Australia	>100μg/ml*	Large plasmid
410	*C. jejuni*	Human	Australia	>1000	–
413	*C. jejuni*	Chicken	Australia	>100μg/ml*	Small plasmid
415	*C. jejuni*	Chicken	Australia	>1000	–
430	*C. jejuni*	Human	Australia	>100μg/ml*	–
435	*C. jejuni*	Human	Australia	>100μg/ml*	–
439	*C. jejuni*	Human	Australia	>100μg/ml*	Large plasmid
440	*C. jejuni*	Human	Australia	>1000	–
458	*C. jejuni*	Human	Australia	>100μg/ml*	–
506	*C. jejuni*	Chicken	Australia	>1000	2 small plasmids & 2 large plasmids
515	*C. jejuni*	Human	Australia	>100μg/ml*	–
520	*C. jejuni*	Human	Australia	>100μg/ml*	–
605	*C. jejuni*	Chicken	Australia	>1000	–
657	*C. coli*	Human	Australia	>100μg/ml*	2 large plasmids
691	*C. coli*	Human	Australia	>100μg/ml*	–
705	*C. jejuni*	Human	Australia	>100μg/ml*	Large plasmid
801	*C. jejuni*	Chicken	Australia	>1000	Large plasmid (≈21-37kb)
886	*C. jejuni*	Human	Australia	>100μg/ml*	–
887	*C. jejuni*	Human	Australia	>1000	Large plasmid
957	*C. jejuni*	Human	Australia	>100μg/ml*	2 large plasmids
961	*C. coli*	Chicken	Australia	>1000	2 small plasmids & large plasmid
4547	*C. jejuni*	Human	Australia	>100μg/ml*	Small plasmid
3015	*C. jejuni*	Chicken	Australia	>1000	–

Table 14. Trimethoprim MICs and plasmid carriage data of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Trimethoprim MIC (μg/ml)	Plasmid carriage & size
4002	*C. jejuni*	Chicken	Australia	>1000	2 small plasmids
5001	*C. jejuni*	Chicken	Australia	>1000	Large plasmid
5565	*C. coli*	Human	Japan	>1000	Large plasmid
7005	*C. jejuni*	Chicken	Australia	>1000	–
7006	*C. coli*	Chicken	Australia	>1000	Small plasmid & large plasmid
8004	*C. coli*	Chicken	Australia	>1000	2 small plasmids & large plasmid
9014	*C. jejuni*	Chicken	Australia	>1000	Large plasmid
9126	*C. jejuni*	Human	Australia	>1000	–
81116	*C. jejuni*	Human	UK	>1000	–
85-239	*C. lari*	Human	USA	>1000	–
85-241	*C. lari*	Human	USA	>1000	–
85-244	*C. lari*	Human	USA	>1000	2 large plasmids
85-256	*C. fetus* subsp. *fetus*	Human	USA	>1000	Large plasmid
0519/010	*C. coli*	Human	Australia	>100μg/ml*	–
0520/072	*C. jejuni*	Human	Australia	>1000	Large plasmid
B5Ft8	*C. jejuni*	Chicken	USA	>1000	–
C50	*C. jejuni*	Chicken	Australia	>1000	–
C163	*C. jejuni*	Chicken	Australia	>1000	–
C170	*C. jejuni*	Chicken	Australia	>1000	–
C491	*C. jejuni*	Chicken	Australia	>1000	Large plasmid
C560	*C. jejuni*	Chicken	Australia	>1000	–
CE01	*C. jejuni*	Chicken	USA	>1000	Large plasmid
CE04	*C. coli*	Chicken	USA	>1000	–
CE05	*C. jejuni*	Chicken	USA	>1000	–
CE06	*C. jejuni*	Chicken	USA	>1000	–
CE07	*C. jejuni*	Chicken	USA	>1000	2 large plasmids
CE7-8	*C. jejuni*	Chicken	USA	>1000	2 large plasmids
CE09	*C. coli*	Chicken	USA	>1000	Large plasmid
CSIRO11	*C. fetus* subsp. *fetus*	Human	Australia	>1000	–

Table 15. Trimethoprim MICs and plasmid carriage data of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Trimethoprim MIC (μg/ml)	Plasmid carriage & size
CSIRO40	*C. coli*	Human	Australia	>1000	2 small plasmids & large plasmid
D117	*C. jejuni*	Human	USA	>1000	Large plasmid
D123	*C. jejuni*	Human	Canada	>1000	Large plasmid
D2189	*C. hyointestinalis*	Human	USA	1000	–
FF1	*C. jejuni*	Human	Australia	>1000	Large plasmid
FF3	*C. jejuni*	Human	Australia	>1000	Large plasmid
FF5	*C. jejuni*	Human	Australia	>100μg/ml*	Large plasmid
FF18	*C. jejuni*	Human	Australia	>1000	–
FF34	*C. jejuni*	Human	Australia	>1000	–
FF45	*C. jejuni*	Human	Australia	>1000	–
JC1	*C. jejuni*	Human	Japan	>1000	–
JC2	*C. jejuni*	Human	Japan	>1000	–
JC3	*C. jejuni*	Human	Japan	>1000	Large plasmid
JC5	*C. jejuni*	Human	Japan	>1000	Large plasmid
JC6	*C. jejuni*	Human	Japan	>1000	2 small plasmids & large plasmid
JC8	*C. jejuni*	Human	Japan	>1000	–
JC10	*C. jejuni*	Chicken	Japan	>1000	2 small plasmids
JC11	*C. jejuni*	Chicken	Japan	>1000	2 large plasmids
JC13	*C. coli*	Cat	Japan	>1000	Large plasmid
JC14	*C. coli*	Dog	Japan	>1000	Large plasmid
PRC13	*C. jejuni*	Chicken	USA	>1000	–
PRC67	*C. jejuni*	Chicken	USA	>1000	2 large plasmids
RM2	*C. jejuni*	Human	Australia	>1000	–
RM3	*C. coli*	Human	Australia	>1000	Large plasmid
RM6	*C. jejuni*	Human	Australia	>1000	N. D.
RM7	*C. jejuni*	Human	Australia	>1000	Large plasmid
RM10	*C. jejuni*	Human	Australia	>1000	–
RM16	*C. jejuni*	Human	Australia	>1000	–

Table 16. Trimethoprim MICs and plasmid carriage data of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Trimethoprim MIC (μg/ml)	Plasmid carriage & size
RM17	*C. jejuni*	Human	Australia	>1000	Large plasmid
RM19	*C. jejuni*	Human	Australia	>1000	–
RM20	*C. jejuni*	Human	Australia	>1000	–
RM103	*C. jejuni*	Human	Australia	>1000	Large plasmid
RM1221	*C. jejuni*	Chicken	USA	>1000	–
TC19	*C. jejuni*	Chicken	USA	>1000	–
NCTC 11168-GS	*C. jejuni*	Human	UK	>1000	–
NCTC 11351	*C. jejuni*	Bovine	USA	>1000	–
NCTC 11366	*C. coli*	Pig	UK	>1000	3 small plasmids & large plasmid
ATCC 33562	*C. sputorum* bv. *sputorum*	Bovine	Belgium	>1000	Large plasmid
ATCC 35223	*C. lari*	Human	UK	>1000	–

* - MICs of strains were previously determined against concentrations of up to 100μg/ml of trimethoprim

N. D. Not Determined

'Small plasmid(s)' referred to plasmids appearing smaller than the 23kb fragment of the λ*Hind*III marker when analysed by agarose gel electrophoresis.

'Large plasmid(s)' referred to plasmids which appeared larger than the 23kb fragment of the λ*Hind*III marker when analysed by agarose gel electrophoresis.

Also, 2 isolates of *C. lari* and one isolate each of *C. fetus* subsp. *fetus* and *C. sputorum* bv. *sputorum* harboured plasmids. Single isolates of feline, canine, porcine and bovine origin were found to harboured plasmids in addition to those of human/clinical or chicken origin. 44 Australian isolates, 10 isolates from the USA, eight Japanese isolates and single isolates from the UK, Canada and Belgium harboured plasmids.

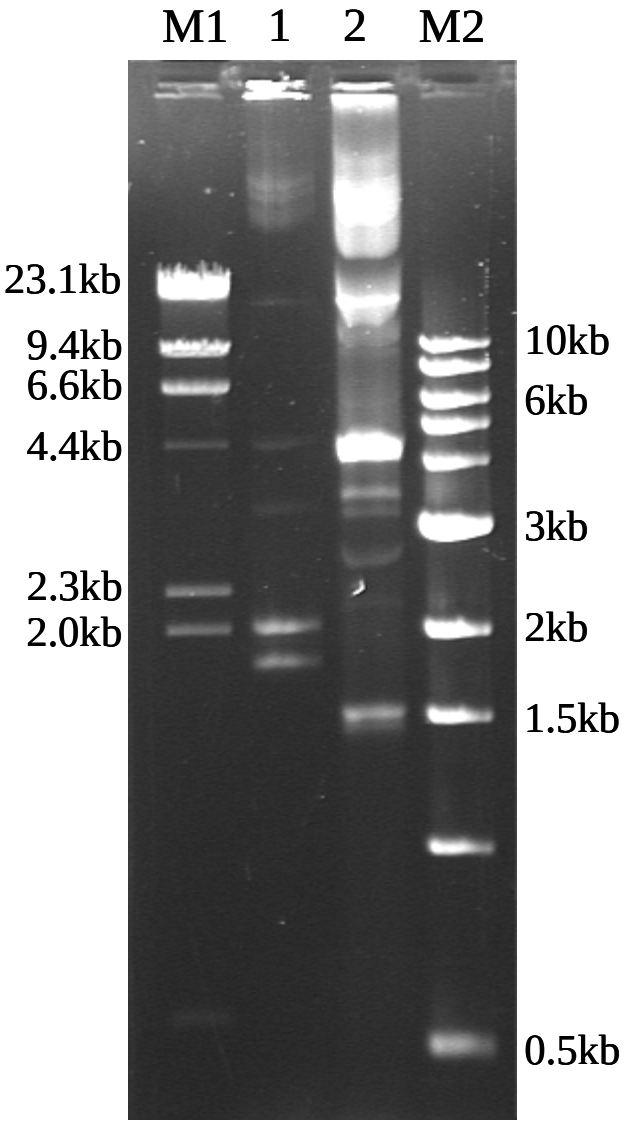

Figure 9. Plasmid profiles of trimethoprim resistant strains of *C. coli*. Lanes: M1 & M2, molecular weight markers *Hin*dIII digested λ DNA and a 1kb ladder respectively; 1 & 2 – plasmids isolated from strains 388 and NCTC 11366.

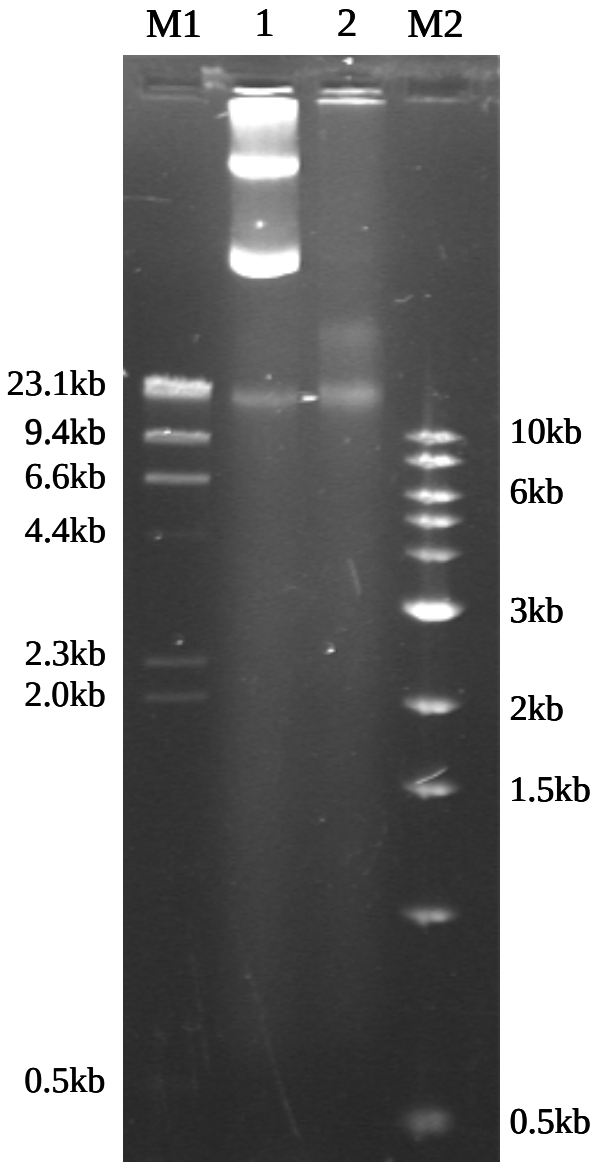

Figure 10. Plasmid profiles of *C. jejuni* and *C. lari* isolates. Lanes: M1 & M2, molecular weight markers *Hin*dIII digested λ DNA and a 1kb ladder respectively; 1 & 2 – plasmids isolated from strains 011 and 957.

Of the 65 plasmid harbouring strains, 42 strains harboured single plasmids of which the majority harboured single large plasmids. Four isolates carried single small plasmids. 23 strains carried multiple plasmids (two to four), in various combinations of small and large plasmids. 10 of the 23 multiple plasmid harbouring strains carried two large plasmids. Multiple plasmid harbouring strains included isolates of *C. jejuni* (13 strains), *C. coli* (eight strains) and *C. lari* (two strains) of chicken (14 strains), human/clinical (8 strains) and porcine (one strain) origin.

56 of the 121 strains examined did not harbour deteacble plasmid DNA. These strains included 25 human/clinical isolates of *C. jejuni*, 21 chicken isolates of *C. jejuni*, two human/clinical isolates of *C. coli*, a single *C. coli* isolate of chicken origin and one *C. jejuni/coli* of chicken origin. Strains that did not harbour detectable plasmids also included three *C. lari* isolates and single isolates of *C. fetus* subsp. *fetus* and *C. hyointestinalis*. 38 Australian isolates, 11 isolates from the USA, three isolates from the UK and Japan, and one Dutch isolate did not harbour plasmid DNA.

In Summary. A selection of over 100 *C. jejuni* and *C. coli* isolates as well as isolates of other *Campylobacter* species were examined for trimethoprim resistance and plasmid carriage. All but one of the isolates examined had trimethoprim MICs of >1000μg/ml, and 65 of 121 strains examined for plasmids harboured single or multiple plasmids, ranging in size from small to large.

4.3 DISCUSSION

In this study a selection of *Campylobacter* isolates were examined for resistance to trimethoprim, and for the presence of plasmids, genetic elements involved in the acquisition and dissemination of antibiotic resistance. High level trimethoprim resistance was observed in all isolates examined for resistance. MICs of >1000μg of trimethoprim per ml were observed for all but one strain. The remaining strain, a single isolate of *C. hyointestinalis*, had a lower, though still high, resistance level.

Further investigation into resistance levels of the *Campylobacter* isolates under study would be of interest. Using higher trimethoprim concentrations such as 1,500, 2000, 2,500μg/ml increased in smaller increments, and a known negative control strain we could determine to what level greater than 1000μg/ml our strains are resistant to. Differences in trimethoprim resistance levels due to species was not evident between isolates of *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* subspecies *fetus* and *C. sputorum* bv. *sputorum*, as the same MIC values were observed. Isolates from varied sources (clinical, poultry, feline, canine, porcine and bovine) and countries of origin did not differ in resistance levels.

Campylobacter strains from Australia, Japan, the USA, the UK, Canada and Belgium were found to be highly resistant to trimethoprim as were Swedish and Canadian *C. jejuni* isolates (Gibreel & Skold, 1998 & Karmarli *et al*, 1981). All Canadian *C. jejuni* isolates examined by Karmarli *et al*, were highly resistant with MICs of 256μg/ml and ≥512 μg/ml, and, 96.4% of strains tested were inhibited by≥512 μg/ml. Gibreel and Skold reported high level resistance (MICs of 500 to 1000μg/ml) in approximately 90% of Swedish clinical *C. jejuni* isolates.

Campylobacter spp. from other countries, including Irish *C. jejuni* and *C. coli* isolates of different origins and a multiple antibiotic resistant clinical *C. jejuni* isolate from Jordan, have been reported to be trimethoprim resistant (Lucey *et al*, 2000a, Lucey *et al*, 2000b & Winstanley *et al*, 1993). The two genome sequenced strains *C. jejuni* NCTC 11168 (11168-GS) and *C. jejuni* RM1221, isolated in the UK and USA respectively, were reported by Fouts *et al*, 2005 to be resistant to trimethoprim when tested against a concentration of 5μg/ml of antibiotic. These strains were found to be highly resistant, with MICs of >1000μg/ml when tested against higher concentrations.

In our study high-level trimethoprim resistance was observed in *Campylobacter* species other than the most commonly isolated members of the genus. Non-*C. jejuni/coli* species such as *C. hyointestinalis*, *C. lari*, *C. fetus* and *C. sputorum* have been demonstrated to be implicated as gastrointestinal pathogens to humans (although some are rare) (Moore *et al*, 2005). Only a small number of non-*C. jejuni/coli* isolates were examined in our study, and a study employing a larger sample would give a clearer picture of the prevalence of this resistance in these species. Antibiotic resistance studies in *C. jejuni* and *C. coli* are far more prevalent. Reports of trimethoprim resistance in isolates of *C. fetus*, *C. hyointestinalis* and *C. sputorum* bv. *sputorum* were unable to be located. And, it appears that there is little known of this resistance and its mechanisms in these species.

Trimethoprim MICs for *Campylobacter* species closed related to *C. jejuni* were reported by Taylor and Courvalin, 1988 to be within the ranges reported for *C. jejuni*. Closely related species presumably included *C. coli* and possibly other thermophilic species, such as *C. lari*. *C. lari*, thermophillic and genetically close to *C. jejuni* and *C. coli*, is most commonly

isolated from human and animal diarrhoea (Fouts *et al*, 2005 & On, 2001). Fouts *et al*, 2005 found that the partially sequenced *C. lari* strain was trimethoprim resistant, when tested against 5μg/ml of antibiotic. It is interesting to note that, although not examined in our study, another thermophilic species, *C. upsaliensis*, was been found to have trimethoprim MICs of ≥128μg/ml (Preston *et al*, 1990).

As previously stated, all *C. jejuni* and *C. coli* isolates are intrinsically resistant to trimethoprim (Gibreel and Skold, 2000, Gibreel and Skold, 1998, Taylor & Courvalin, 1988 & Karmali *et al*, 1981) and MICs range from 256μg/ml to ≥512μg/ml (Taylor & Courvalin, 1988). As high level resistance in all 83 isolates, including isolates of different origin and species, was observed, with all but one strain resistant to concentrations greater than 1000μg/ml, it could be proposed in accordance with Karmali's observations that trimethoprim resistance in these isolates is intrinsic.

Assuming that all *Campylobacter* strains are intrinsically resistant to trimethoprim, strains and resistance levels would not be influenced by initial isolation and subsequent cultivation on trimethoprim containing media such as Skirrow's medium (as used in our laboratory). Or, although intrinsically resistant to a certain level, exposure to the antibiotic may increase resistance levels. And if other resistance mechanisms are also involved, prior exposure to the antibiotic may select for more resistant variants and or also increase resistance levels. A detailed study employing 'fresh' samples, antibiotic free isolation media and an examination of trimethoprim resistance levels may help to answer these questions. However, exposure of isolates to trimethoprim in poultry or clinical settings may be an unknown factor that could also influence results.

Plasmids may play a role in the acquisition and dissemination of trimethoprim resistance genes and associated resistance phenotypes among campylobacters. 53.7%, just over half of the *Campylobacter* isolates examined in this study harboured plasmids. About two thirds (64.6%) of plasmid harbouring isolates carried single plasmids, the majority of which were large plasmids. The remaining 35.4% of plasmid harbouring strains carried multiple plasmids. Between two to four plasmids in varying combinations were observed. Two large plasmids were harboured by 43.5% of multiple plasmid harbouring strains.

Plasmid harbouring strains were of poultry, clinical, feline, canine, porcine and bovine origin, and, were isolated in Australia, the USA, Japan, the UK, Canada and Belgium. A number of studies have reported the presence of plasmids in isolates of *Campylobacter*. Plasmids have been detected in strains from Canada, the USA, Brazil, Poland, the UK Portugal, Spain, Israel, Taiwan, Japan, India, Thailand and Malaysia. Plasmid carriage rates and the number of plasmids harboured by strains vary between reports. Plasmids have been found in between 13 to 53% of *C. jejuni*, with the majority being resistance plasmids (Bacon *et al*, 2000 & Gibreel *et al*, 2004a). As previously stated, plasmid incidence in *C. jejuni* also varies from one geographical area to another and from source to source (Lekowska-Kochaniak *et al*, 1996).

Plasmids were carried by isolates of *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* subsp. *fetus* and *C. sputorum* bv. *sputorum*. Little information is available on plasmid carriage in non-*C. jejuni/coli* species. However, plasmids have been detected in some isolates of *C. fetus*, *C. lari* and *C. hyointestinalis* by other investigators. Fouts *et al*, 2005 reported that the trimethoprim resistant, partially sequenced, *C. lari* strain harboured a single plasmid of

approximately 46kb in size. A small cryptic plasmid from a *C. hyointestinalis* isolate was analysed by Waterman *et al*, 1993, indicating the presence of plasmids in *C. hyointestinalis*. Varga, 1991 reported that isolates of *C. fetus* subsp. *fetus* harboured plasmids.

Plasmid-mediated trimethoprim resistance, due to the production of an additional, trimethoprim resistant, DHFR enzyme encoded by foreign genes, is the most clinically important mechanism of resistance. It is also the most common mechanism in enterobacteria (Amyes & Smith, 1974 in Adrian *et al*, 1998). But in contrast, there are only three known plasmid-encoded resistance genes in gram-positive organisms, *dfrA* and *dfrD* (Coque *et al*, 1999). The genes encoding some plasmid-borne resistant DHFR enzymes may be temporarily located on the chromosome due to transposon movement, but are still referred to as plasmid-borne or transferable DHFRs (Huovinen *et al*, 1995).

As of 1995, most of the genes coding for characterized DHFR enzymes were located within integrons as gene cassettes (Huovinen *et al*, 1995). Integrons are in turn located within transposons and/or plasmids, mobile genetic elements involved in the dissemination of antibiotic resistance genes among different bacterial species and genera (Heikkila *et al*, 1990 & Recchia & Hall, 1995 in Adrian & Klugman, 1997, Huovinen *et al*, 1995, Coque *et al*, 1999).

The role of plasmids in trimethoprim resistance in *Campylobacter* is unknown. Only one study has reported the presence of plasmids in *Campylobacter* strains found to be trimethoprim resistant. Other studies have not stated whether strains examined harboured plasmids. Fouts *et al*, 2005 reported the presence of plasmids in both partially sequenced *C.*

coli and *C. lari* strains. However, the two completely sequenced *C. jejuni* strains did not harbour plasmid DNA (Fouts *et al*, 2005).

The same trimethoprim resistance levels were observed among isolates that harboured plasmids and those that did not harbour detectable plasmid DNA. It is likely that not all *Campylobacter* isolates harbour plasmids, as rates of less than 100% have been reported (Bacon *et al*, 2000 & Gibreel *et al*, 2004a and Pratt & Korolik, 2005). Diversity in plasmid carriage rates could indicate that plasmids are not involved in trimethoprim resistance. And as *Campylobacter* may be intrinsically resistant to trimethoprim, it is less likely that plasmids play a key role in this resistance. Resistance (*dfr1*) genes and genetic elements, such as integrons that are not plasmid associated may play a greater role in trimethoprim resistance.

In conclusion, high level trimethoprim resistance was observed in all isolates examined. This high level resistance was observed in Australian isolates of *C. jejuni* and *C. coli* and isolates from other countries. Additionally, in our study high-level trimethoprim resistance was also observed in *Campylobacter* species other than the most commonly isolated members of the genus, including *C. lari*, *C. fetus* subspecies *fetus* and *C. sputorum* bv. *sputorum*. Just over half of the *Campylobacter* isolates examined in this study harboured plasmids, and this may indicate that plasmids are not involved in trimethoprim resistance in these species.

CHAPTER 5

TRIMETHOPRIM RESISTANCE GENES

5. TRIMETHOPRIM RESISTANCE GENES

The genetic basis of trimethoprim resistance in *Campylobacter* spp. isolates has only been examined in a small number of studies. Two *dfr* genes, *dfr1* and *dfr9*, were found to encode resistant DHFR enzymes, conferring trimethoprim resistance in Swedish isolates of *C. jejuni* (Gibreel & Skold, 1998 and Gibreel & Skold, 2000).

Nothing was known of the presence and prevalence of trimethoprim resistance encoding *dfr* genes, including the previously described *Campylobacter* associated genes, in Australian campylobacters. Previously identified trimethoprim resistant *C. jejuni* and *C. coli* isolates, along with a small selection of a variety of resistant thermophilic and non-thermophillic *Campylobacter* spp. isolates were examined for the presence of *dfr1* and *dfr9*.

5.1 DETECTION OF *dfr* GENES

5.1.1 dfr 1

The 83 randomly selected *Campylobacter* spp. isolates examined in the previous chapter for trimethoprim resistance and the additional 38 *C. jejuni* and *C. coli* strains which were previously found to be trimethoprim resistance were chosen for analysis. Strains examined are listed in **Table 3**, **4**, **5**, **6** and **7** in **Materials and Methods**.

In total, 122 trimethoprim resistant strains were examined for the presence of the *dfr1* gene using PCR analysis as described in **Materials and Methods**. Strains included 94 isolates of *C. jejuni*, 16 *C. coli* isolates, three isolates only identified to *C. jejuni/coli* level, five *C. lari* isolates, two *C. fetus* subsp. *fetus*, and single isolates of *C. hyointestinalis* and *C. sputorum*

bv. *sputorum*. The majority of isolates were of either human/clinical or chicken origin. In addition, representative isolates of bovine, feline, canine and porcine origin were also analysed. *dfr1* PCR results for a selection of strains are shown in **Figure 11**.

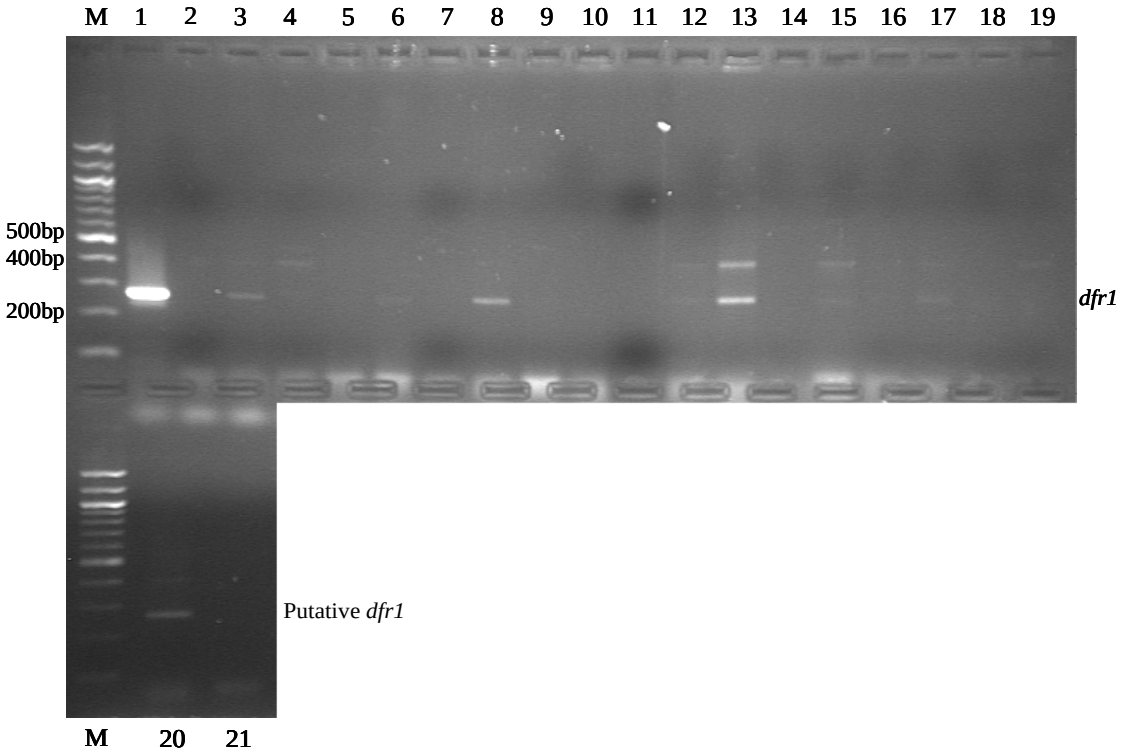

Figure 11. Agarose gel electrophoresis of *dfr1* PCR results from a selection of trimethoprim resistant *C. jejuni*, *C. coli*, *C. lari* and *C. fetus* subsp. *fetus* isolates. The *dfr1* fragment is ≈254bp, putative *dfr1* bands are between ≈240-250bp and ≈260-270bp and products other than putative *dfr1* are ≈400bp and ≈450bp. Lanes: M, molecular weight marker (100bp ladder); 1, positive control strain *E. coli* ColE1::Tn7; 2-20, strains 801, 5565, 011, 85-239, 85-241, 85-244, ATCC 35223, CSIRO 11, 7005, 7006, 8004, 9014, CSIRO 40, C50, C163, C170, C491, C560, JC14; 21, water control.

Products of the expected *dfr1* size (254bp) along with products slightly smaller (≈240-250bp) or larger (≈260-270bp) were amplified from strains, and were considered 'putative' *dfr1* as products were not DNA sequenced. Putative *dfr1* products were amplified from 19 of the 122 strains examined for this gene. Putative *dfr1* positive isolates are listed in **Table 17**.

Table 17. *C. jejuni*, *C. coli* and *C. lari* isolates from which putative *dfr1* was amplified

Strain	**Species**	**Source**	**Country**	***dfr1* gene**
0-10	*C. jejuni*	Chicken	USA	Putative *dfr1*[#]
007	*C. jejuni*	Chicken	Australia	Putative *dfr1*
8	*C. jejuni*	Human	Australia	Putative *dfr1*[#]
011	*C. lari*	Chicken	Australia	Putative *dfr1*[#]
017	*C. jejuni*	Chicken	Australia	Putative *dfr1*[#]
173	*C. jejuni/coli*	Human	Australia	Putative *dfr1*
293	*C. coli*	Human	Australia	Putative *dfr1*[#]
413	*C. jejuni*	Chicken	Australia	Putative *dfr1*
439	*C. jejuni*	Human	Australia	Putative *dfr1*[#]
440	*C. jejuni*	Human	Australia	Putative *dfr1*
957	*C. jejuni*	Human	Australia	Putative *dfr1*[#]
5565	*C. coli*	Human	Japan	Putative *dfr1*[#]
8004	*C. coli*	Chicken	Australia	Putative *dfr1*[#]
9014	*C. jejuni*	Chicken	Australia	Putative *dfr1*[#]
85-241	*C. lari*	Human	USA	Putative *dfr1*
C50	*C. jejuni*	Chicken	Australia	Putative *dfr1*[#]
C170	*C. jejuni*	Chicken	Australia	Putative *dfr1*[#]
JC14	*C. coli*	Dog	Japan	Putative *dfr1*[#]
ATCC 35223	*C. lari*	Human	UK	Putative *dfr1*

[#] - Products other than *dfr1* were also produced in PCR experiments

Putative *dfr1* positive strains consisted of 11 isolates of *C. jejuni*, four isolates of *C. coli*, one isolate only identified to *C. jejuni/coli* level and three isolates of *C. lari*, including one type strain. Isolates were of human/clinical, chicken and canine origin, and, were isolated in Australia, the USA, Japan and the UK.

In addition to putative *dfr1* amplicons, 13 of the 19 positive strains produced products of sizes other than that of putative *dfr1*. Refer to **Appendix 1, Table 19, 20, 21, 22** and **23** for a detailed list of products amplified from strains examined and approximate sizes. These products ranged from ≈110-120bp to ≈600bp in size and eight of the 13 strains produced a product of ≈400bp. An example of products amplified other than putative *dfr1* can be seen in **Figure 11**.

Products other than putative *dfr1* were amplified from 40 of 103 strains negative for putative *dfr1*. As for positive strains that produced such products, the most frequent amplicon was ≈400bp in size with 21 of the 40 putative *dfr1* negative strains producing a product of this size. Products amplified from putative *dfr1* negative strains had the same range as those amplified from putative *dfr1* positive strains. Genome sequenced strain *C. jejuni* NCTC 11168 (11168-GS) produced a product other than putative *dfr1*. No product was amplified from the other genome sequenced strain *C. jejuni* RM1221, and type strains *C. jejuni* NCTC 11351 and *C. coli* NCTC 11366.

5.1.2 dfr 9

The 122 trimethoprim resistant *Campylobacter* isolates analysed for the presence of *dfr1* were examined for the presence of the *dfr9* gene by PCR analysis as described in **Materials**

and Methods. *dfr9* PCR results for a selection of strains are shown in **Figure 12**. All 122 strains examined for the presence *dfr9* were negative for this gene.

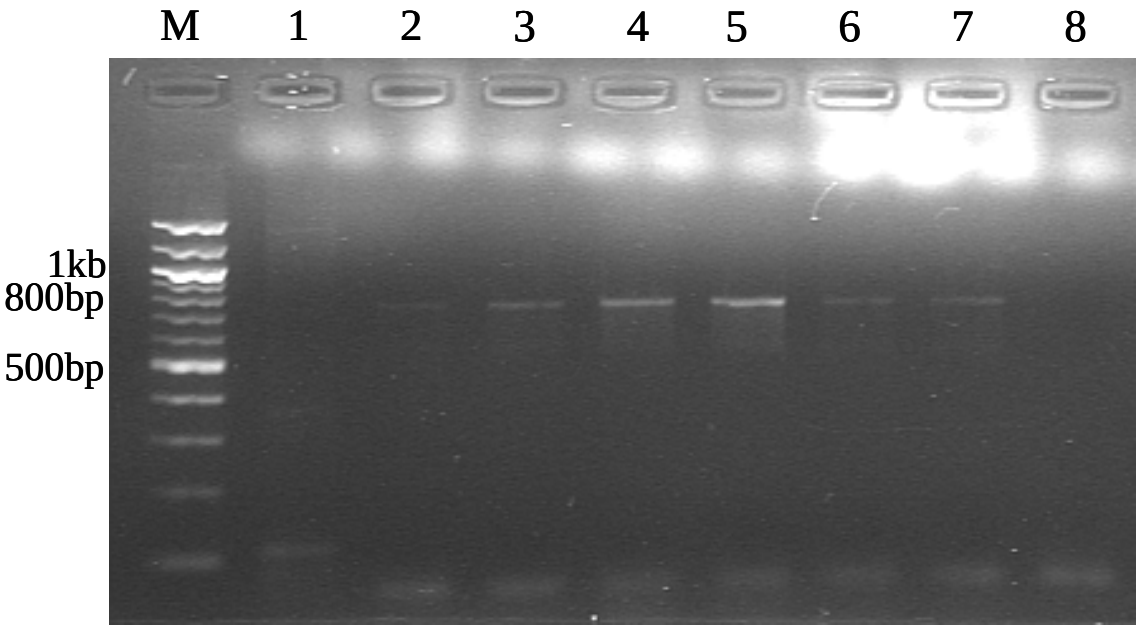

Figure 12. Agarose gel electrophoresis of *dfr9* PCR results from a selection of trimethoprim resistant *C. jejuni* isolates. Products amplified other than the expected *dfr9* product are ≈800bp. Lanes: M, molecular weight marker (100bp ladder); 1, Non-*Campylobacter* strain *E. coli* ColE1::Tn7; 2-7, strains 019, 4547, 9014, NCTC 11168-GS, N. A., 4002; 8, water control. N.A. – Not Applicable

15 strains produced products other than the predicted *dfr9* product (398bp). Refer to **Appendix 1, Table 19, 20, 21, 22** and **23** for a detailed list of amplification products and approximate sizes. Strains that produced products other than *dfr9* included 11 isolates of *C. jejuni* and single isolates of *C. coli*, *C. lari*, *C. hyointestinalis* and *C. sputorum* bv.

sputorum. Strains were of chicken, human/clinical, canine and bovine origin and were isolated in Australia, Japan, the USA, the Netherlands, the UK and Belgium.

Amplified products other an appropriate *dfr9* fragment ranged in size from≈200 -250bp to ≈800-850bp with products of between≈700 -750bp, ≈800bp and ≈800-850bp more frequently amplified. Type strains - genome sequenced strain *C. jejuni* NCTC 11168 (11168-GS) and *C. sputorum* bv. *sputorum* ATCC 33562 produced products other than the expected *dfr9* band.

In Summary. 122 trimethoprim resistant isolates of *C. jejuni*, *C. coli* and other *Campylobacter* species were examined for *dfr1* and *dfr9* genes. Putative *dfr1* products were amplified from 19 of the 122 strains examined for this gene, and all 122 strains examined for the presence *dfr9* were negative for this gene. Products of sizes other than that of putative *dfr1* and the expected size of *dfr9* were also amplified from strains examined.

5.2 DISCUSSION

The prevalence of trimethoprim resistance encoding (*dfr*) genes was previously unknown in Australian campyloabcters prior to this study. Only a small number of the total trimethoprim resistant isolates examined in this study harboured a known *Campylobacter* associated *dfr* gene. 15.6% of the 122 strains examined were positive for putative *dfr1*, a *dfr1*-like product. The low rate of putative *dfr1* prevalence observed in this study is in contrast to the high rate of prevalence reported amongst Swedish *C. jejuni* isolates. Gibreel & Skold, 1998 found that 73% of 41 isolates examined from two geographical regions were positive for *dfr1*.

The putative *dfr1* positive, trimethoprim resistant isolates examined from our collection were of clinical (47.4%), poultry (47.4%) and canine origin (5.3%). Putative *dfr1* was detected in Australian *Campylobacter* isolates and in isolates from other countries, indicating the presence of *dfr1* in campylobacters from countries other than Sweden, the country in which *dfr1* was first identified in *Campylobacter* isolates.

Trimethoprim resistant, putative *dfr1* positive strains identified from our collection were of only thermophilic *Campylobacter* species, with more than half of positive strains isolates of *C. jejuni*. A putative *dfr1* fragment was detected in this study also in isolates of *C. lari*, including one reference strain. As *C. lari* is closely related to *C. jejuni* and *C. coli* (Fouts *et al*, 2005 & On, 2001) and may occupy similar niches, such as the gastrointestinal tract of food animals (chickens, swine, and cattle) or other birds such as seagulls (Aarestrup *et al*, 1997, Takkinen & Ammon, 2003 and Fouts *et al*, 2005), it is not so surprising to find *dfr1* in this species. It would be interesting to determine the presence and prevalence of this resistance gene in atypical/non-thermophilic *Campylobacter* species such as *C. fetus* and *C. hyointestinalis* using a larger sample of isolates than that examined in this study.

Products amplified from strains during *dfr1* PCR analysis were considered putative, as the exact identity of these fragments was not determined through DNA sequencing and comparison with known *dfr1* sequences. Such products may be due to deletion or addition of base pairs within the gene sequence amplified by PCR. DNA sequencing and comparisons with known sequences could determine identity and size of products amplified.

In addition to the amplification of putative *dfr1* amplicons, products of a size other than putative *dfr1*, ranging in size from ≈110-120bp to≈600bp, were amplified from trimethoprim resistant strains examined from our collection. Such products were amplified from both putative *dfr1* positive and putative *dfr1* negative isolates. Products larger than putative *dfr1* fragments may represent variants of *dfr1* containing a different number of repeat regions and/or repeats of a different size, similar to that reported by Gibreel & Skold, 2000, or non-specific amplicons. Products smaller than that of putative *dfr1* may similarly be of a non-specific nature, or may be truncated variants of this gene. Due to time limitations, DNA sequencing and comparisons to gene databases could not be carried out to determine the identity of these amplified products.

Although the *dfr1* gene was putatively detected in trimethoprim resistant isolates examined, the other reported *Campylobacter* associated *dfr* gene *dfr9* was not detected. Due to importation restrictions and the unavailability of a known *dfr9* harbouring strain, a positive control strain could not be included in PCR experiments detecting this gene. The lack of detection of *dfr9* in the *Campylobacter* isolates examined is in contrast to the findings of Gibreel and Skold, 1998, where it was reported that 46% of Swedish *C. jejuni* strains examined carried the *dfr9* gene. Some Swedish strains were also found to harbour both *dfr1* and *dfr9* (Gibreel and Skold, 1998). The lack of detection of *dfr9* in the 122 trimethoprim resistant isolates examined, may indicate that this gene is not widespread and possibly may not be present in Australian *Campylobacter* spp.

Although amplicons of an appropriate *dfr9* size were not amplified from the *Campylobacter* isolates examined, products of sizes other than the expected size of *dfr9* were amplified

from 12.3% of strains, including genome sequenced strain, *C. jejuni* NCTC 11168. Such products may be of a non-specific nature or may be associated with the *dfr9* gene, or, with other *dfr* genes. Redesigning of the PCR protocol to reduce the amplification of non-specific products, and the identification of amplified products could not be carried out due to time limitations. Employment of a known *dfr9* harbouring strain and a larger sample size may help in determining the presence of this gene in trimethoprim resistant strains from our collection.

The significant role of *dfr* genes in trimethoprim resistance, the low rate of putative *dfr1* prevalence and the lack of detection of *dfr9*, implies that there may be other possibly more prevalent *dfr* genes present in the trimethoprim resistant *Campylobacter* isolates examined in this study. Inter-regional diversity in *dfr* gene prevalence has been observed in a number of bacteria, including *Campylobacter* spp. (Blahna *et al*, 2006 & Gibreel and Skold, 1998). *dfr* genes other than *dfr1* have been found to be more common in gram negative isolates in countries such as South Africa and Korea, which is in contrast to the high prevalence of *dfr1* in *E. coli* isolates from Europe and Canada (Adrian *et al*, 1998, Yu *et al*, 2004 & Blahna *et al*, 2006). Differences in prevalence have been proposed to be due to limited contact between continents allowing for different alleles to become common by genetic drift, or, clonal expansion by isolates containing different *dfr* alleles (Blahna *et al*, 2006), and such events may have lead to the presence of *dfr* genes other than *dfr9* may in the isolates examined from our collection.

It is possible that the trimethoprim resistant isolates examined in this study acquired *dfr* genes, other than *dfr1* and *dfr9*, from other bacterial species. *Campylobacter* isolates

inhabit similar niches to *E. coli*, and may acquire DNA from this species, an example of which is the location of *dfr9* on the *C. jejuni* chromosome in an identical configuration to that in porcine *E. coli* (Gibreel and Skold, 1998). Australian *Campylobacter* spp. isolates examined may harbour genes such as *dfr5*, *dfr12*, *dfr17* and *dfrB2* as detected in Australian isolates of *E. coli* by Sidjabat *et al*, 2006 & White *et al*, 2001.

Additionally, strains may harbour *dfr* genes of a gram positive origin, such as the two plasmid mediated *dfr* genes from *Staphylococcal* species (Woodford, 2005, Coque *et al*, 1999, Adrian & Klugman, 1997, Dale *et al*, 1995a, Dale *et al*, 1995b). Several genes of a putative gram positive origin have been detected in campylobacters, including *tetO*, aminoglycoside resistance genes, transposon and insertion sequence homologues, and recently a streptomycin resistance gene from a lactic acid coccus (O'Connor *et al*, 2007, Nirdnoy *et al*, 2005, Manavathu *et al*, 1988, Sougakoff *et al*, 1987 & Taylor *et al*, 1983), hinting at the possibility of gram positive dfr genes in the trimethoprim resistant isolates examined in this study. Determination of the presence of a variety of known *dfr* genes could provide a clearer picture of *dfr* gene prevalence in *Campylobacter* species.

In conclusion, the trimethoprim resistance gene, *dfr1*, was detected at a low prevalence in a selection of 122 trimethoprim resistant *Campylobacter* spp. isolates examined. The second *Campylobacter* associated *dfr* gene, *dfr9*, was not detected in the strains examined. The presence of other genes encoding trimethoprim resistance in isolates that did not harbour these two genes has yet to be determined.

CHAPTER 6

INTEGRONS & ASSOCIATED ELEMENTS

6. INTEGRONS & ASSOCIATED ELEMENTS

Integrons carrying antibiotic resistance gene cassettes play an important role in the acquisition and dissemination of antibiotic resistance, including resistance to trimethoprim (Barlow *et al*, 2004 & Hansson *et al*, 2002). The trimethoprim resistance gene, *dfr1*, is harboured as a gene cassette by both class 1 and class 2 integrons (Skold, 2001 & Huovinen *et al*, 1995).

Integrons had previously been detected in campylobacters from other countries (O'Halloran *et al*, 2004, Lee *et al*, 2002, Lucey *et al*, 2000a, Gibreel & Skold, 2000 & Gibreel & Skold, 1998), but nothing was known of the presence and prevalence of integrons in Australian *Campylobacter* spp. A selection of trimethoprim resistant *Campylobacter* isolates were examined for the presence of class 1 and class 2 integrons encoded by the *intI1* and *intI2* genes, and for gene cassettes inserted in class 1 integrons.

6.1 DETECTION OF *intI* GENES

6.1.1 intI1

Of the trimethoprim resistant *Campylobacter* isolates analysed for *dfr1* and *dfr9*, 108 isolates were examined for the presence of *intI1* by PCR analysis, as described in **Materials and Methods**, section 2.5.4. Strains examined consisted of 81 isolates of *C. jejuni*, 15 *C. coli* isolates, three isolates only identified to *C. jejuni/coli* level, five isolates of *C. lari*, two isolates of *C. fetus* subsp. *fetus* and a single isolate of *C. hyointestinalis* and *C. sputorum* bv. *sputorum*. Most isolates were of either human/clinical origin or chicken origin. A small number of isolates of bovine origin and single isolates of feline, canine and

porcine origin were also analysed. Results of the *intI1* PCR for a selection of strains are shown in **Figure 13** and **14**.

Products of the expected *intI1* size (280bp) and slightly larger (≈290bp) were amplified and were all considered 'putative' *intI1* as products were not DNA sequenced. Putative *intI1* fragments were amplified from six of the 108 strains examined for this gene and results of these strains are displayed in **Table 18**. All putative *intI1* positive strains were isolates of *C. jejuni*, were isolated in Australia, Japan, the UK and Canada, and were of human/clinical origin and chicken origin. One putative *intI1* positive isolate was also positive for putative *dfr1*.

Table 18. Trimethoprim resistant *C. jejuni* & *C. coli* strains from which *intI1*/putative *intI1* & putative *intI2* were amplified

Strain	**Species**	**Source**	**Country**	***intI1* gene**	***intI2* gene**
336	*C. jejuni*	Chicken	Australia	Putative *intI1**	-
9014	*C. jejuni*	Chicken	Australia	-*	Putative *intI2**
81116	*C. jejuni*	Human	UK	Putative *intI1**	-*
C170	*C. jejuni*	Chicken	Australia	Putative *intI1**	-
D123	*C. jejuni*	Human	Canada	Putative *intI1**	-*
JC1	*C. jejuni*	Human	Japan	Putative *intI1**	-
JC2	*C. jejuni*	Human	Japan	Putative *intI1**	-
JC14	*C. coli*	Dog	Japan	-*	Putative *intI2*

* - Products other than putative *intI1* and putative *intI2* were amplified from strains in *intI1* or *intI2* PCR experiments

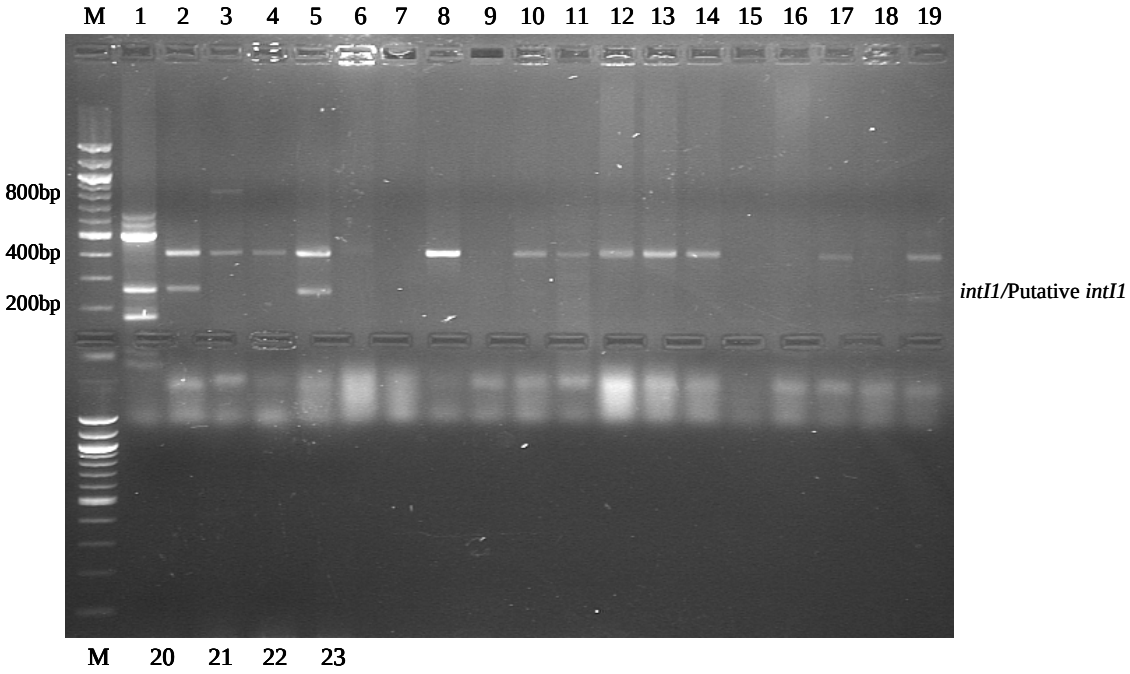

Figure 13. Agarose gel electrophoresis of *intI1* PCR results from a selection of trimethoprim resistant *C. jejuni*, *C. coli* and *C. sputorum* bv. *sputorum* isolates. The *intI1* fragment is 280bp, putative *intI1* bands are ≈280bp and ≈290bp and other fragments produced range from ≈250-270bp to ≈850bp. Lanes: M, molecular weight marker (100bp ladder); 1, positive control strain *E. coli* ColE1::Tn7; 2-22, strains 81116, JC14, D117, D123, 3-9, CE01, CE04, CE05, CE06, CE07, CE7-8, CE09, PRC67, B5Ft8, ATCC 33562, C50, C163, C170, C491, C560, 440; 23, water control. N.B. A putative *intI1* product is not visible for strain C170 (lane 19), but is visible in the following figure.

Single or multiple products of a size other than putative *intI1* were amplified from putative *intI1* positive strains and strains negative for putative *intI1*. For a detailed list of products and approximate sizes amplified from strains examined refer to **Appendix 2, Table 24, 25, 26, 27** and **28**. All positive strains produced amplicons of sizes other than putative *intI1*.

These products ranged in size from ≈250 -270bp to ≈560bp, with four of the six putative *intI1* positive isolates producing a product of ≈400-410bp.

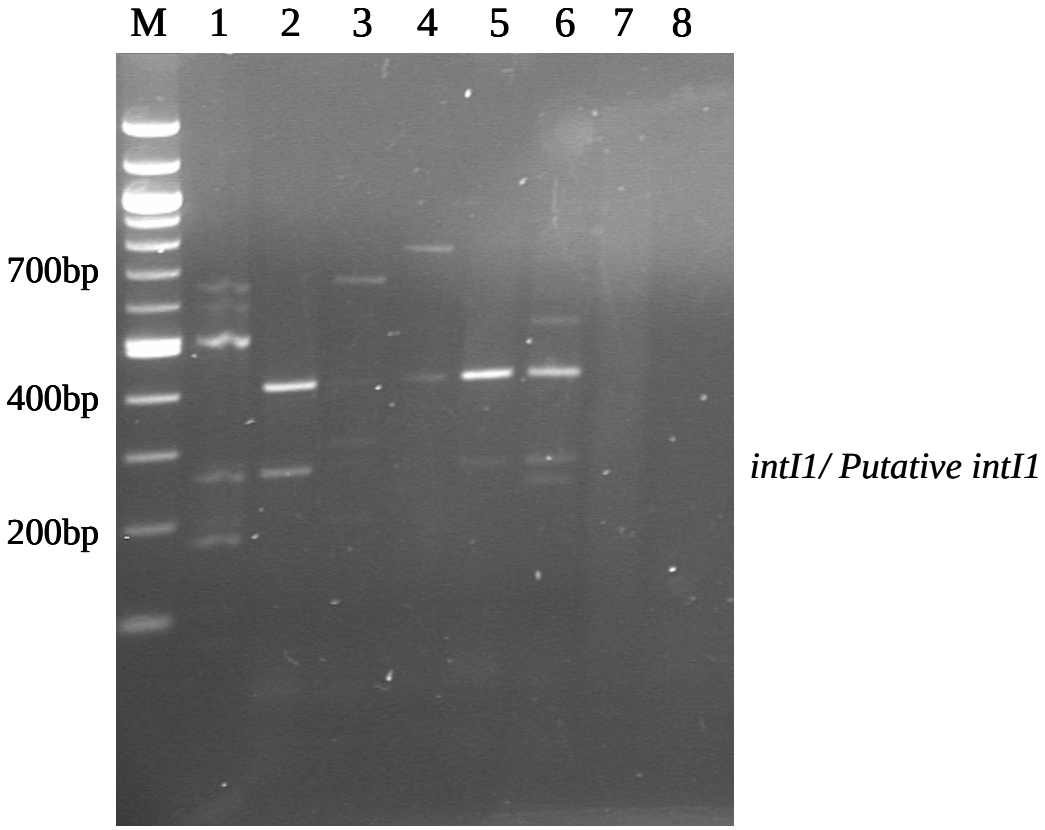

Figure 14. Agarose gel electrophoresis of *intI1* PCR results from a selection of trimethoprim resistant *C. jejuni* strains. The *intI1* fragment is 280bp, putative *intI1* fragments are ≈280bp and ≈290bp and other products amplified range from ≈250-270bp to ≈800bp. Lanes: M, molecular weight marker (100bp ladder); 1, positive control strain *E. coli* ColE1::Tn*7*; 2-6, strains 81116, 131, 351, 336, C170; 7 & 8, water control.

Of the 102 strains negative for putative *intI1*, 88 strains produced products other than putative *intI1* ranging in size from ≈130bp to ≈850bp. Products of ≈400bp and ≈400-410bp

were the most frequently amplified and were produced by 47 and 25 strains respectively. The estimated size and number of products other than putative *intI1* for some strains was compiled from multiple PCR experiments.

6.1.2 intI2

The 120 trimethoprim resistant *Campylobacter* isolates examined for *dfr1* and *dfr9* were examined for the presence of *intI2* by PCR analysis as described in **Materials and Methods**, section 2.5.4. Strains included 93 isolates of *C. jejuni*, 15 *C. coli* isolates, three isolates only identified to *C. jejuni/coli* level, five *C. lari* isolates, two *C. fetus* subsp. *fetus*, and single isolates of *C. hyointestinalis* and *C. sputorum* bv. *sputorum*. The majority of isolates were of either human/clinical or chicken origin. In addition isolates of bovine, feline, canine and porcine origin were also analysed. The *intI2* PCR results of a selection of strains are shown in **Figure 15**.

As for *intI1* detection a product of a similar size to that of *intI2* but slightly larger than *intI2* (≈240bp) was observed and this was termed 'putative *intI2*.' Of the 120 isolates examined only two strains were positive for putative *intI2*, consisting of one *C. jejuni* isolate of chicken origin and a *C. coli* isolate of canine origin. Strains were isolated in Australia and Japan respectively and were both positive for putative *dfr1*. Refer to **Table 18** for details of putative *intI2* positive strains. Remaining strains were negative for putative *intI2*, including type strains *C. jejuni* NCTC 11351, *C. sputorum* bv. *sputorum* ATCC 33562 and *C. lari* ATCC 35223.

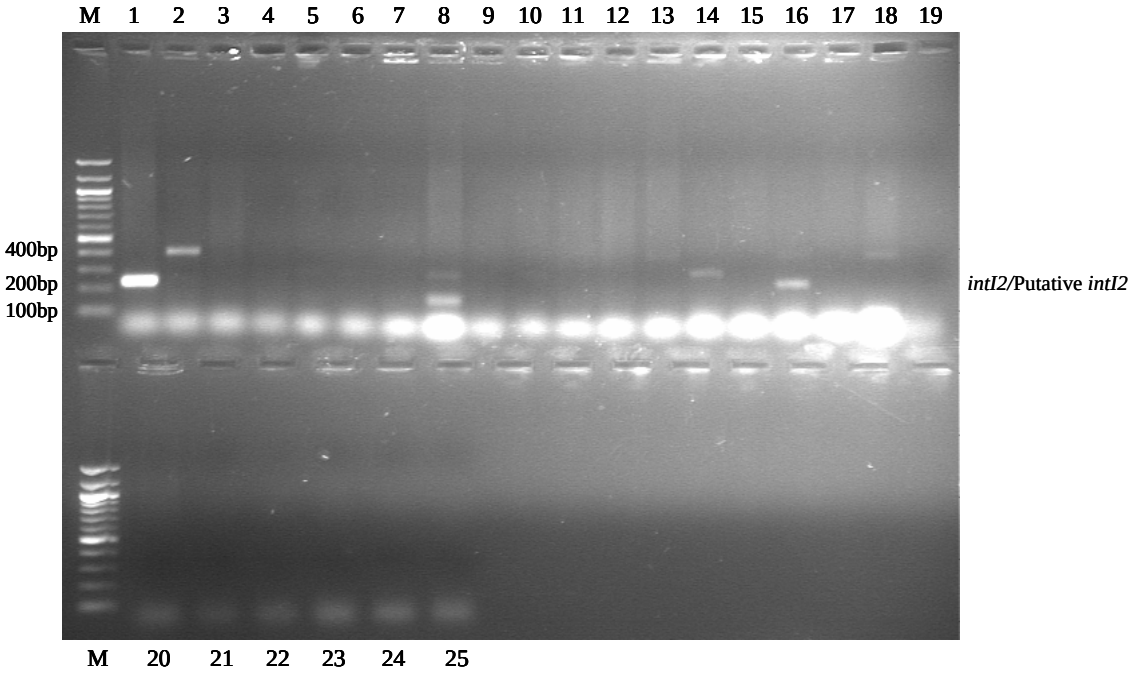

Figure 15. Agarose gel electrophoresis of *intI2* PCR results from a selection of trimethoprim resistant *C. jejuni* and *C. coli* strains. The *intI2* fragment is 233bp, putative *intI2* fragments are ≈240bp and products other than *intI2* range from ≈120bp to ≈400-410bp. Lanes: M, molecular weight marker (100bp ladder); 1, positive control strain *E. coli* ColE1::Tn7; 2-24, strains 81116, NCTC 11351, JC2, 008, 410, 440, CE7-8, JC3, JC5, JC6, JC8, JC10, JC11, JC13, JC14, 5565, D123, B5Ft8, CE01, CE04, CE05, CE06, CE07; 25, water control.

Amplicons of sizes other than that of putative *intI2* were amplified from a putative *intI2* positive and isolates negative for putative *intI2*. For a detailed list of products and approximate sizes refer to **Appendix 2, Table 24, 25, 26, 27** and **28**. The single putative *intI2* positive strain, from which products other than putative *intI2* were amplified, produced products of≈290 -300bp, ≈400-410bp and≈1 -1.05kb. Products other than *intI2*,

ranging in size from ≈120bp to ≈1.1kb were produced by 21 putative *intI2* negative strains, including sequenced type strain NCTC 11168-GS.

6.2 GENE CASSETTE AMPLIFICATION

For the amplification of gene cassettes inserted into class 1 integrons, 69 trimethoprim resistant *Campylobacter* isolates were chosen. Strains included 50 isolates of *C. jejuni*, 12 *C. coli* isolates, two isolates only identified to *C. jejuni/coli* level, three isolates of *C. lari* and two isolates of *C. fetus* subsp. *fetus*. Isolates were of were of human/clinical (34 strains) and chicken origin (31 strains) and one isolate each was of canine, porcine and bovine origin. Included in the analysis were genome sequenced strains *C. jejuni* RM1221 and *C. jejuni* NCTC 11168 (11168-GS).

Both putative *intI1* positive strains and putative *intI1* negative strains were examined for gene cassettes. Strains analysed included six putative *intI1* positive strains (including one strain that was also positive for putative *dfr1*) and 63 putative *intI1* negative isolates. The putative *intI1* negative isolates consisted of 13 strains positive for putative *dfr1* and putative *intI2*, 49 strains that produced single or multiple products (other than putative *intI1*) in the *intI1* PCR, and one strain that did not produce product of any type.

Of the 49 putative *intI1* negative isolates that produced product in the *intI1* PCR, 26 strains produced ≈400bp products, 14 strains produced ≈400 -410bp products, one strain each produced products of ≈410bp, ≈410-420bp and ≈800bp and five isolates that produced two products and one strain that produced five products. Gene cassette PCR results for a selection of putative *intI1* positive and putative *intI1* negative isolates are displayed in

Figure 16 and **17**. Refer to **Table 29**, **30** and **31** in **Appendix 3** for detailed results of each strain examined.

When examined, 55 strains produced gene cassette amplicons. Strains included mostly isolates of *C. jejuni*, along with isolates of *C. coli*, those only identified to *C. jejuni/coli* level and isolates of *C. lari* and *C. fetus* subsp. *fetus* from human, chicken and canine origin. Five putative *intI1* positive strains (including one strain positive for putative *dfr1*), 13 putative *intI1* negative isolates positive for putative *dfr1*, and, 37 isolates negative for putative *intI1* produced amplicons.

Amplicons produced by strains ranged in number from one to ten. Two, three and four amplicons were the most frequently observed, with ten strains producing two amplicons, nine strains producing three amplicons and four amplicons amplified from eight strains. A larger number of amplicons (seven, eight, nine and ten) were produced by six strains, two strains, three strains and one strain respectively. Amplicons produced ranged in size from ≈210bp to >1.5kb. Dominant amplicons of 440bp, 450bp, 500bp, 550bp, and 1kb were observed.

A 500bp amplicon was the most frequently observed and was amplified from 17 strains. One or more dominant amplicons were amplified from 36 of the 55 strains that produced gene cassette product. 11 strains produced one dominant amplicon and amplicons of other sizes; five strains produced two dominant amplicons only, and, 11 other strains produced two dominant amplicons along with amplicons of other sizes. One strain produced three

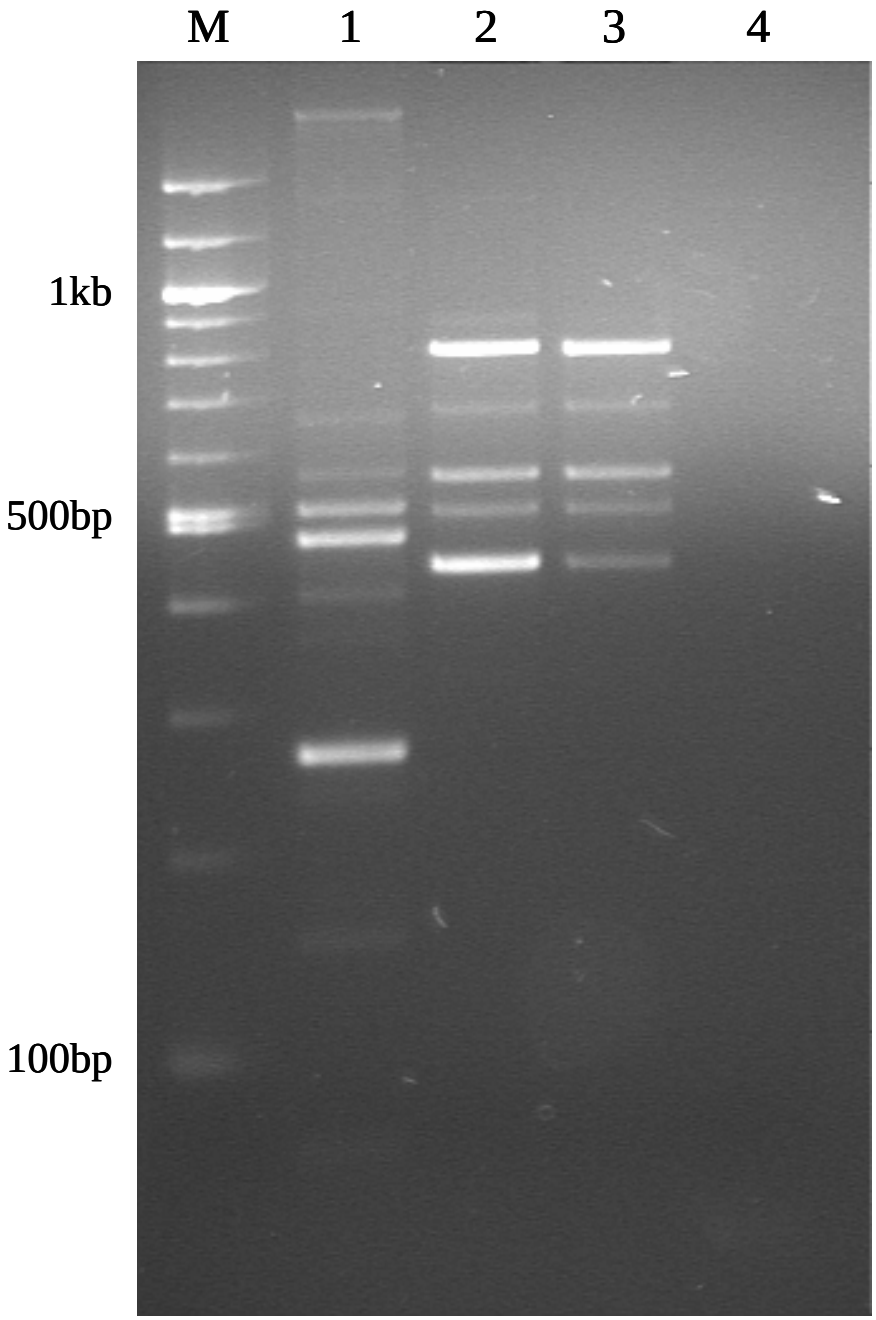

Figure 16. Gene cassette products amplified from a selection of putative *intI1* positive *C. jejuni* isolates. Lanes: M, molecular weight marker (100bp ladder); 1, Non-*Campylobacter* strain *E. coli* ColE1::Tn7; 2-3, strains 81116 & JC2; 4, water control.

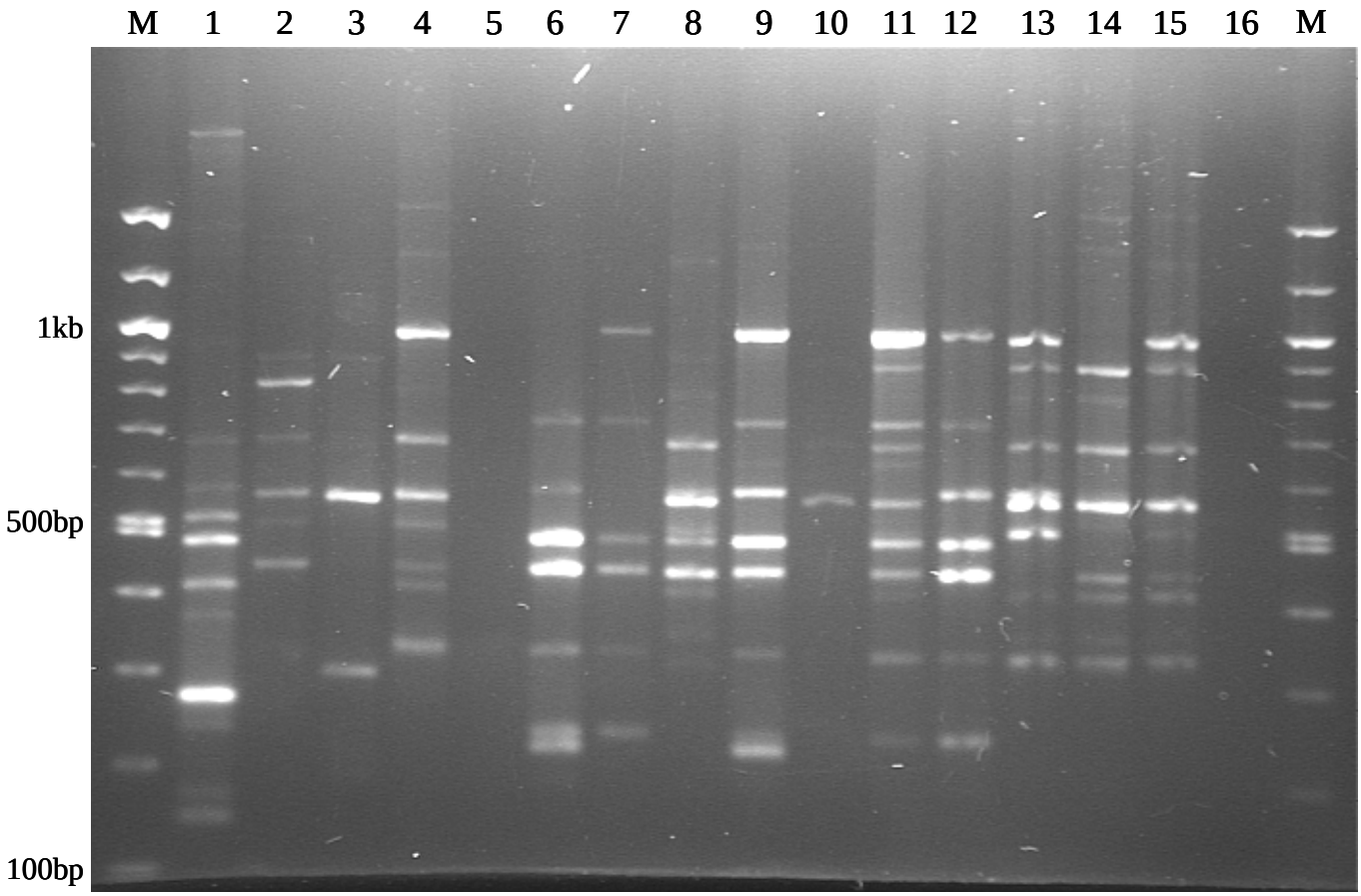

Figure 17. Gene cassette products amplified from a selection of putative *intI1* positive and putative *intI1* negative *C. jejuni, C. coli, C. lari* and *C. fetus* subsp. *fetus* isolates. Lanes: M, molecular weight marker (100bp ladder); 1, Non-*Campylobacter* strain *E. coli* ColE1::Tn7; 2-15, strains 81116, 35223, 85-256, 801, 887, 961, 4002, 8004, 9014, 9126, RM3, RM7, RM17, RM20; 16, water control. N.B. Strain 801 subsequently produced one gene cassette amplicon and 9014 produced two additional products.

dominant amplicons only and eight strains produced three dominant amplicons and products of other sizes. Products of sizes other than the dominant amplicons were amplified from the remaining 19 strains.

All amplified products larger than 1.0kb in size were considered potential gene cassettes, as DNA sequences of this length or greater could potentially contain complete open reading frames (ORFs) (O'Halloran *et al*, 2004). The same size cut-off as O'Halloran *et al* was employed, where amplified fragments of ≥1.0kb were considered of interest, since the average size of bacterial coding sequence is ≈800bp. Details of *Campylobacter* strains that produced gene cassette products of interest, and product sizes, are listed in **Table 32** in **Appendix 3**. 24 strains produced products ≥1.0kb in size, ranging from ≈950bp -1kb to >1.5kb. Strains included isolates of *C. jejuni*, *C. coli*, *C. lari* and *C. fetus* subsp. *fetus*, and strains were of human and chicken origin. Strains included only one putative *intI1* positive strain, three putative *intI1* negative, putative *dfr1* positive isolates, and 20 putative *intI1* negative isolates.

Gene cassette product was not amplified from 14 isolates, including NCTC11168-GS. Isolates included one putative *intI1* positive *C. jejuni* strain, a clinical isolate from Japan, and, putative *intI1* negative strains of consisting of 11 *C. jejuni* isolates and two *C. coli* isolates from human, chicken, bovine and porcine sources.

In Summary. Trimethoprim resistance isolates of *C. jejuni*, *C. coli* and other *Campylobacter* species were examined for the *intI1* and *intI2* genes and gene cassette sequences inserted into class 1 integrons. Putative *intI1* fragments were amplified from six of the 108 strains examined for *intI1*, and putative *intI2* amplicons were amplified from two of the 120 isolates examined for *intI2*. Products of sizes other than that of putative *intI1 and intI2* were also amplified from strains examined. Gene cassette amplicons were amplified from both *intI1* positive and negative strains.

6. 3 DISCUSSION

Integrons and corresponding gene cassettes have been implicated in the acquisition and dissemination of antibiotic resistance, including resistance to trimethoprim. Nothing was known of the presence or prevalence of class 1 and class 2 integrons in *Campylobacter* species in Australia.

Only 5.56% of the trimethoprim resistant *Campylobacter* isolates examined for the *intI1* gene in this study, putatively harboured *intI1* encoding the class I integron integrase enzyme. All putative *intI1* positive strains were isolates of *C. jejuni* of clinical and poultry origin. The putative detection of the *intI1* gene in strains from our collection, including isolates from Australia, confirms the previous identification of *intI1* in *Campylobacter* isolates (O'Halloran *et al*, 2004, Lee *et al*, 2002, Gibreel and Skold, 2000 and Lucey *et al*, 2000a). The low rate of prevalence of putative *intI1* in the strains examined, however, is in contrast to the findings of Lee *et al*, 2002 and Gibreel and Skold, 2000, where 21% and 22% of clinical and poultry isolates of *C. jejuni* strains carried *intI1*.

In addition to the detection of the more highly prevalent *intI1* gene, isolates from our collection were examined for the *intI2* gene encoding the class 2 integron integrase enzyme. Only 1.67% of the 120 trimethoprim resistant isolates examined for *intI2* were positive for putative *intI2*. Putative *intI2* positive strains consisted of one *C. jejuni* isolate of poultry origin, and one *C. coli* isolate of canine origin, and were both positive for putative *dfr1*. Similarly *intI2* was also detected at a low rate (2.44%) in *C. jejuni* isolates by Gibreel and Skold, 2000.

A difference in the prevalence rates of putative *intI1* and putative *intI2* in trimethoprim resistant isolates examined for these genes was observed, where putative *intI1* was detected in six strains, and putative *intI2* was only detected in two strains. The *intI1* gene was also found to be more prevalent than *intI2* in Swedish isolates of *C. jejuni* (Gibreel and Skold, 2000), although a larger disparity in prevalence was observed (22% for *intI1* and 2.44% for *intI2*). The *intI1* gene has also been reported to be the more prevalent integrase gene in other gram negative bacteria, such as *Enterobacteriaceae* (including *E. coli*) and *Acinetobacter baumannii* (Jones *et al*, 2003, White *et al*, 2001, Blahna *et al*, 2006, Ploy *et al*, 2000 & Goldstein *et al*, 2001).

In contrast to the observations of this study and that of Gibreel and Skold, 2000, *intI1* and *intI2* were not detected in Dutch multiple antibiotic resistant *Campylobacter* isolates examined for these genes (van Essen-Zandbergern *et al*, 2007). Such diverse prevalence rates for *intI1* and *intI2* indicates that these integron genes are present in *Campylobacter* spp. isolates at differing rates.

Strains positive for *intI1* and *intI2* from our collection were considered 'putative' positive isolates, as the identity of these amplicons was not determined through DNA sequencing and comparisons to known *intI1* and *intI2* sequences. Fragments of the expected *intI1* size are likely to represent appropriate *intI1* sequences amplified using known PCR methods (Lee *et al*, 2002 and Bass *et al*, 1999). Putative amplicons may be due to deletion or addition of base pairs within the *intI1* or *intI2* gene sequences amplified by PCR. However, due to time limitations, further analysis of the identity of these putative amplicons could not

be conducted. DNA sequencing and comparisons with known *intI1* and *intI2* sequences could determine the identity and size of products amplified.

In addition to putative *intI1* and putative *intI2* amplicons, other products of varying sizes were amplified from *intI1* and *intI2* positive strains as well as negative strains. Products other than putative *intI2* were amplified from genome sequenced strain *C. jejuni* NCTC 11168-GS, and are possibly of a non-specific nature as sequences related to integrases have yet to be identified in this strain (Gundogdu1 *et al*, 2007). Products other than putative *intI1* and putative *intI2* amplified from other isolates may also be of a non-specific nature.

Conversely, additional products amplified from *intI1* and *intI2* PCR analyses may be related to *intI1* and *intI2* or other integrase genes such as *intI3* and superintegron integrases. DNA sequence analysis and comparisons with known *intI* sequences would determine if these products are indeed related to integron integrase genes. Additional amplicons may also be due to the presence of insertion sequences in the integrase genes (Fluit and Schmitz, 2004). Insertion sequences have been detected in *Campylobacter* isolates (Parkhill *et al*, 2000, Gibreel *et al*, 2004b, Fouts *et al*, 2005 & Nirdnoy *et al*, 2005), and such genetic elements may be present in the *intI1* and *intI2* genes.

An association between insertion sequences and the *intI1* and *intI2* genes may have led to a deleted form of these genes (Fluit and Schmitz, 2004), and may be represented by products smaller than putative *intI1* and putative *intI2* as proposed by Lucey *et al*, 2000a. Products smaller than the expected size of *intI1* were similarly amplified from *Campylobacter* isolates by Lucey *et al*, 2000a, which was proposed to be due to the presence of a deleted

form of *intI1*. Larger fragments may also be due to the presence of insertion sequences in the *intI1* or *intI2* gene as reported by Shi *et al*, 2006 for the *Vibrio cholerae* superintegron integrase gene. Further analysis, including DNA sequencing, would shed light on the presence or absence of insertion sequences associated with *intI1* or *intI2* genes in the strains examined.

Gene cassettes inserted into the integron platform play a key role in a variety of antibiotic resistances and *dfr* genes encoding trimethoprim resistance are frequently present in integrons as inserted gene cassettes (Skold, 2001 & White & Rawlinson, 2001, White *et al*, 2000). As gene cassettes are an integral part of integrons and play a role in trimethoprim resistance, a selection of trimethoprim resistant putative *intI1* positive and putative *intI1* negative *Campylobacter* spp. isolates were examined for the presence of gene cassettes inserted into class 1 integrons.

More than three quarters (79.7%) of isolates examined, including putative *intI1* positive and putative *intI1* negative strains, produced gene cassette amplicons. Such amplicons may represent sequences inserted into the variable region of class 1 integrons. The amplification of gene cassette products from isolates in our collection confirms the previous identification of gene cassettes in class 1 integrons from *Campylobacter* isolates of various origins (O'Halloran *et al*, 2004, Lee *et al*, 2002, Lucey *et al*, 2000a & Gibreel and Skold, 2000).

Unique combinations of number and size of gene cassette amplicons were produced by trimethoprim resistant isolates analysed for gene cassettes in this study. Between one and ten gene cassette amplicons were produced by strains and ranged in size from ≈210bp to

>1.5kb. Similarly, amplicons ranging in size from 230bp to 1.47kb, and, 300bp to 1.4kb were amplified by O'Halloran *et al*, 2004 & Lucey *et al*, 2000a. Due to time limitations and the large number of gene cassette products amplified from strains it was decided not to pursue the identification of individual products.

Large gene cassette amplicons ranging in size from ≈950bp-1kb to >1.5kb were amplified from 24 of the selected trimethoprim resistant isolates examined. Interestingly an amplicon of 1kb in size was frequently amplified, and this may constitute a commonly occurring gene or ORF amongst strains from our collection. Such large gene cassette amplicons are of interest due to the potential to contain complete ORFs, as the average size of bacterial coding sequence is ≈800bp (O'Halloran *et al*, 2004).

These large gene cassette amplicons may contain similar genes/ORFs to those previously identified in integrons from *Campylobacter* isolates, such as the trimethoprim resistance gene, *dfr1*, *aadA2* genes encoding aminoglycoside resistance, and *aacA4* genes encoding aminoglycoside (tobramycin and gentamicin) resistance (O'Halloran *et al*, 2004, Lee *et al*, 2002 & Gibreel and Skold, 2000).

Alternatively, large amplicons may contain other integron associated antibiotic resistance genes, such as those conferring resistance to trimethoprim, aminoglycosides, β-lactams, chloramphenicol and streptothricin, that have been found as gene cassettes in integrons in other bacterial species (Henriques *et al*, 2006 & Lu *et al*, 2003). Conversely, amplicons may not constitute amplified inserted gene cassettes, but instead may contain insertion sequences or non-coding DNA sequences with a possible biological role other than protein

coding, such as transcribed RNAs or binding sites for regulatory proteins (Rosser and Young, 1999 & Holmes *et al*, 2003).

Smaller gene cassette amplicons (<1.0kb) were also amplified from strains under study, and these may contain short ORFs such as that identified by O'Halloran *et al*, 2004; non-coding DNA sequences, or may represent "empty" integron structures (lacking an integrated gene cassette) where only conserved segments are amplified (Heir, 2004, Holmes *et al*, 2003 & Chang *et al*, 2000). The frequent amplification of small amplicons of 440bp, 450bp, 500bp and 550bp in size was observed from strains examined, and this, along with the amplification of small gene cassette products in general may be due to the bias of *Taq* polymerase towards amplification of smaller products as proposed by Plante *et al*, 2003 and Stokes *et al*, 2001.

Campylobacter spp. isolates from our collection that produced gene cassette amplicons included 83.3% of putative *intI1* positive strains, and most (79.4%) putative *intI1* negative strains, including all putative *intI1* negative, putative *dfr1* positive isolates, examined for such products. Interestingly, the two putative *intI2* positive isolates (negative for putative *intI1*) produced gene cassette amplicons, and these products may be related to gene cassettes harboured by class 2 integrons. Amplification of gene cassette products from a high number of putative *intI1* positive strains was in contrast to the findings of Lee *et al*, 2002 where only approximately 20% of *intI1* positive *C. jejuni* isolates examined produced gene cassette amplicons.

It is interesting to note that in addition to amplification from putative *intI1* positive strains, gene cassette products were also amplified from putative *intI1* negative strains. The amplification of products that may correspond to sequences inserted into class 1 integrons from strains that appear to lack the *intI1* gene is similar to that reported by Gibreel and Skold, 2000, where most *C. jejuni* isolates harbouring *dfr1* as a gene cassette were found to not contain a corresponding integrase gene. In the putative *intI1* negative isolates from our collection that produced gene cassette amplicons, the integrase gene may have been lost by genetic drift as proposed by Gibreel and Skold, 2000.

The remaining 20% of selected isolates examined for gene cassettes, did not produce gene cassette amplicons, including putative *intI1* negative isolates and one putative *intI1* positive strain. The lack of amplification of gene cassette products from an putative *intI1* positive isolate corresponds with the findings of other investigators, and may be due to the lack of a 3'-CS region or a 5'-CS region, or, the presence of DNA sequences in the variable region that were too large to be amplified by the PCR method employed (Yao *et al*, 2007, Henriques *et al*, 2006, Pan *et al*, 2006, Barlow *et al*, 2004 and White *et al*, 2001). The lack of amplification of gene cassette products from putative *intI1* negative isolates is similar to that reported by van Essen-Zandbergen *et al*, 2007, in which both class 1 integron genes and corresponding inserted gene cassettes were not detected in isolates of *Campylobacter*.

In conclusion, from the results presented in this study, it is likely that both class 1 and class 2 integrons are not highly prevalent in the trimethoprim resistant *Campylobacter* isolates examined from our collection. Gene cassette amplicons were amplified from many strains examined, including strains positive and negative for putative *intI1*. Further study of gene

cassette amplicons will determine the identity of such products and the role of these products in antibiotic resistance, including trimethoprim resistance, in *Campylobacter* spp.

CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Campylobacter species are the most common cause of foodborne disease in Australia and many countries throughout the world. Although Campylobacteriosis is usually self-limiting, approximately 10% of infections require hospitalisation. Antibiotic therapy may be prudent for such severe cases and in the young, the elderly and the immunocompromised, such as AIDS patients. Antibiotic resistant *Campylobacter* spp. isolates however may prolong illness and increase the risk of invasive disease. Therefore, an understanding of the mechanisms, genetics and prevalence of antibiotic resistance in *Campylobacter* spp. is essential to help combat the more severe illnesses and complications caused by resistant isolates of these organisms. Also such information would contribute to knowledge of these clinically, agriculturally and environmentally important organisms.

Little information was known about tetracycline and trimethoprim resistance in Australian campylobacters, including the presence and distribution of previously described resistance genes and associated genetic elements, prior to this study. Similarly, the presence and distribution of integrons and associated elements (gene cassettes) was not known in Australian isolates of *Campylobacter* species.

High level tetracycline resistance was observed in Australian isolates of *C. jejuni* and *C. coli*, and was due to the presence of the previously described *Campylobacter* tetracycline resistance determinant, TetO, encoded by the *tetO* gene. The findings of this study show that tetracycline resistance in Australian isolates of these species is due to the same determinants as that observed for resistant isolates from other countries. Although previous studies have described the significant role of plasmids in tetracycline resistance, resistance

in the majority of *Campylobacter* isolates examined was due to chromosomally encoded *tetO*, including those that harboured plasmids.

The recent identification of transposon and insertion sequence genes on *tetO* carrying plasmids points to the possibility that such an association may have led to the localisation of *tetO* on the chromosome of resistant isolates, including isolates that harboured large plasmids. These plasmids may have once harboured *tetO* but subsequently lost this gene to the chromosome due to transposon or insertion sequence induced movement. The movement of genetic elements and *tetO* if associated may also explain resistant isolates that did not harbour plasmids, where *tetO* moved from plasmid to chromosome with subsequent loss of the plasmid. Recombination between a *tetO* harbouring plasmid and the chromosome or the integration of such a plasmid into the chromosome may also explain these isolates.

The *tetO* carrying plasmids harboured by some isolates confirmed that *tetO*, and thus tetracycline resistance, was carried by plasmids (Tracz *et al*, 2005, Gibreel *et al*, 2004a; Gibreel *et al*, 2004b, Bacon *et al*, 2000, Lee *et al*, 1994, Taylor *et al*, 1988 & Ng *et al*, 1987). The conjugative transfer of such plasmids from six isolates, similarly confirmed the observations of conjugative transfer of resistance and the *tetO* gene, by other investigators (Batchelor *et al*, 2004, Gibreel *et al*, 2004a, 2000, Velazquez *et al*, 1995, Prasad *et al*, 1994, Taylor *et al*, 1988 & Sagara *et al*, 1987). However, the low carriage rate of *tetO* harbouring plasmids amongst strains from our collection is in contrast to that observed by some previous studies (Gibreel *et al*, 2004a & Ng *et al*, 1987).

In light of the recent identification of an association between *tetO* and genes related to transposon and insertion sequence elements, predominantly chromosomally encoded *tetO* in isolates examined and the possible diminished role of plasmids in this resistance, it is likely that mobile genetic elements other than conjugative plasmids play a role in dissemination of tetracycline resistance in *C. jejuni* and *C. coli*. Further research into the association of such elements with *tetO* and *tetO* carrying plasmids would lead to a better understanding of the acquisition and dissemination mechanisms of antibiotic resistance, including tetracycline resistance, in *Campylobacter* spp.

In this study, high level trimethoprim resistance was observed in a different selection of Australian *C. jejuni* and *C. coli* isolates than that examined for tetracycline resistance. High level trimethoprim resistance was also observed in *C. jejuni* and *C. coli* isolates from other countries, including the two *C. jejuni* genome sequenced strains, and in a small selection of isolates of less common species such as *C. lari*, *C. fetus*, *C. hyointestinalis* and *C. sputorum*. As all strains examined in this study exhibited high level resistance and *C. jejuni* and *C. coli* isolates are considered intrinsically resistant to trimethoprim (Gibreel and Skold, 2000, Gibreel and Skold, 1998, Taylor & Courvalin, 1988 & Karmali *et al*, 1981), it is likely that trimethoprim resistance in these isolates is intrinsic in nature.

Intrinsic resistance to trimethoprim has been reported in a number of other bacterial species including *Pediococcus cerevisiae*, *Bacteroidis fragilis*, *Clostridium* spp., *Moraxella catarrhalis*, a *Neisseria* sp., a *Nocardia* sp. and a *Lactobacillus* sp. In these species, such resistance is due to the presence of a less susceptible DHFR enzyme or naturally insensitive

DHFRs (Skold, 2001, Huovinen, 2001, Coque *et al*, 1999, Huovinen *et al*, 1995 & Huovinen, 1987).

Intrinsic trimethoprim resistance in *Campylobacter* spp. may be through resistance mechanisms such as cell wall impermeability, efflux pumps, naturally insensitive target enzymes and/or chromosomal DHFR gene mutations. Eight putative drug efflux pumps, in addition to the characterised CmeABC and CmeDEF efflux systems, were identified in *C. jejuni* by Ge *et al*, 2005. These uncharacterised efflux pumps may contribute to intrinsic trimethoprim resistance by extruding the drug from the cell, before the trimethoprim elicits its effects.

In the *Campylobacter* isolates examined for trimethoprim resistance, the same level of resistance was observed for plasmid harbouring strains and those that did not harbour detectable plasmid DNA, with the exception of one isolate. Only just over 50% of trimethoprim resistant campylobacters examined harboured plasmids. These observations imply that plasmids are unlikely to play a significant role in trimethoprim resistance, especially if it is intrinsic in nature.

Chromosomally encoded resistance mechanisms may therefore play a role in trimethoprim resistance in the Campylobacter isolates examined in this study. The determination of the presence and sequence of the chromosomal DHFR gene would be useful. High level resistance may be due to mutational changes in the intrinsic (chromosomal) DHFR gene, and regulatory mutations leading to over-expression of DHFR and a decreased affinity for trimethoprim (Adrian and Klugman, 1997).

Such mutational changes have been reported in isolates of *E. coli*, *Haemophilus influenzae*, *S. pneumoniae* and *S. aureus* (Coque *et al*, 1999). Interestingly, mutational changes in the chromosomal dihydropteroate synthase gene targeted by sulphonamides, which are used separately or in combination with trimethoprim, leads to resistance to this antibiotic in *C. jejuni* (Gibreel & Skold, 1999). Such changes in a chromosomal DHFR gene in *Campylobacter* isolates, if present, may impart resistance to trimethoprim.

Trimethoprim resistance in the isolates examined may alternatively be due to the presence of one or more *dfr* genes encoding drug insensitive DHFR enzymes. Although these genes play a significant role in this resistance, only approximately 16% of resistant strains examined putatively harboured a known *Campylobacter* associated *dfr* gene, *dfr1*, and the second *Campylobacter* associated gene, *dfr9*, was not detected amongst resistant isolates. The detection of putative *dfr1* corresponds with the previous identification of *dfr1* in *Campylobacter* spp. isolates and demonstrates that this gene is present in Australian isolates of *C. jejuni* and *C. coli*, and also in isolates of the closely related, thermophilic species, *C. lari*.

The low prevalence of putative *dfr1* and the lack of detection of *dfr9* in the resistant isolates examined from our collection implies that other *dfr* genes, such as those detected in other bacterial species inhabiting similar environments, and/or of a gram positive origin, may be present. However, if trimethoprim resistance in *Campylobacter* spp. is intrinsic, acquired *dfr* genes may not play such a key role in this resistance, but instead play a more accessory role. Acquired *dfr* genes may only be present in selected isolates that have acquired these

genes through horizontal gene transfer involving mobile genetic elements such as transposons and integrons.

Integrons harbouring *dfr* genes as inserted gene cassettes play an important role in trimethoprim resistance in other bacterial species and also may do so in campylobacters. Interestingly, class 1 and class 2 integron integrases were not highly prevalent in the trimethoprim resistant *Campylobacter* isolates examined, with only a small number of isolates positive for putative *intI1* and putative *intI2*. The detection of putative *intI1* and putative *intI2* corresponds with the previous identification of these genes in *Campylobacter* spp. isolates, and indicates that these integron integrase genes are present in Australian *C. jejuni* and *C. coli* isolates.

Only a small number of strains positive for putative *dfr1* were also found to carry putative *intI1* or putative *intI2*. In the remaining putative *dfr1* carrying isolates the integrase gene may have been lost by genetic drift as proposed by Gibreel and Skold, 2000. Alternatively, as proposed previously, other *dfr* genes may be present in resistant strains and these genes may be located within class 1 integrons. *dfr* genes other than *dfr1*, such as *dfr5*, *dfr12*, *dfr17*, *dfrB2* have been detected in class 1 integrons in other bacterial species (White & Rawlinson, 2001). Identification of gene cassette amplicons produced by trimethoprim resistant strains may determine if other *dfr* genes are present as gene cassettes in these isolates, and also determine the role integrons play in trimethoprim resistance in campylobacters.

Interestingly in the closely related bacterium *H. pylori*, which is also naturally resistant to trimethoprim like *Campylobacter* spp., *dfr1* (harboured by transposon Tn*7* carrying *intI2*) has been proposed to not have an important role in trimethoprim resistance (Crespo *et al*, 2005). This finding has interesting implications for the role of *dfr* genes and associated integrons in trimethoprim resistance in *Campylobacter* spp. Given the intrinsic nature of trimethoprim resistance in campylobacters and the low prevalence of class 1 and class 2 integrons in the isolates examined in this study, *dfr* genes and the integron platforms that harbour these genes, may only play an accessory role in this resistance.

The identification by Poly *et al*, 2004 of ORFs with significant homology to putative integrases from the closely related bacterium *Wolinella succinogenes* in a *C. jejuni* strain hints at the possibility of other classes of integron integrases being present in *Campylobacter* spp. Such putative integrases may be present in isolates examined from our collection. The putative integrases of *W. succinogenes* are of a suspected bacteriophage origin (Baar *et al*, 2003) and have yet to be characterised. The role these integrons play in antibiotic resistance in *Campylobacter* spp., if any, has yet to be elucidated, along with the prevalence of these integrons among *Campylobacter* strains.

Additionally, integrons that have yet to be characterised may be present in some *Campylobacter* isolates, such as those examined from our collection. For example, new, uncharacterised integrase genes have been isolated from environmental samples, including polluted and unpolluted soil and heavy metal contaminated mine tailings (Nemergut *et al*, 2004 & Nield *et al*, 2001). Nemergut *et al* detected 14 previously undescribed integrase genes, including six novel gene lineages and gene cassettes, and Nield *et al* identified new

integrons including novel *intI* genes, inserted putative gene cassettes, *attI* sites and possible promoters. Although the above mentioned studies examined DNA samples of environmental origin, such exploratory techniques using PCR based methods may also recover new genes from other areas, including clinical and agricultural sources.

7.1 FUTURE WORK

Through this study, knowledge and an understanding of tetracycline and trimethoprim resistance in Australian *C. jejuni* and *C. coli* and other *Campylobacter* species was gained. However, much is still yet to be determined including the role of mobile genetic elements other than plasmids in tetracycline resistance, the mechanisms involved in intrinsic trimethoprim resistance in campylobacters, the role of integrons in this resistance, and the presence of other integron classes in *Campylobacter* spp.

To increase the understanding of tetracycline resistance in Australian *Campylobacter* species, future work could aim at determining the role of other factors influencing this resistance. Further analysis could include determining the presence/absence of transposons and/or insertion sequence elements on *tetO* carrying plasmids and the chromosome of resistant isolates using DNA sequencing upstream and downstream of *tetO*, and an anslysis of conjugative *tetO* carrying plasmids for the conjugation associated type IV secretion system

A clear understanding of the nature and mechanisms involved in trimethoprim resistance in *Campylobacter* spp. is yet to be obtained. Firstly analysis into the intrinsic nature of this resistance in Campylobacter, including the presence/absence of a *Campylobacter*

chromosomal DHFR gene, which may govern the intrinsic nature of trimethoprim resistance in these species, would be essential. Further to this continued analysis of isolates from a variety of countries for other *dfr* genes, such as those identified in gram negative isolates and gram positive staphylococcal *dfr* genes would lead to a better understanding of the prevalence and the influence of *dfr* genes in trimethoprim resistance in campylobacters. Also continuing analysis of PCR amnplicons obtained from isolates examined in this study would assist in determining the extent of *dfr* gene presence and influence and such analysis could entail DNA sequencing and identification of putative *dfr1* PCR amplicons products other than putative *dfr1* and *dfr9*.

Continuing analysis of *Campylobacter* spp. isolates for integons and integrases would lead to a greater understanding of the prevalence of these genetic elements in these species, which is still not totally known. Continued analysis of PCR amplicons obtained from strains examined in this study, including DNA sequencing and identification of putative *intI1* and putative *intI2* PCR amplicons and products other than putative *intI1* and *intI2*, and examination of other strains would assist in determining the presence of these integron integrase genes in these species.

Examination of *Campylobacter* isolates from our collection for the presence of other classes of integrase genes, such as the putative integrases of *W. succinogenes*. Methods such as PCR analysis, Southern hybridisation or PCR protocols detecting conserved segments of integrase genes could be employed to determine if uncharacterised integrases are present. Integrase sequences native to campylobacters or the ε-protobacteria such as W. succinogenes may be present in isolates examined and have yet to be discovered. Such

native superintegrons have been described in other bacterial classes and may be present in the ε-protobacterial class. DNA sequencing and identification of large (>1.0kb in size) gene cassette amplicons and small amplicons amplified from both putative *intI1* positive and negative isolates examine from out collection may lead to further insights into the role of integrons (through the carriage of antibiotic resistance gene cassettes) in antibiotic resistance in Campylobacter spp.

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APPENDIX

APPENDIX

APPENDIX 1

Table 19. *dfr1* & *dfr9* results of trimethoprim resistant *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	*dfr1* gene	*dfr9* gene
0-10	*C. jejuni*	Chicken	USA	Putative *dfr1*, ≈370bp	-
004	*C. jejuni*	Chicken	Australia	≈370bp	-
007	*C. jejuni*	Chicken	Australia	Putative *dfr1*	-
8	*C. jejuni*	Human	Australia	Putative *dfr1*, ≈380bp	-
008	*C. jejuni*	Chicken	Australia	-	-
011	*C. lari*	Chicken	Australia	Putative *dfr1*, ≈400bp, ≈450bp	≈700-750bp
013	*C. jejuni*	Chicken	Australia	-	-
017	*C. jejuni*	Chicken	Australia	Putative *dfr1*, ≈400bp	-
018	*C. coli*	Chicken	Australia	-	-
019	*C. jejuni*	Chicken	Australia	-	≈800bp
3-9	*C. jejuni*	Chicken	USA	≈400bp	-
32	*C. jejuni*	Human	Australia	-	-
93	*C. jejuni*	Human	Australia	≈400bp	-
108	*C. jejuni*	Human	Australia	-	≈800-850bp
108	*C. jejuni*	Chicken	Netherlands	≈400bp	≈800-850bp
131	*C. jejuni*	Chicken	Australia	≈110-120bp, ≈400-410bp	-
133	*C. jejuni*	Chicken	Australia	≈400bp	-
134	*C. jejuni*	Chicken	Australia	≈400bp	-
141		Chicken	Australia	-	-
142		Chicken	Australia	-	-
173		Human	Australia	Putative *dfr1*	-
231	*C. jejuni*	Chicken	Australia	≈380bp	-
235	*C. jejuni*	Chicken	Australia	≈410bp	-

Table 20. *dfr1* & *dfr9* results of trimethoprim resistant *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	*dfr1* gene	*dfr9* gene
239	*C. jejuni*	Human	Australia	-	-
293	*C. coli*	Human	Australia	Putative *dfr1*, ≈500bp, ≈600bp	-
303	*C. jejuni*	Chicken	Australia	-	-
336	*C. jejuni*	Chicken	Australia	-	-
351	*C. jejuni*	Human	Australia	-	-
354	*C. jejuni*	Human	Australia	≈400-410bp	-
375	*C. jejuni*	Human	Australia	-	-
388	*C. coli*	Human	Australia	-	-
405	*C. jejuni*	Chicken	Australia	-	-
410	*C. jejuni*	Human	Australia	≈450bp	-
413	*C. jejuni*	Chicken	Australia	Putative *dfr1*	-
415	*C. jejuni*	Chicken	Australia	≈440bp, ≈600bp	-
430	*C. jejuni*	Human	Australia	≈400bp	-
435	*C. jejuni*	Human	Australia	≈400-410bp	-
439	*C. jejuni*	Human	Australia	Putative *dfr1*, ≈110-120bp, ≈400-410bp	-
440	*C. jejuni*	Human	Australia	Putative *dfr1*	-
458	*C. jejuni*	Human	Australia	≈400bp	-
506	*C. jejuni*	Chicken	Australia	-	-
515	*C. jejuni*	Human	Australia	-	-
520	*C. jejuni*	Human	Australia	≈400bp	-
605	*C. jejuni*	Chicken	Australia	≈380bp, ≈440bp	-
657	*C. coli*	Human	Australia	≈400bp	-
691	*C. coli*	Human	Australia	≈300bp	-
705	*C. jejuni*	Human	Australia	-	-
801	*C. jejuni*	Chicken	Australia	-	-
886	*C. jejuni*	Human	Australia	≈400-410bp	-
887	*C. jejuni*	Human	Australia	-	-
957	*C. jejuni*	Human	Australia	Putative *dfr1*, ≈110-120bp, ≈400-410bp	≈700-750bp
961	*C. coli*	Chicken	Australia	-	-
4547	*C. jejuni*	Human	Australia	≈400bp	≈800bp

Table 21. *dfr1* & *dfr9* results of trimethoprim resistant *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	**Species**	**Source**	**Country**	***dfr1* gene**	***dfr9* gene**
3015	*C. jejuni*	Chicken	Australia	≈450bp	-
4002	*C. jejuni*	Chicken	Australia	≈450bp	≈800-850bp
5001	*C. jejuni*	Chicken	Australia	-	-
5565	*C. coli*	Human	Japan	Putative *dfr1*, ≈400bp	-
7005	*C. jejuni*	Chicken	Australia	-	-
7006	*C. coli*	Chicken	Australia	-	-
8004	*C. coli*	Chicken	Australia	Putative *dfr1*, ≈400bp	-
9014	*C. jejuni*	Chicken	Australia	Putative *dfr1*, ≈400bp	≈800bp
9126	*C. jejuni*	Human	Australia	-	-
81116	*C. jejuni*	Human	UK	≈400bp	-
85-239	*C. lari*	Human	USA	-	-
85-241	*C. lari*	Human	USA	Putative *dfr1*	-
85-244	*C. lari*	Human	USA	-	-
85-256	*C. fetus* subsp. *fetus*	Human	USA	≈420bp	-
0519/010	*C. coli*	Human	Australia	-	-
0520/072	*C. jejuni*	Human	Australia	≈450bp	≈800-850bp
B5Ft8	*C. jejuni*	Chicken	USA	-	-
C50	*C. jejuni*	Chicken	Australia	Putative *dfr1*, ≈400bp	-
C163	*C. jejuni*	Chicken	Australia	-	-
C170	*C. jejuni*	Chicken	Australia	Putative *dfr1*, ≈400bp	≈700-750bp
C491	*C. jejuni*	Chicken	Australia	-	-
C560	*C. jejuni*	Chicken	Australia	≈400bp	-
CE01	*C. jejuni*	Chicken	USA	-	-
CE04	*C. coli*	Chicken	USA	-	-
CE05	*C. jejuni*	Chicken	USA	-	-
CE06	*C. jejuni*	Chicken	USA	-	-
CE07	*C. jejuni*	Chicken	USA	-	-
CE7-8	*C. jejuni*	Chicken	USA	≈400bp	-
CE09	*C. coli*	Chicken	USA	≈400bp	-
CSIRO11	*C. fetus* subsp. *fetus*	Human	Australia	-	-

Table 22. *dfr1* & *dfr9* results of trimethoprim resistant *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	**Species**	**Source**	**Country**	***dfr1* gene**	***dfr9* gene**
CSIRO40	*C. coli*	Human	Australia	-	-
D117	*C. jejuni*	Human	USA	≈400bp	-
D123	*C. jejuni*	Human	Canada	≈400bp, ≈520bp	-
D2189	*C. hyointestinalis*	Human	USA	-	≈510bp
FF1	*C. jejuni*	Human	Australia	≈450bp	-
FF3	*C. jejuni*	Human	Australia	-	-
FF5	*C. jejuni*	Human	Australia	≈400bp	-
FF18	*C. jejuni*	Human	Australia	-	-
FF34	*C. jejuni*	Human	Australia	-	-
FF45	*C. jejuni*	Human	Australia	-	-
JC1	*C. jejuni*	Human	Japan	-	-
JC2	*C. jejuni*	Human	Japan	≈400bp	-
JC3	*C. jejuni*	Human	Japan	-	-
JC5	*C. jejuni*	Human	Japan	≈400bp	-
JC6	*C. jejuni*	Human	Japan	≈400bp	≈700-750bp
JC8	*C. jejuni*	Human	Japan	-	-
JC10	*C. jejuni*	Chicken	Japan	-	-
JC11	*C. jejuni*	Chicken	Japan	-	-
JC13	*C. coli*	Cat	Japan	-	-
JC14	*C. coli*	Dog	Japan	Putative *dfr1*, ≈400bp	≈220bp
PRC13	*C. jejuni*	Chicken	USA	-	-
PRC67	*C. jejuni*	Chicken	USA	-	-
RM2	*C. jejuni*	Human	Australia	-	-
RM3	*C. coli*	Human	Australia	-	-
RM6	*C. jejuni*	Human	Australia	-	-
RM7	*C. jejuni*	Human	Australia	≈430bp	-
RM10	*C. jejuni*	Human	Australia	-	-
RM16	*C. jejuni*	Human	Australia	≈430bp	-
RM17	*C. jejuni*	Human	Australia	-	-
RM19	*C. jejuni*	Human	Australia	-	-

Table 23. *dfr1* & *dfr9* results of trimethoprim resistant *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	**Species**	**Source**	**Country**	***dfr1* gene**	***dfr9* gene**
RM20	*C. jejuni*	Human	Australia	-	-
RM103	*C. jejuni*	Human	Australia	-	-
RM1221	*C. jejuni*	Chicken	USA	-	-
TC19	*C. jejuni*	Chicken	USA	≈400bp	-
NCTC 11168-GS	*C. jejuni*	Human	UK	≈400-410bp	≈800bp
NCTC 11351	*C. jejuni*	Bovine	USA	-	-
NCTC 11366	*C. coli*	Pig	UK	-	-
ATCC 33562	*C. sputorum* bv. *sputorum*	Bovine	Belgium	-	≈200-250bp
ATCC 35223	*C. lari*	Human	UK	Putative *dfr1*	-

APPENDIX 2

Table 24. *intI1*& *intI2* results of *C. jejuni, C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	*intI1* gene	*intI2* gene
0-10	*C. jejuni*	Chicken	USA	≈400bp	-
004	*C. jejuni*	Chicken	Australia	≈400bp	≈130-180bp, ≈300bp
007	*C. jejuni*	Chicken	Australia	≈400bp	-
8	*C. jejuni*	Human	Australia	≈400bp	-
008	*C. jejuni*	Chicken	Australia	≈400bp	-
011	*C. lari*	Chicken	Australia	≈400bp, ≈700bp	≈300bp
013	*C. jejuni*	Chicken	Australia	≈400bp	-
017	*C. jejuni*	Chicken	Australia	≈400bp	-
018	*C. coli*	Chicken	Australia	≈400-410bp	-
019	*C. jejuni*	Chicken	Australia	≈400bp	-
3-9	*C. jejuni*	Chicken	USA	-	-
32	*C. jejuni*	Human	Australia	≈400bp	N. A.
93	*C. jejuni*	Human	Australia	≈400-410bp	≈320-350bp
108	*C. jejuni*	Human	Australia	≈400bp	-
108	*C. jejuni*	Chicken	Netherlands	≈400bp	-
131	*C. jejuni*	Chicken	Australia	≈200-210bp, ≈290-300bp, ≈310bp, ≈410bp, ≈690bp	-
133	*C. jejuni*	Chicken	Australia	≈400bp, ≈800-850bp	-
134	*C. jejuni*	Chicken	Australia	≈400bp, ≈800-850bp	≈410-420bp
141		Chicken	Australia	-	-
142		Chicken	Australia	N. A.	-
173		Human	Australia	≈430bp	-
231	*C. jejuni*	Chicken	Australia	≈400bp	-
235	*C. jejuni*	Chicken	Australia	≈410-420bp	-
239	*C. jejuni*	Human	Australia	≈400-410bp	-
293	*C. coli*	Human	Australia	≈400bp	-
303	*C. jejuni*	Chicken	Australia	-	-
336	*C. jejuni*	Chicken	Australia	Putative *intI1*, ≈400-420bp	-

N. A. – Not Applicable.

Table 25. *intI1*& *intI2* results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	**Species**	**Source**	**Country**	***intI1* gene**	***intI2* gene**
351	*C. jejuni*	Human	Australia	≈410-420bp, ≈800bp	-
354	*C. jejuni*	Human	Australia	≈400bp	-
375	*C. jejuni*	Human	Australia	≈400bp	-
388	*C. coli*	Human	Australia	N. A.	-
405	*C. jejuni*	Chicken	Australia	≈400-410bp	-
410	*C. jejuni*	Human	Australia	≈400bp	-
413	*C. jejuni*	Chicken	Australia	N. A.	≈500bp, ≈780bp, ≈1kb
415	*C. jejuni*	Chicken	Australia	≈420bp, ≈850bp	-
430	*C. jejuni*	Human	Australia	N. A.	-
435	*C. jejuni*	Human	Australia	N. A.	-
439	*C. jejuni*	Human	Australia	N. A.	-
440	*C. jejuni*	Human	Australia	≈400-410bp	-
458	*C. jejuni*	Human	Australia	N. A.	-
506	*C. jejuni*	Chicken	Australia	≈400bp	-
515	*C. jejuni*	Human	Australia	≈400bp	≈300-310bp
520	*C. jejuni*	Human	Australia	≈400bp	-
605	*C. jejuni*	Chicken	Australia	≈420bp, ≈850bp	≈420bp
657	*C. coli*	Human	Australia	≈400bp	-
691	*C. coli*	Human	Australia	≈400-410bp	-
705	*C. jejuni*	Human	Australia	N. A.	-
801	*C. jejuni*	Chicken	Australia	≈410bp	-
886	*C. jejuni*	Human	Australia	N. A.	-
887	*C. jejuni*	Human	Australia	≈400-410bp	≈150-160bp
957	*C. jejuni*	Human	Australia	N. A.	-
961	*C. coli*	Chicken	Australia	≈400-410bp	-
4547	*C. jejuni*	Human	Australia	N. A.	-
3015	*C. jejuni*	Chicken	Australia	≈400-410bp	-
4002	*C. jejuni*	Chicken	Australia	≈400bp	-
5001	*C. jejuni*	Chicken	Australia	≈400-410bp	≈1kb
5565	*C. coli*	Human	Japan	≈400bp	-

N. A. – Not Applicable.

Table 26. *intI1*& *intI2* results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	**Species**	**Source**	**Country**	***intI1* gene**	***intI2* gene**
7005	*C. jejuni*	Chicken	Australia	≈410bp	-
7006	*C. coli*	Chicken	Australia	-	-
8004	*C. coli*	Chicken	Australia	≈400-410bp	-
9014	*C. jejuni*	Chicken	Australia	≈400-410bp	Putative *intI2*, ≈290-300bp, ≈400-410bp, ≈1-1.05kb
9126	*C. jejuni*	Human	Australia	≈400-410bp	-
81116	*C. jejuni*	Human	UK	Putative *intI1*, ≈400-410bp, ≈500-510bp	≈400-410bp, ≈1-1.05kb
85-239	*C. lari*	Human	USA	≈400bp	-
85-241	*C. lari*	Human	USA	-	≈500bp, ≈600bp
85-244	*C. lari*	Human	USA	≈400bp	-
85-256	*C. fetus* subsp. *fetus*	Human	USA	≈400bp	-
0519/010	*C. coli*	Human	Australia	≈400-410bp	-
0520/072	*C. jejuni*	Human	Australia	≈410bp	-
B5Ft8	*C. jejuni*	Chicken	USA	-	-
C50	*C. jejuni*	Chicken	Australia	≈400bp	≈1.1kb
C163	*C. jejuni*	Chicken	Australia	≈400-410bp	-
C170	*C. jejuni*	Chicken	Australia	≈250-270bp, Putative *intI1* ≈400-410bp, ≈560bp	-
C491	*C. jejuni*	Chicken	Australia	≈200bp, ≈250bp, ≈400bp	-
C560	*C. jejuni*	Chicken	Australia	≈200-250bp, ≈400bp	-
CE01	*C. jejuni*	Chicken	USA	≈160-170bp, ≈340-350bp, ≈400-410bp	≈1-1.01kb
CE04	*C. coli*	Chicken	USA	≈400-410bp	-
CE05	*C. jejuni*	Chicken	USA	≈390-400bp	-
CE06	*C. jejuni*	Chicken	USA	≈410bp	-
CE07	*C. jejuni*	Chicken	USA	≈400-410bp	-

N. A. – Not Applicable.

Table 27. *intI1*& *intI2* results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	*intI1* gene	*intI2* gene
CE7-8	*C. jejuni*	Chicken	USA	≈400-410bp	≈120bp, ≈260-270bp
CE09	*C. coli*	Chicken	USA	≈400-410bp	≈150bp, ≈300bp
CSIRO11	*C. fetus* subsp. *fetus*	Human	Australia	≈800bp	-
CSIRO40	*C. coli*	Human	Australia	-	-
D117	*C. jejuni*	Human	USA	≈410bp	-
D123	*C. jejuni*	Human	Canada	Putative *intI1*, ≈400-410bp	≈400bp
D2189	*C. hyointestinalis*	Human	USA	≈300-310bp	≈500bp, ≈700bp
FF1	*C. jejuni*	Human	Australia	≈400bp	-
FF3	*C. jejuni*	Human	Australia	-	-
FF5	*C. jejuni*	Human	Australia	N. A.	-
FF18	*C. jejuni*	Human	Australia	≈400-410bp	≈300-310bp
FF34	*C. jejuni*	Human	Australia	-	-
FF45	*C. jejuni*	Human	Australia	≈400bp	-
JC1	*C. jejuni*	Human	Japan	Putative *intI1*, ≈410bp	-
JC2	*C. jejuni*	Human	Japan	Putative *intI1*, ≈400-410bp	-
JC3	*C. jejuni*	Human	Japan	≈400bp	-
JC5	*C. jejuni*	Human	Japan	≈370p	-
JC6	*C. jejuni*	Human	Japan	≈400bp	-
JC8	*C. jejuni*	Human	Japan	≈400bp	-
JC10	*C. jejuni*	Chicken	Japan	-	-
JC11	*C. jejuni*	Chicken	Japan	≈410bp	≈300bp
JC13	*C. coli*	Cat	Japan	-	-
JC14	*C. coli*	Dog	Japan	≈400-410bp, ≈850bp	Putative *intI2*
PRC13	*C. jejuni*	Chicken	USA	N. A.	-
PRC67	*C. jejuni*	Chicken	USA	≈400-410bp	-
RM2	*C. jejuni*	Human	Australia	-	-
RM3	*C. coli*	Human	Australia	≈400-410bp	-
RM6	*C. jejuni*	Human	Australia	-	-
RM7	*C. jejuni*	Human	Australia	≈400bp	-

N. A. – Not Applicable.

Table 28. *intI1*& *intI2* results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	**Species**	**Source**	**Country**	***intI1* gene**	***intI2* gene**
RM10	*C. jejuni*	Human	Australia	≈130bp	-
RM16	*C. jejuni*	Human	Australia	≈400bp	-
RM17	*C. jejuni*	Human	Australia	≈400bp	-
RM19	*C. jejuni*	Human	Australia	≈400bp	-
RM20	*C. jejuni*	Human	Australia	≈400bp	-
RM103	*C. jejuni*	Human	Australia	≈400bp	-
RM1221	*C. jejuni*	Chicken	USA	≈400bp	N. A.
TC19	*C. jejuni*	Chicken	USA	N. A.	≈160-180bp, ≈300bp
NCTC 11168-GS	*C. jejuni*	Human	UK	≈400bp	≈320bp
NCTC 11351	*C. jejuni*	Bovine	USA	≈400bp	-
NCTC 11366	*C. coli*	Pig	UK	≈400bp	-
ATCC 33562	*C. sputorum* bv. *sputorum*	Bovine	Belgium	-	-
ATCC 35223	*C. lari*	Human	UK	≈400bp	-

N. A. – Not Applicable.

APPENDIX 3

Table 29. Gene cassette PCR results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Amplicons	Amplicon Number
0-10	*C. jejuni*	Chicken	USA	510	1
004	*C. jejuni*	Chicken	Australia	450, 550	2
007	*C. jejuni*	Chicken	Australia	610	1
8	*C. jejuni*	Human	Australia	450, 550	2
008	*C. jejuni*	Chicken	Australia	420-430, 520-540, 910-950	3
011	*C. lari*	Chicken	Australia	350, 500, 550, 620, 800, 1kb, 1.2kb	7
013	*C. jejuni*	Chicken	Australia	450, 1kb	2
017	*C. jejuni*	Chicken	Australia	430-440, 550	2
018	*C. coli*	Chicken	Australia	450, 500, 1kb	3
019	*C. jejuni*	Chicken	Australia	500, 550, 900	3
32	*C. jejuni*	Human	Australia	-	-
93	*C. jejuni*	Human	Australia	450, 500, 570, 700, 1kb	5
108	*C. jejuni*	Human	Australia	440-450, 500, 610	3
108	*C. jejuni*	Chicken	Netherlands	-	-
131	*C. jejuni*	Chicken	Australia	430-440, 520, 620, 890-900	4
133	*C. jejuni*	Chicken	Australia	430-440, 510, 620, 890-900, 1.3kb	5
134	*C. jejuni*	Chicken	Australia	510, 620, 890-900, 1.3kb	4
142		Chicken	Australia	610	1
173		Human	Australia	600-610, 710	2
231	*C. jejuni*	Chicken	Australia	420-430, 550, 710, 810	4
235	*C. jejuni*	Chicken	Australia	440-450, 560-570	2
239	*C. jejuni*	Human	Australia	-	-
293	*C. coli*	Human	Australia	250, 260, 430, 480-490, 550, 950	6
303	*C. jejuni*	Chicken	Australia	410, 450, 500, 570	4

Table 30. Gene cassette PCR results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Amplicons	Amplicon Number
336	*C. jejuni*	Chicken	Australia	440, 500, 560	3
351	*C. jejuni*	Human	Australia	510, 620, 890-900, 1kb	4
354	*C. jejuni*	Human	Australia	520-540	1
375	*C. jejuni*	Human	Australia	450, 500	2
405	*C. jejuni*	Chicken	Australia	420-430, 530-540	2
410	*C. jejuni*	Human	Australia	-	-
415	*C. jejuni*	Chicken	Australia	470-480, 600, 950-1kb	3
440	*C. jejuni*	Human	Australia	520-530	1
506	*C. jejuni*	Chicken	Australia	270-280, 500-510, 520, 1kb	4
515	*C. jejuni*	Human	Australia	-	-
520	*C. jejuni*	Human	Australia	430, 490, 530, 630-650	4
605	*C. jejuni*	Chicken	Australia	440-450, 600	2
657	*C. coli*	Human	Australia	-	-
691	*C. coli*	Human	Australia	230, 320, 430, 460, 530, 700, 1kb	7
801	*C. jejuni*	Chicken	Australia	420	1
887	*C. jejuni*	Human	Australia	220, 230, 330, 440, 490, 580, 720-730	7
961	*C. coli*	Chicken	Australia	230, 330, 450, 500, 720-730, 1kb	6
3015	*C. jejuni*	Chicken	Australia	-	-
4002	*C. jejuni*	Chicken	Australia	410, 440, 500, 510, 550, 580, 670, 1.25kb	8
5565	*C. coli*	Human	Japan	290, 400, 500, 550, 900, 1.2kb	6
8004	*C. coli*	Chicken	Australia	210, 330, 440, 490, 580, 720, 1kb, 1.4kb	8
9014	*C. jejuni*	Chicken	Australia	440, 500, 560	3
9126	*C. jejuni*	Human	Australia	230, 330, 410, 450, 500, 550, 670, 720, 890-900, 990-1kb	10
81116	*C. jejuni*	Human	UK	450, 500, 550, 680, 810, 900	6
85-244	*C. lari*	Human	USA	280-290, 510, 800, 1.1kb	4
85-256	*C. fetus* subsp. *fetus*	Human	USA	330, 410-420, 450, 500-510, 560, 680, 1kb, 1.3kb, >1.5kb	9

Table 31. Gene cassette PCR results of *C. jejuni*, *C. coli* and non-*C. jejuni/coli* isolates examined

Strain	Species	Source	Country	Amplicons	Amplicon Number
0519/010	*C. coli*	Human	Australia	210, 250, 430, 470, 530, 1kb	6
C170	*C. jejuni*	Chicken	Australia	370-380, 450-460, 500, 560-570, 610, 730, 930-950	7
CE04	*C. coli*	Chicken	USA	510-520, 620-630, 750	3
CSIRO11	*C. fetus* subsp. *fetus*	Human	Australia	400, 500, 1kb, 1.3kb, >1.5kb	5
D123	*C. jejuni*	Human	Canada	610, 720, 850, 930-950, 1.4kb	5
JC1	*C. jejuni*	Human	Japan	-	-
JC2	*C. jejuni*	Human	Japan	450, 500, 550, 680, 810	5
JC6	*C. jejuni*	Human	Japan	-	-
JC8	*C. jejuni*	Human	Japan	-	-
JC14	*C. coli*	Dog	Japan	440, 550	2
RM3	*C. coli*	Human	Australia	230, 330, 440, 490-500, 580, 720, 1kb	7
RM7	*C. jejuni*	Human	Australia	320, 410, 500-510, 540, 690, 900, 1kb	7
RM17	*C. jejuni*	Human	Australia	320, 410, 440, 550, 670, 800, 900, 1.4kb, >1.5kb	9
RM20	*C. jejuni*	Human	Australia	330, 410-420, 440, 500-510, 550, 670, 900, 1kb, 1.3kb	9
RM1221	*C. jejuni*	Chicken	USA	-	-
NCTC 11168-GS	*C. jejuni*	Human	UK	-	-
NCTC 11351	*C. jejuni*	Bovine	USA	-	-
NCTC 11366	*C. coli*	Pig	UK	-	-
ATCC 35223	*C. lari*	Human	UK	300, 550, 900	3

Table 32. *Campylobacter* isolates that produced gene cassette products of >1.0kb in size

Strain	Species	Source	Country	Amplicon(s) Size
011	*C. lari*	Chicken	Australia	1kb, 1.2kb
013	*C. jejuni*	Chicken	Australia	1kb
018	*C. coli*	Chicken	Australia	1kb
93	*C. jejuni*	Human	Australia	1kb
133	*C. jejuni*	Chicken	Australia	1.3kb
134	*C. jejuni*	Chicken	Australia	1.3kb
351	*C. jejuni*	Human	Australia	1kb
415	*C. jejuni*	Chicken	Australia	950-1kb
506	*C. jejuni*	Chicken	Australia	1kb
691	*C. coli*	Human	Australia	1kb
961	*C. coli*	Chicken	Australia	1kb
4002	*C. jejuni*	Chicken	Australia	1.25kb
5565	*C. coli*	Human	Japan	1.2kb
8004	*C. coli*	Chicken	Australia	1kb, 1.4kb
9126	*C. jejuni*	Human	Australia	990-1kb
85-244	*C. lari*	Human	USA	1.1kb
85-256	*C. fetus subsp. fetus*	Human	USA	1kb, 1.3kb, >1.5kb
0519/010	*C. coli*	Human	Australia	1kb
CSIRO11	*C. fetus subsp. fetus*	Human	Australia	1kb, 1.3kb, >1.5kb
D123	*C. jejuni*	Human	Canada	1.4kb
RM3	*C. coli*	Human	Australia	1kb
RM7	*C. jejuni*	Human	Australia	1kb
RM17	*C. jejuni*	Human	Australia	1.4kb, >1.5kb
RM20	*C. jejuni*	Human	Australia	1kb, 1.3kb

APPENDIX 4