# Identification and genotyping of *Campylobacter* spp. strains isolated from a captive wildlife population in New Zealand

A thesis submitted in fulfilment
of the requirements for the degree
of
**Masters of Science**
at
**Auckland University of Technology**
by
**Niki Sowerby**

———————

2015

School of Applied Science
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# Abstract

The Auckland Zoological Park holds 120 species and over 850 animals within a 17-hectare park in central Auckland. Species include non-primate mammals, avians, primates and reptiles, including a mixture of exotic and native species. Little is known about the epidemiology of *Campylobacter* in wildlife populations, as previous and current studies focus on domestic and food producing animals, as well as *Campylobacter* in medical settings. This study set out to determine and identify *Campylobacter* in a healthy captive wildlife population and present the isolated organisms virulence potential by investigating the presence of putative virulence genes (*flaA*, *cadF*, *cdtA*, *cdtB*, *cdtC* and *gyrA*). The genes investigated are commonly associated with multifactorial processes that are involved with *Campylobacter* infection. Based on sequencing profiles of the virulence genes investigated, phylogenetic relationships were demonstrated between the different *Campylobacter* strains isolated from such a wide variety of animals.

Over a 9-month period (December 2013 to August 2014) 202 faecal samples were collected from a variety of animal species of the Auckland Zoo's captive population, for evaluation of the presence of *Campylobacter*. From the 202 samples collected, *Campylobacter* was isolated from 17 (8.9%), where *Campylobacter jejuni* was the most frequently isolated *Campylobacter* species, with a recovery rate of 52.9% in the present study. Upon isolation, isolates were then investigated for the presence of specific genes that are commonly associated with pathogenesis of *Campylobacter* infection. The genes were selected on the basis of their involvement in motility, adhesion, invasion and toxin production, which are all associated with the multifactorial bacterial pathogenic mechanisms.

The absence of one of these virulence factors can limit and reduce the organism's pathogenicity and virulence potential. Of the 6 genes investigated, flagella A (*flaA*) genes were found in 100%, *Campylobacter* adhesion factor (*cadF*) was found in 58.8%, gyrase A (*gyrA*) in 70.6%, and cytotoxin A, B and C (*cdtA*, *cdtB* and *cdtC*) in 70.6%, 47.1% and 35.3% respectively. Sequencing of these genes revealed both homology and heterogeneity of gene sequences between the different

*Campylobacter* species, demonstrating both genetic conservation and variation respectively.

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# Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Signed .....

Date .....

# Acknowledgements

I would like to thank Richard Campbell at New Zealand Veterinary Pathology, (NZVP) along with Dr Richard Jakob-Hoff and the NZCCM Vet team at the Auckland Zoo. Without their support this project would not have been possible. Now that this is over, you can all stop calling me the poo lady.

Thank you to Dr Gerardo Herrera and Ian Bruce at NZVP Palmerston North, Dr Anne Midwinter at Massey University Palmerston North, Dr Miomir Vidicki at Auckland University of Technology, as well as Darren Welch and Kevin Smith at North Shore Hospital for your technical help and support throughout my research.

Thanks to all my family, Nadia and friends who put up with my absence on numerous occasions while I was stuck at my desk working. Im sure I have missed a lot of birthdays and other important dates in the process. And I know you must all be sick of hearing about *Campylobacter* by now, but on the plus side, you are all very educated on the topic.

Lastly, a big thank you to Dr Fabrice Merien for all the help and advice throughout this project. Thanks for always being on hand and available, and never rolling your eyes (at least not to my face) at my numerous questions. Without your encouragement and support this thesis would not have progressed further than an idea on an A3 piece of paper.

# 1 Introduction

## 1.1 *Campylobacter* biology

*Campylobacter* is a genus of Gram-negative, motile, spiral-shaped, non-spore forming bacteria belonging to the *Campylobacteraceae* family (Markey, Leonard, Archambault, Cullinane, & Maguire, 2013). *Campylobacter* species (spp.) are fastidious organisms that have specific growth requirements. They are microaerophilic, requiring an oxygen concentration of 3-15% and a carbon dioxide concentration of 3-5% (Van Vliet & Ketley, 2001). These organisms are thermotolerant with growth capabilities in temperatures between 37-42°C. However, despite this narrow temperature range *Campylobacter* spp. have the ability to grow in a wide range of hosts (Mihaljevic et al., 2007). They are small in size, ranging from 0.2-0.9µm in width, and 0.5-5µm in length (Epps et al., 2013). In comparison to other enteric pathogens, *Campylobacter* spp. have a relatively small genome size. For example, *Campylobacter jejuni* and *Campylobacter coli* have a genome of around 1600-1700kb, compared to *Escherichia coli* that has a genome size of around 4500kb (Van Vliet & Ketley, 2001).

*Campylobacter* spp. are ubiquitous in the environment, and form long term associations with animals hosts (Young, Davis, & DiRata, 2007) as commensal enteric organisms. *Campylobacter* spp. are zoonotic pathogens (T. Humphrey, O'Brien, & Madsen, 2007) and are commonly associated with human gastroenteritis. Within the genus *Campylobacter*, there are 20 species, 8 subspecies and 2 biovars (Table 1) (Debruyne et al., 2010; T. Humphrey et al., 2007) that can be found widely distributed throughout the world. *Campylobacter* spp. can be found as both commensals in the intestinal tracts of many warm blooded animals and humans, as well as a pathogenic organisms which can lead to illness in the host. In humans, *Campylobacter* spp. are known to cause infection, such as gastroenteritis, periodontal disease and in severe cases abortion and septicaemia whereas many others are non-pathogenic (Allos, 2001).
*C. jejuni* and *C. coli* are among the *Campylobacter* spp. that are most associated with gastroenteritis (Manser & Dalziel, 1985), although more recently,

*Campylobacter upsaliensis* has also been reported as human pathogen. (T. Humphrey et al., 2007). Similarly, those that hold importance in a veterinary setting include *C. jejuni*, *C. coli* and *C. upsaliensis* (M. Andrzejewska, B. Szczepańska, J J. Klawe, D. Śpica, & Chudzińska, 2013).

**Table 1. Members of the *Campylobacteraceae* family**. Their known reservoirs and disease associations in humans (Debruyne et al., 2010; T. Humphrey et al., 2007; Inglis, Hoar, Whiteside, & Morck, 2007).

| Species | Known source | Disease associations in humans |
|---|---|---|
| *C. canadensis* | Whooping crane birds | No known disease |
| *C. coli* | Pigs, poultry, cattle, sheep, birds | Gastroenteritis, septicaemia |
| *C. concisus* | Human | Periodontal disease, gastroenteritis |
| *C. curvus* | Human | Periodontal disease, gastroenteritis |
| *C. fetus subsp. fetus* | Cattle, sheep | Septicaemia, gastroenteritis, abortion, meningitis |
| *C. fetus* subsp. *venerealis* | Cattle | Septicaemia |
| *C. gracilis* | Man | Periodontal disease, empyema, abscesses |
| *C. helveticus* | Cats, dogs | None at present |
| *C. hyointestinalis* subsp. *hyointestinalis* | Pigs, cattle, hamsters, deer | Gastroenteritis |
| *C. hyointestinalis* subsp. *lawsonii* | Pigs | None at present |
| *C. hyoilei* | Pigs | None at present |
| *C. jejuni* septicaemia subsp. *doylei* | Man | Gastroenteritis, gastritis, |
| *C. jejuni* subsp. *jejuni* | Poultry, pigs, cattle, sheep, water, birds, mink, rabbits, dogs, cats, insects | Gastroenteritis, septicaemia, meningitis, abortion, proctitis Guillain- *Barré* syndrome |
| *C. lari* | Birds (including poultry), water, dogs, cats, monkeys horse, seals | Gastroenteritis, septicaemia |
| *C, lari* subsp. *concheus* | | |
| *C, lari* subsp. *lari* | | |
| *C. mucosalis* | Pigs | None at present |
| *C. rectus* | Man | Periodontal disease |
| *C. showae* | Man | Periodontal disease |
| *C. sputorum* bv. Sputorum | Man, cattle, pigs | Abscesses, gastroenteritis |
| *C. sputorum* bv. Faecalis | Sheep, bulls | None at present |
| *C. subantarcticus* | Birds | No known disease |
| *C. upsaliensis* | Dogs, cats | Gastroenteritis, septicaemia, abscesses |
| *C. insulaenigrae* | Seals, porpoises | None at present |
| *C. lanienae* | Cattle, pigs, humans | None at present |
| *C. hominis* | Humans | Gastroenteritis in immunocompromised |

# 2 Epidemiology of *Campylobacter* Infections

## 2.1 Reservoirs

The different species of *Campylobacter* exhibit different preferences to reservoirs in which they are found, such as soil and water (Sandberg, Bergsjø, Hofshagen, Skjerve, & Kruse, 2002). However, *Campylobacter* is most commonly found in warm blooded animals, including birds, domestic and production animals such as cats, dogs, cattle, sheep and pigs without producing any symptoms of disease (Blaser, 1997; Epps et al., 2013; T. Humphrey et al., 2007). Incidences of *Campylobacter* infections in humans is high due to the high prevalence of *Campylobacter* found in animals used as food sources.

In developing countries, the most common sources for *Campylobacter* infections are contaminated food sources and the environment (Epps et al., 2013). In particular, food sources from animal origin: meat (cattle, sheep and pig), poultry and unpasteurised milk, where infection sources from the environment are contaminated water reservoirs (Horrocks, Anderson, Nisbet, & Ricke, 2009) and soil. Given the high incidence of *Campylobacter* among animals, it is no surprise that the organism has been found to contaminate the environment (Blaser, 1997).

*Campylobacter* spp. hold importance in the food industry as a majority of *Campylobacter* related illnesses originate from the consumption of contaminated foodstuffs. In particular, raw and undercooked meat, poultry, fresh vegetables and unpasteurized milk have been a predominant source for the acquisition of *Campylobacter* infections (Horrocks et al., 2009).

## 2.2 Incidence in humans

In the 70s, *Campylobacter* garnered much attention as it became apparent that these organisms, specifically *C. jejuni* and *C. coli*, were the causes of acute diarrhoea (M. B. Skirrow, 1994). In humans, *Campylobacter* related gastroenteritis (campylobacteriosis) is the leading cause of bacterial related illnesses every year. *C. jejuni*, *C. upsaliensis* (Snelling, Matsuda, Moore, & Dooley, 2005) and *C. coli* (Manser & Dalziel, 1985) are the species most commonly associated with gastroenteritis among humans. Around 95% of clinical

cases of campylobacteriosis in the United Kingdom are reported to be caused by *C. jejuni* and *C. coli* (Snelling et al., 2005). In the United States *C. jejuni* accounts for 80-90% of *Campylobacter* related illness (Gharst, Oyarzabal, & Hussain, 2013).

There are approximately 2.4 million reported *Campylobacter* related illnesses in the United States (Horrocks et al., 2009) annually. In the United Kingdom, 1% of reported cases of gastroenteritis are caused by *Campylobacter* (Snelling et al., 2005). This is the same for the rest of the European population, in which *Campylobacter* affects 1% on an annual basis (E. V. Taylor et al., 2013). Campylobacteriosis is the most common notified disease in New Zealand (NZ) (Lane & Briggs, 2014). In fact, NZ has one of the highest national incidence rates of campylobacteriosis per capita in the developed world, with 400 cases per 100,000 people (Sharon V R. Epps et al., 2013). Incidences in NZ is 1.5 to 3 times higher then those reported in Australia, England and Wales (Lane & Briggs, 2014).

Because surveillance of gastroenteritis illnesses tends to be more common only in the developed world and higher income countries compared to developing countries, it is believed that worldwide incidences are actually far higher than what is currently reported. Humphrey et al. (2007) suggest that for every case reported, nine go unreported. However, estimates have been made through community studies reporting incidences in developing countries (African continent, Eastern Mediterranean, Southeast Asia) to be around 5 to 20% annually (Coker, Isokpehi, Thomas, Amisu, & Obi, 2002).

## 2.3 Incidence in animals

*Campylobacter* is a commensal enteric organism in many domestic animals including dogs, cats and birds (J. Stanley et al., 1992), as well as farm animals including cattle, sheep (Butzler, 2004) and pigs, without showing ill effect. However, given certain conditions, such as stress and dietary changes this organism can cause gastroenteritis. Incidence of *Campylobacter* related illnesses has been on the rise over the past decade, with an increase of those acquired by animals. Estimations on the prevalence of *Campylobacter* in animals that are encountered on a daily basis within the household is important to help limit the

transmission of *Campylobacter* from pets to their human owner (M. Andrzejewska et al., 2013).

## 2.4 Domestic animals

*Campylobacter* have been routinely isolated from domestic animals, such as cats and dogs, which can be asymptomatic carriers. The incidence of *Campylobacter* in cats and dogs varies depending on factors such as age, animal species and seasonality (M. Andrzejewska et al., 2013). Estimates have indicated that domestic animal incidence rates can range anywhere between 11-92% (Horrocks et al., 2009). *Campylobacter* have been isolated in both healthy and diarrhoeic cats and dogs, who are often sources of infection among pet owners.

### 2.4.1 *Campylobacter* in cats

In cats, common *Campylobacter* organisms isolated have included *C. jejuni, C. coli, C. upsaliensis* and *C.helveticus* (Shen, Feng, Dewhirst, & Fox, 2001). *C. jejuni, C. coli* and *C. upsaliensis* have all been associated with campylobacteriosis in humans, whereas *C. helveticus*, which shows a considerable degree of similarity in phenotypic characteristics (J. Stanley et al., 1992) to *C. upsaliensis* has not been associated with human illness. In fact, *C. helveticus* has only been isolated in healthy cats and on rare occasions, dogs (J. Stanley et al., 1992).

The incidence of *Campylobacter* is said to be 14.5%- 32% in healthy cats. This range can be seen in various studies, such as that performed by Shen et al. (2001), who isolated *Campylobacter* from 20.7% of 227 felines tested in the United States. Co-colonisation was apparent in 37% of positive samples. Of the total *Campylobacter* spp. isolated, 86% were *C. helveticus*, 47% were *C. upsaliensis* and 6% *C. jejuni* (Shen et al., 2001). Co-colonisation of *Campylobacter* seen here is known to occur in cats and dogs, as well as pigs and on rare occasions, humans (Koene, Houwers, Dijkstra, Duim, & Wagenaar, 2009).
In supposedly healthy stray cats, Gargiulo et al. (2008) found slightly lower rates (16.8%) of *Campylobacter*. However, all species isolated from this study were *C. jejuni* (Gargiulo et al., 2008). Andrzejewska et al. (2013) isolated *Campylobacter* with an even lower incidence of only 9.6% of cats examined in their study. *Campylobacter* infections in cats have most commonly been associated with *C. jejuni*, though it is generally more problematic in kittens (Shen et al., 2001).

The presence of *Campylobacter* isolated from healthy cats in these studies, correspond with suggestions that *Campylobacter* spp. isolated in cats are commensal enteric organisms. The differences in species and rates of isolation may be a culmination of factors such as age, seasonal variances (M. Andrzejewska et al., 2013) as well as living environments. For example, Gargiulo et al. (2008), provided evidence in which environmental factors potentially enhanced isolation of *Campylobacter* spp. The prevalence of *Campylobacter* found in cats during their study, was higher in harbour areas, compared to those isolated in urban areas (Gargiulo et al., 2008). This may be a result of increased cross contamination of *Campylobacter* from sea birds species that are commonly found in harbour/coastal areas and also harbour the organism.

### 2.4.2 *Campylobacter* in dogs

Similar to cats, it is also well established that dogs harbour *Campylobacter* within the intestinal tracts, where the organism has been associated with both healthy and diarrhoeic dogs. Dogs are so often referred to as more than just pets within the family household, making this closeness dogs have with family members a cause for concern with the transmission of *Campylobacter* to human owners (Leonard et al., 2011). Common *Campylobacter* spp. found in dogs include *C. upsaliensis, C. jejuni* and *C. coli*. Investigations have revealed that *C. upsaliensis* is most commonly isolated from dogs. In fact, reports have indicated that the first isolate of *C. upsaliensis* was from canine faeces in 1983 (Birthe, Karl, Michael, Christian, & Mogens, 2004). Though, unlike cats, only on rare occasions (2%) has *C. helveticus* been isolated from dogs (J. Stanley et al., 1992).

Out of 240 dogs tested, Leonard et al. (2011) isolated 52 *Campylobacter* spp. Of these positive samples, 88.5% were *C. upsaliensis,* 13.5% were *C. jejuni,* with only one dog with co-colonisation of both *Campylobacter* spp. (Leonard et al., 2011). This provides evidence to back up earlier reports that co-colonisation does occur in dogs. Further evidence of this, is seen in *Campylobacter* excretion pattern studies in dogs by Hald et al. (2004), who also detected co-colonisation in 3.4% of 366 canine faecal samples tested. Although the prevalence is small, evidence suggests that it can occur.

*Campylobacter* is more prevalent in young dogs, with puppies being more likely to harbour *C. upsaliensis* (Parsons et al., 2010). Higher rates of infection occur in puppies between the ages of 9-15 months (Birthe et al., 2004). As with humans, the lower incidence of *Campylobacter* in older dogs may also be due to immunity acquired from early exposure as puppies.

The presence of *Campylobacter* in dogs, particularly puppies, poses a risk as a source of infection to those in the household. There is an increased risk for children between the ages of 0-5 years acquiring *Campylobacter* related illnesses (Birthe et al., 2004). Speciation of *Campylobacter* found in dogs may provide important information in order to limit and control cross-contamination from household dogs within a family.

## 2.5 *Campylobacter* in birds

Birds are well known reservoirs and asymptomatic carriers of *Campylobacter,* and it appears that *C. jejuni* in particular has evolved to preferentially colonise the avian gastrointestinal tract (Newell, 2002). A likely explanation is the favourable metabolic rate and body temperature, 42°C, exhibited by birds that may predispose them to act as reservoirs for *Campylobacter* (Horrocks et al., 2009). *C. jejuni* is the most prevalent species of *Campylobacter* that colonises avian hosts, followed by *C. coli* and *C. lari* respectively. Incidence in birds may vary from 0-100% depending on bird species, feeding habits and location. For example, readings have stated incidence rates of 21.6% (J. Waldenström et al., 2002) in wild migrating birds, 25.3% among birds of prey (Ludovico Dipineto et al., 2014), 33% and 50% (Mohan, 2015) in wild urban birds and aquatic birds respectively. Certain birds appear to be colonised more frequently, where others not at all (J. Waldenström et al., 2002).

In poultry birds, chickens, turkeys, ducks, quails and pigeons are the bird species most frequently colonised (Saleha, Mead, & Ibrahim, 1998) by *Campylobacter*. All in all chickens are a major source of *Campylobacter* related infection in humans, where consumption of contaminated chicken products has lead to numerous foodborne illnesses. In the UK alone, estimates of the prevalence of *Campylobacter* in raw chicken products are as high as 80% (Conlan, Coward, Grant, Maskell, & Gog, 2007; S. Humphrey et al., 2014). Due to potential human

health implications of chicken consumption, the majority of research studies on *Campylobacter* are focussed on poultry. Poultry are accountable for around 50-70% of human infections (Epps et al., 2013).

High incidences of *Campylobacter* found in poultry plants, contamination during the evisceration processes, and overcrowding of poultry plants are the most likely causes. Horizontal transfer of *Campylobacter* can rapidly spread across broiler plants causing mass infection. Humphrey et al. (2014) concluded that these mass infections are caused by the shedding of *Campylobacter* in faeces that harbour the organism, as well as the coprophagic nature of chickens (S. Humphrey et al., 2014), in which faecal matter contaminated with *Campylobacter* is consumed. Chicks are born free of *Campylobacter* and it is only on rare occasions that *Campylobacter* was isolated from chicks earlier then 2-3 weeks old (Mead, 2002). Though, once infected by *Campylobacter* the organism spreads rapidly through the flock which can test positive for *Campylobacter* within 3 days (Newell, 2002).

Questions are also being raised as to the possibilities of birds acting as vectors for *Campylobacter*, in which faecal contamination in the environment has lead to the transmission of *Campylobacter* in humans, domestic animals and livestock (J. Waldenström et al., 2002). Urban birds such as ducks, geese and swans are known to cause heavy environmental faecal contamination (Mohan, 2015), therefore could very well act as vectors for *Campylobacter*.

## 2.6 *Campylobacter* in production animals

Cattle, sheep and pigs are also known to harbour *Campylobacter* spp. where production animal food products have been a source of infection. Unpasteurised milk for instance, has been implicated as a major source of *Campylobacter* outbreaks, more than poultry products which are generally associated with point source/endemic infections.(Blaser, 1997).

The occurrence of *Campylobacter* in cattle is present in lower numbers than what is found in poultry, with an incidence varying between 0-80% (Epps et al., 2013). Calves may become colonised at as young as 4-months old (K. Stanley & Jones, 2003), where adults tend to exhibit lower incidence rates. *C. jejuni* is the most

predominant species isolated from cattle, where it is mainly found in the lower gastrointestinal tract compared to the rumen (Horrocks et al., 2009). This may be due of the low pH levels that are found in the rumen (Epps et al., 2013), which is an unfavourable environment for the organism to flourish.

In cattle ready for slaughter, Hezron et al (2010) isolated *Campylobacter* in 5.6% of samples taken. Of those, 88.9% were *C. jejuni* and 11.1% *C. coli* (Nonga, Sells, & Karimuribo, 2010). However, *Campylobacter* was isolated from carcasses in 9.3% of the samples taken (Nonga et al., 2010). This is indicative of contamination of the carcass during the evisceration processes, which could increase the volume of contaminated raw meat that is readily available to consumers. During studies of the incidence of *Campylobacter* in grazing cattle, Humphrey & Beckett (1987) found an incidence of 10-70% of *Campylobacter* among 10 of 12 herds investigated for the organism (T. J. Humphrey & Beckett, 1987). Although these figures correlate with Epps et al. (2013), it is worth noting that of these 12 herds, the 2 *Campylobacter* negative herds were both provided with water from the mains supply, whereas the 10 herds that tested positive for *Campylobacter* all had access to river or stream water (T. J. Humphrey & Beckett, 1987).

Given that *Campylobacter* is so often found in surface water, this may in fact be the source of *Campylobacter* isolated from the 10 herds. Cows drink roughly 45-70 litres of water per day (T. J. Humphrey & Beckett, 1987), therefore permitting exposure to *Campylobacter.* This may suggest that the presence of *Campylobacter* in cattle is due to environmental sources, without affecting their health. Variables that may be taken into account for incidence load of *Campylobacter* in cattle include herd size, age and diet (Nonga et al., 2010). Reports have found that cattle that are forage fed (dairy) have a lower incidence of *Campylobacter* (Horrocks et al., 2009) compared to those from feedlot herds, 7.3% versus 68% respectively (Epps et al., 2013). Access to shared feed and water troughs may be a source of transmission of *Campylobacter* contributing to the higher incidence of the organism seen in feedlot cattle.

Pigs are also known to naturally harbour *Campylobacter*, often found in fairly high carriage rates, 50-100% (Jensen, Dalsgaard, Baggesen, & Nielsen, 2006). Unlike in most other animals, the predominating species of *Campylobacter* isolated is *C. coli*, ranging from 90-100% (Rosef, Paulauskas, & Haslekas, 2009). *C. jejuni* is also commonly isolated, but is generally found in lower numbers, and can coexist with *C. coli* (Jensen et al., 2006). A study in Vietnam found isolation rates of *Campylobacter* of 44.9 % in 188 pigs across 343 farms (Carrique-Mas et al., 2014). Other reports have stated that, *C. jejuni* was in fact isolated at a higher rate than *C. coli* with 38.6% and 14.1 % respectively (Carrique-Mas et al., 2014). Unlike Carrique-Mas et al. (2014) study, Manser et al. (1985), isolated *Campylobacter* from 117 (66%) out of 178 pigs. All species isolated were in fact *C. coli* (Manser & Dalziel, 1985). The higher isolation of *C. jejuni* isolated in Carrique-Mas et al. (2013) study, may be due to environmental factors, farm type and husbandry practices. It has been said that pigs found in outdoor habitats as opposed to more 'conventional' pig farms may often permit a differing occurrence of *Campylobacter* spp. (Jensen et al., 2006).

The incidence of *Campylobacter* in sheep appears to be poorly understood, though some studies have suggested common species isolated from sheep include *C. fetus* subspecies *fetus* (Horrocks et al., 2009), *C. jejuni* and *C. coli* (Mehmet Nuri Açik & Çetinkaya, 2006). Both *C. jejuni* and *C. Coli* are more commonly isolated from the intestines whereas *C. fetus* subspecies *fetus* is associated with pregnant ewes and is the main cause of abortions (Horrocks et al., 2009) among sheep. Carriage among sheep varies like in other animals, depending on age, herd size, and environmental conditions of pasture (K. Stanley & Jones, 2003). Açık et al. (2006) found isolation rates of *Campylobacter* among sheep up to 49.5%, with *C. jejuni* being only slightly more predominant than *C. coli*. Jones et al. (1999) found larger variation rates of anything between 0-100% of *Campylobacter* shedding in sheep.

Jones et al. (1999) performed a year long study of *Campylobacter* in sheep. They found that colonisation of *Campylobacter* in lambs reached 100% by day five (Jones, Howard, & Wallace, 1999). Having been born free of the organism, this provided evidence that horizontal transfer via the environment or the ewe

occurred very early on during lambing. In fact, peaks of *Campylobacter* isolation can often be seen during lambing (K. Stanley & Jones, 2003). The large variation of *Campylobacter* shedding during this study appeared to be dependant on age, diet, season and even pasture location. During the summer months, grazing sheep were found to be shedding *Campylobacter* at a rate of 30%, where *Campylobacter* shedding were in fact at 0% when the sheep were fed on hay and silage, but reached 100% during lambing and weaning (Jones et al., 1999).

## 2.7 *Campylobacter* in wild animal populations

Like domestic and production animal populations, exotic wild animals can also be healthy carriers of *Campylobacter* (Maged M. Taema et al., 2008). Just like in the domestic and production counterparts, given the opportune time *Campylobacter* can also be a major cause of gastroenteritis in exotic animals. Few studies exist on the presence of *Campylobacter* in exotic animals in the wild. Studies appear to be based on those in a captive setting.

Captive non-human primates can also be a common source of the organism, with a prevalence of *Campylobacter* ranging from 9% to 41.7% (Misawa et al., 2000; Stirling et al., 2008). Common *Campylobacter* spp. isolated included *C. jejuni,* which interestingly Stirling et al. (2008) isolated from a variety of new world monkey species such as tamarins, where Naoaki et al. (2000) most commonly isolated *C. hyointestinalis* from larger primate species, simians and chimpanzees. Previous reports have also suggested that *Campylobacter* is one of the most frequently isolated pathogens from both diarrhoeic and healthy captive non-human primates (Maged M. Taema et al., 2008).

Findings on the incidence of *Campylobacter* in the big cats (lions, tigers, cheetahs) are difficult to retrieve. However, studies on causes of gastroenteritis in these animals have found to be *Helicobacter pylori*-like organisms (Eaton et al., 1991; Jakob, Stolte, Valentin, & Schröder, 1997) as opposed to *Campylobacter* spp. However, given the carnivorous eating habits of these large cats, we could assume *Campylobacter* may be transmitted from the carcasses. Behaviour, environment and diet of an animal species may dictate the ability for *Campylobacter* (Maged M. Taema et al., 2008) to infect these animals.

Further study is required to increase our knowledge and understanding of the epidemiology of *Campylobacter* in exotic wildlife populations. Not only to fill the gaps of missing information but also to aid in future management plans for husbandry practices, wildlife disease and conservation programs.

## 2.8 Transmission

*Campylobacter* infections in humnas have been commonly acquired through poor handling techniques, particularly of poultry (Allos, 2001). In the kitchen, where cross-contamination of food may occur during food handling process. Where contaminated meat or poultry is introduced to other food stuff during preparation, often a result of food preparation flow. Many household acquired *Campylobacter* infections (up to 90% within the developed world) occur during the summer months due to bad food preparation and undercooked meats in outdoor cooking facilities (Epps et al., 2013).

Direct transmission can also play a role in acquiring *Campylobacter* related illnesses. The organism is zoonotic, in that it can be transferred from animal to humans directly. As can be seen in Table 1, different animals harbour a variety of *Campylobacter* spp. Where an occupation allows for close or direct contact with animals or animal products, such as veterinarians, farmers, zoo workers, meat and poultry slaughter house and process workers (M.B. Skirrow, 1991) the risk of acquiring *Campylobacter* related infections is increased. The presence in domestic animals such as cats and dogs is of interest to the public health sectors (Koene et al., 2009) as household pets often harbour *Campylobacter*. Both dogs and cats are known to harbour *C. upsaliensis*, *C. jejuni* and *C. coli* (Nietfeld, 2013) and are often a source of infection among owners. Children, older and immunosuppressed people are most at risk from this organism.

# 3 Virulence Factors

The ability of *Campylobacter* to colonise both man and animals is not fully understood (T. Humphrey et al., 2007). To colonise its host, *Campylobacter* must overcome both mechanical and immunological barriers that are found within the gastrointestinal tract (Young et al., 2007). In order to establish and maintain a presence within the gastrointestinal tract *Campylobacter* must facilitate various mechanisms and strategies (Manja Boehm et al., 2011) to permit longevity in its host. Colonisation is influenced by various mechanisms that are proposed to play a role in the pathogenesis of *Campylobacter* (Rizal, Kumar, & Vidyarthi, 2010). These include motility, adherence and toxin production, which are controlled by a set of putative virulence genes. Studying these genes may provide us with clues of *Campylobacter's* role in disease (Datta, Niwa, & Toh, 2003).

## 3.1 Flagella

A virulence factor commonly associated with *Campylobacter* is motility, which is achieved by flagella, and is one of the best studied (T.M. Wassenaar & Blaser, 1999). Motility is crucial for *Campylobacter* to (i) to penetrate the mucus layer that covers the intestinal epithelial cells (M. Andrzejewska, J J. Klawe, B. Szczepańska, & Spica, 2011), (ii) reach attachment sites (Solomon & Hoover, 1999) and (iii) permit chemotactic behaviour (Dasti, Tareen, Lugert, Zautner, & Groß, 2010), triggering movement of the bacteria to more favourable conditions within the gastrointestinal tract.

The flagellar filament is composed of a basal body, hook and filament (Konkel et al., 2004). In *C. jejuni* two copies of flagella genes *flaA* and *flaB* can be found, and are situated adjacent to one another (T. M. Wassenaar, Bleumink-Pluym, & van der Zeijst, 1991). Both genes are 1.7kb in length (Nuijten, van den Berg, Formentini, van der Zeijst, & Jacobs, 2000) but differ in their regulation. The *flaA* gene is regulated by $\sigma^{28}$ (Konkel et al., 2004), whereas the *flaB* gene is regulated by $\sigma^{54}$ (Alm, Guerry, & Trust, 1993). Both genes show 95% sequence identity (T. M. Wassenaar et al., 1991) and are found highly conserved among *Campylobacter* isolates.

The high frequency in which these flagellum genes are found, can be seen in a study performed by Andrzejewska et al. (2011), who set out to determine the prevalence of putative virulence genes found in *Campylobacter* isolated from children and domestic animals. It was evidenced that all of 49 *Campylobacter* strains isolated from children with enteritis contained *flaA* gene, and out of 30 isolates from cats and dogs, only one *C. coli* isolated from a dog did not possess the *flaA* gene (M. Andrzejewska et al., 2011). Similarly, investigating putative virulence genes isolated from *C. jejuni* strains Datta et al. (2003) also detected *flaA* gene in 100% of the samples they tested. This included strains from various sources, human (56), poultry meat (21), broiler faeces (21) and bovine faeces (13) (Datta et al., 2003). The high prevalence of *flaA* genes found in *Campylobacter* from various isolates clearly indicates that *flaA* is highly conserved in *Campylobacter.*

Although there are two genes, *flaA* and *flaB* within the flagellum construct, only one is transcribed (Fischer & Nachamkin, 1991; King & Clayton, 1991). It is believed *flaA* is the major and preferred subunit (Nuijten et al., 2000) of *Campylobacter* flagellum and the gene necessary for motility. Investigating the function of both *flaA* and *flaB* genes, Wassenaar et al. (1991) inactivated each gene separately to determine their respective roles in motility. It was established that a strain that carried an intact *flaA* gene exhibited long flagella and was motile. Those that exhibited *flaB* but lacked a functioning *flaA* gene had shorter flagella and were non-motile (T. M. Wassenaar et al., 1991) and also lacked invasive ability. A function for *flaB* was not discovered, as this gene is actually not transcribed. Therefore, *flaB* protein is not required for motility (Nuijten et al., 2000) or invasion (T. M. Wassenaar et al., 1991). The function of *flaB* in motility is unknown, but it is speculated that it may play a role as a depot for antigenic variation (Nuijten et al., 2000).

These findings suggest that the presence of *flaA* and its expression is a precursor for mobility and ultimately invasion of *Campylobacter* within the host cells. Strains lacking *flaA* and motile flagella show a decreased invasive ability, thus indicating the importance of flagella as a virulence factor for pathogenicity of

*Campylobacter* infection. Although motility is a necessary feature of *Campylobacter* virulence, it is not sufficient for invasion of the host.

## 3.2 Adhesion

Another well-documented *Campylobacter* virulence factor required for pathogenesis is adhesion of the organism to host intestinal epithelial cells. There are numerous genes that play a role in various adhesion mechanisms, such as *Campylobacter* adhesion factor (*CadF*), surface exposed lipoprotein *JlpA* and permease protein *PEB1* (Manja Boehm et al., 2011) among others. Adhesion of *Campylobacter* is one of the initial steps for development of campylobacteriosis (Scott et al., 2010), allowing attachment of the organism to host cells and enabling the potential for further pathogenesis. It has been found that *C. jejuni* isolated from humans with diarrhoea and fever adhere more frequently to mammalian cells in culture when compared to isolates from asymptomatic carriers (Fauchere et al., 1986; M E. Konkel, M D. Corwin, L A. Joens, & Cieplak, 1992). This finding suggests that isolates lacking the ability to adhere may also lack the ability for increased pathogenic potential.

CadF is an outer membrane protein that binds fibronectin (Fn) (M E. Konkel, S A. Gray, B J. Kim, S G. Garvis, & Yoon, 1999), allowing attachment to the intestinal epithelial host cell and is one of the most commonly studied virulence factor associated with *Campylobacter.* Fn is a large glycoprotein of the extra cellular matrix (ECM), where it mediates numerous cellular interactions (Pankov & Yamada, 2002), including adherence for colonising bacteria (Chagnot, Listrat, Astruc, & Desvaux, 2012). This provides an ideal target for the binding of CadF to the ECM resulting in *Campylobacter* colonisation. Like in many other bacterial pathogens such as *Staphylococcus aureus* and *Escherichia coli*, CadF is a common adhesion target (Marshall R. Monteville & Konkel, 2002) that has been identified in *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* (Hirayama et al., 2009), indicating that this protein is also found highly represented among *Campylobacter* species.

In three separate studies, *cadF* was found to be present in 100% of the *Campylobacter* isolates studied. Of the 111 *C. jejuni* isolates from humans,

poultry meat, broiler faeces and bovine faeces investigated for the prevalence of several *Campylobacter* virulence genes, including *cadF*, Datta et al. (2003) detected *cadF* in all isolates. Similarly, Rizal et al. (2010) determined the presence of *cadF* in 100% of the 78 *C. jejuni* isolates from both poultry and humans. The third investigation performed by Andrzejewska et al. (2011) also found *cadF* in 100% of *C. jejuni* and *C. coli* isolates from both domestic animals and children's clinical samples. Interestingly, the high prevalence of *cadF* in theses studies indicates that the gene is conserved among a broad range of strains isolated from a broad range of hosts. It corroborates the earlier statement that *cadF* is highly conserved among *Campylobacter* species. Understanding the role of the genes involved in colonisation capacity among various hosts may provide vital information for epidemiological studies.

The importance of *cadF* in the function of adhesion was demonstrated by Krause-Gruszczynska et al. (2007). The authors determined attachment in human INT-407 cells using *C. jejuni* isolates with $cadF^{+}$ and $cadF^{-}$ mutant strains. In mutant strains lacking the *cadF* gene, the cadF protein was not synthesised and adherence of these isolate to INT-407 cells, was significantly reduced (50%) compared to the $cadF^{+}$ isolates (Malgorzata Krause-Gruszczynska et al., 2007). This finding suggests that *cadF* is a necessary gene required for adherence and ultimately colonisation of *Campylobacter*. The binding of pathogenic organisms is not only an important virulence determinant into the initial steps of the development of the disease, but is also necessary for attachment to prevent bacteria being washed away by mechanical cleansing mechanisms during peristalsis (M E. Konkel, S G. Garvis, S. L. Tipton, Jr D E.Anderson, & Cieplak, 1997).

PEB1 is another putative virulence protein commonly associated with *C. jejuni* and *C. coli* (Pei & Blaser, 1993) that mediates adherence to intestinal epithelial cells (Maria Del Rocio Leon-Kempis, Edward Guccione, Francis Mulholland, Michael P. Williamson, & Kelly, 2006). Analysis of the protein has revealed that PEB1 is located within an operon that is homologous to ATP-binding transporters that are currently seen in many other Gram-negative organisms found in the periplasmic cytoplasm (Pei et al., 1998). PEB1 is most abundantly found in the periplasmic cytoplasm, though a portion can also be found surfaced exposed

(Maria Del Rocio Leon-Kempis et al., 2006), where PEB1 is also implicated in exporting other proteins to the outer membrane (O Cróinín & S.Backert, 2012).

PEB1 is conserved among *C. jejuni* isolates and has been seen in nearly 80% of *C. jejuni* infected patients (Pei et al., 1998). Flanagan et al. (2009) found the presence of PEB1 to be higher having isolated the protein in 100% from 97 of *C. jejuni* strains from human, poultry, cervine, porcine and canine samples. The 100% isolation rate suggests that PEB1 is highly conserved among *C. jejuni* species (Flanagan, Neal-McKinney, Dhillon, Miller, & Konkel, 2009), though its presence in other *Campylobacter* species is not widely known. Conversely, the role of PEB1 in adhesion was also demonstrated by Flanagan et al. (2009), where PEB1$^-$ knock out mutants yielded 0% colonisation rates in chicken intestines.

Another less studied gene responsible for adhesion of *Campylobacter* is the surface-exposed lipoprotein *jlpA* (O Cróinín & S.Backert, 2012). Like *cadF* and PEB1, *jlpA* also promotes adherence to intestinal epithelial cells (Scott et al., 2009). However, unlike *cadF*, which is highly conserved among *Campylobacter* species, *jlpA* has only been isolated from *C. jejuni*. Through molecular testing procedures, Jin et al. (2000) determined the location of *jlpA* in very close proximity to the *hipO* gene, which are both found within a 15 kb region within *C. jejuni* (Jin et al., 2001). Interestingly, *hipO* gene is also a species-specific gene, found only in *C. jejuni* isolates.

Jin et al. (2000) not only evidenced *jlpA* in 100% *C. jejuni* strains investigated, but failed to isolate the gene in all other *Campylobacter* strains *C. coli*, *C. lari*, *C. sputorum* and *C. upsaliensis* (Jin et al., 2001). Biswas et al. (2013) also detected *jlpA* in high isolation rates within *C. jejuni* from both human sources and cattle. In human clinical isolates *jlpA* was found in 96.1% and in cattle isolates, *jlpA* was determined in 90% (D. Biswas, Hannon, Townsend, Potter, & Allan, 2011). Although *jlpA* appears to be present in high rates, this gene does appear to be species specific.

Although adhesion is not the sole effector in *Campylobacter* infection pathogenesis, attachment of the organism to host epithelial cells is an important

function and vital step in disease manifestation. This was reflected in circumstances where adherence defective mutants yielded significantly lower colonisation rates (Backert & Hofreuter, 2013). Without effective colonisation, *Campylobacter* bacteria are limited in their disease potential.

## 3.3 Invasion

Invasion is another step of the multifactorial process involved in *Campylobacter* infection. The organism must not only adhere to the host intestinal epithelial cells, but must achieve internalisation too. Once *Campylobacter* has crossed the mucus layer protecting the intestinal epithelial cells, adherence takes place which ultimately leads to invasion of cells, which is considered to be an essential step for the pathogen (Van Vliet & Ketley, 2001). In fact, correlations have been made between the pathogen's invasiveness and the severity of disease (Dasti et al., 2010; Fauchere et al., 1986). The more invasive *Campylobacter* species have been associated with more severe illnesses including the presence of bloody diarrhoea (Malagón, Arcía, & Heredia, 2010). Invasion is also considered to be the trigger for inflammation of intestinal cells, which also is associated with severity of infection (Van Vliet & Ketley, 2001).

A key difference between *Campylobacter* isolates that often cause disease in human hosts compared to isolates that are found as a commensal in animal hosts, such as chickens, is the organism's invasiveness towards the epithelial cells in the human host (O Cróinín & S.Backert, 2012; Young et al., 2007). Studies are limited to gene markers for invasion of *Campylobacter*, but they have revealed a few genes that are known to mediate invasion of *Campylobacter*, adding to the virulence potential of this micro-organism. Two relevant examples are an invasion-associated marker (Iam) (Rizal et al., 2010) and *Campylobacter* invasion antigen (CiaB) (Konkel, Kim, Rivera‐Amill, & Garvis, 1999).
*Iam* is associated with invasive strains of *Campylobacter* and numerous studies have identified the gene particularly in *C. jejuni* and *C. coli*. Isolation rates are highly variable going from 6.8% (Sanad et al., 2011) to 91.6% (M. Andrzejewska et al., 2011). During clinical investigations of children with diarrhoea, Andrzejewska et al. (2011) found *iam* incidence to be higher in *C. coli* compared to *C. jejuni* (91.6% and 32.4% respectively). Further investigations also detected

*iam* from *Campylobacter* isolated from both cats and dogs, with isolates from cats being more frequently found to exhibit the gene compared to dogs. Unlike the *Campylobacter* isolated from children, *C. jejuni* (88.8% in cats and 30% in dogs) was found to exhibit higher incidences of *iam* in both animal species compared to *C. coli* (50% in cats, 14.2% in dogs).

During further investigation of *C. jejuni*, Rizal et al. (2010) detected *iam* in 77.7% of the samples investigated from chickens, and in 60% of *C. jejuni* isolates from human sources. In cattle, Sanad et al. (2011) detected the gene in only 6.8% of *C. jejuni* samples isolated from cattle. Sanad et al. (2011) detected *iam* in 54% of *C. jejuni* from human sources. The isolation of *iam* appears to vary between sources, however its presence found across various sources indicates that the gene is conserved across *C. jejuni* and *C. coli* isolates. It has been revealed that *Campylobacter* has the ability to bind to both human and non-human cells, though is internalised more efficiently in human cells (Rivera-Amill, Kim, Seshu, & Konkel, 2001).

*ciaB* has also been identified in *Campylobacter*. It encodes a protein that is required for internalisation (Konkel et al., 1999) followed by translocation into the host cell cytoplasm (O Cróinín & S.Backert, 2012; Rivera-Amill et al., 2001). Like *iam*, *ciaB* has been found in high incidence rates within *Campylobacter* species. For example, *C. jejuni* isolated from human clinical specimens were found to harbour *ciaB* in 92.3% of the 51 isolates investigated (D. Biswas et al., 2011). Compared to the lower distribution of *iam* in cattle, Biswas et al. (2011) isolated *ciaB* in high frequency (94%) in *Campylobacter* isolated from cattle, suggesting a high conservation of *ciaB* among *C. jejuni* isolates.

Investigating *ciaB* gene's effect on the internalisation of *Campylobacter*, Konkel et al. (1999), who first isolated the gene in *C. jejuni*, produced a *ciaB* mutant strain to determine the organism's invasive abilities. They found a significant reduction in internalisation of the mutant strains by the human INT-407 cells, demonstrating that insertional deletions in a *ciaB* mutant do result in a non-invasive phenotype (Konkel et al., 1999). This also demonstrates the role that the CiaB protein plays in invasion of *Campylobacter* into a host cell. The presence of

*Campylobacter* in patient tissue biopsies has demonstrated the ability of the pathogen to get internalised within the host cells (Dasti et al., 2010), causing subsequent host cell inflammation and damage. The ability of *Campylobacter* not only to enter but to survive within a host cell supports the major role of invasion proteins such as Iam and CiaB in diarrheal disease (Rivera-Amill et al., 2001).

## 3.4 Toxin production

The ability to produce toxins within a host cell is another putative virulence factor found in pathogenic bacteria. Gram-negative bacteria such as *E. coli*, *Shigella* and *Campylobacter*, particularly *C. jejuni* and *C. coli* (Liesbeth M. Ceelen, Annemie Decostere, Richard Ducatelle, & Haesebrouck, 2006), exhibit this ability and are known to produce a number of toxic products (Eyigor, Dawson, Langlois, & Pickett, 1999a). The multi-subunit Cytolethal Distending Toxin (CDT) (Masahiro Asakura et al., 2007) is the most studied toxin produced by *Campylobacter*.

The CDT protein is composed of 3 subunits CdtA, CdtB and CdtC (Eyigor et al., 1999a; M N. Acik, M. Karahan, B. Karagulle, H. Ongor, & Cetinkaya, 2013; Masahiro Asakura et al., 2007). The three units form a tripartite holotoxin that are required for cytotoxic activity (Talukder et al., 2008). Each subunit is required for various mechanisms, but only CdtB is the active unit responsible for the cytotoxic activity (Maria Lara-Tejero & Galán, 2001). Conversely, the mechanisms of CdtA and CdtC are still not completely understood. Although, studies have revealed that without CdtA and CdtC, CdtB is unable to transport itself to the target site (Liesbeth M. Ceelen et al., 2006), it is hypothesised that CdtA and CdtC are involved in and required for transportation of CdtB (Maria Lara-Tejero & Galán, 2001). Without these two subunits, CdtB is unable to express its toxic effects. Ceelan et al. (2006) also suggest that CdtA and CdtC are involved in the binding of the holotoxin to the target cell surface, as they found that CdtB was unable to bind without CdtA and CdtC.

The CDT toxin causes disruption in the cell cycle, which eventually leads to cell death. Once inside the target cell, the main mechanism of CDT is to block the cell cycle in the $G_2$ phase preventing cell division (de Carvalho et al., 2013). This leads to cell distension and then to cell disintegration (Eyigor, Dawson, Langlois,

& Pickett, 1999b). The CDT holotoxin has also been found to be well conserved among *Campylobacter* species, including *C. jejuni*, *C. coli, C. lari* and *C. fetus* (Eyigor et al., 1999b).

Isolation rates of the three CDT genes vary from 0-100%. Findik et al. (2010) investigated the prevalence of *cdtA*, *cdtB* and *cdtC* genes found in *Campylobacter* species isolated from various sources. Of the 168 *C. jejuni* strains isolated, *cdtA*, *cdtB* and *cdtC* were all found in 95%, 98% and 93% respectively (Findik et al., 2011). Similarly Ripabelli et al. (2008) found the CDT gene cluster (3 gene subunit) in 93.1% of the 29 *C. jejuni* isolates tested, and in 97.2% of the 36 *C. coli* isolates. Interestingly, further investigation found that only 27.6% of 29 *C. jejuni* isolates and 2.8% of 36 *C. coli* isolates showed evidence of toxin production (Ripabelli, Tamburro, Minelli, Leone, & Sammarco, 2010). These studies provide evidence that the CDT gene clusters are found conserved among *Campylobacter* isolates, particularly in *C. jejuni*. CDT production is said to be associated with *Campylobacter* strains that have been associated with human enteritis (de Carvalho et al., 2013).

The widespread presence of virulence genes that are found so conserved among *Campylobacter* spp., regardless of the source, confirms that animals both domestic and production are important reservoirs for pathogenic strains (Ripabelli et al., 2010).

## 3.5 Molecular investigation

A better understanding of an organism's host niche and lineage structure can be an important aspect in epidemiological studies, reducing disease (Sheppard et al., 2011) and investigating how an organism such as *Campylobacter* is found in such a variety of host species. Molecular and genetic analysis has become a useful tool in the epidemiology of *Campylobacter*, where molecular methods such as multilocus sequence typing (MLST), fragment length polymorphism (RFLP) and microarrays have been successfully used. These procedures are able to aid in the determination of genes and their sequences, to investigate virulence factors, genetic diversity of *Campylobacter* spp. (Normand, Boulianne, & Quessy, 2008), mutation rates, synonymous and non-synonymous polymorphisms (Ioannidou et

al., 2013) and population structure (Korczak, Zurfluh, Emler, Kuhn-Oertli, & Kuhnert, 2009).

Every pathogen species has a unique genome sequence (Sung, 2012). A better understanding of an organism's genome and specific gene sequences can help provide a better comprehension of the relationships between genotypes and disease (Thusberg & Vihinen, 2009). Next generation sequencing has enabled increased molecular testing capabilities, where computer based programs coupled with biological data have enabled greater investigative power, comparing gene sequences with those from known databases. This has enabled high-throughput sequencing experiments to generate genome profiles for investigation of small sequence regions of a genome (Sung, 2012).

Gilpin et al. (2006) performed a clinical study in New Zealand and found that 75% of the 183 isolates evaluated through serotyping and Pulsed-Field Gel Electrophoresis, were able to be grouped in clusters of 2-26 isolates that had indistinguishable patterns. It is believed that the isolates that were not found as part of a cluster were most likely to have come from overseas origins, from patients who had recently travelled (Gilpin et al., 2006; Taboada, Clark, Sproston, & Carrillo, 2013). Studies like this aid in defining common exposure and source of outbreaks and get a better understanding of the disease (Gilpin et al., 2006). Investigating genetic properties that are unique and conserved among *Campylobacter* strains have become ideal targets for these molecular investigations and can also be useful for identification purposes.

# 4 Clinical Manifestations of *Campylobacter* Infections

*Campylobacter* is the leading cause of gastroenteritis worldwide, surpassing *Salmonella*, *Shigella* and *Escherichia coli* (Nachamkin, 2002). *C. coli* and *C. jejuni* are the most frequently isolated species in cases of campylobacteriosis, of which approximately 80% are due to *C. jejuni* (Nayak, Stewart, & Nawaz, 2005). Incubation period of *Campylobacter* infection is generally 24-48 hours, but can be as long as 10 days (Butzler, 2004). Clinical symptoms of campylobacteriosis are generally indistinguishable (Nachamkin, 2002) from other gastroenteritis causing organisms such as *Salmonella*. Symptoms may include abdominal pain, fever and general malaise (Van Vliet & Ketley, 2001), with diarrhoea persisting for 2-3 days (Butzler, 2004). *Campylobacter* infection is usually self-limiting, but severity may depend on host responses. For instance, those that are immunocompromised tend to suffer more severe symptoms (T.M. Wassenaar & Blaser, 1999).

Clinical manifestations of *Campylobacter* infections differ in developing countries compared to the developed nations. In developing countries, infection is more commonly seen in those 5 years and under, whereas in developed countries the common age of those infected 14 to 24-years old (Matthias Zilbauer, Nick Dorrell, Brendan W. Wren, & Bajaj-Elliott, 2008). Lower incidence rates seen in adult populations in developing countries may be due to immunity build up in adults from early exposure to the organisms. Studies of the epidemiology of *Campylobacter* infections have revealed 2 different manifestations of the disease (Young et al., 2007), which appear to be due to geographical locations and socio-economic backgrounds. In developing countries, clinical manifestations of *Campylobacter* infections are presented with inflammatory diarrhoea, stomach cramping, fever, bloody stools, while those in developing countries generally appear to be more asymptomatic (Van Vliet & Ketley, 2001). Influencing factors for this may come down to host immunity and other demographic factors (Silva et al., 2011). Intriguingly, people who contract *Campylobacter* infections abroad, still suffer the same symptoms that they would in their home country of origin,

rather than the origin of where they contracted the illness (Matthias Zilbauer et al., 2008).

Outbreaks of *Campylobacter* infections are rare, and are usually a result of unpasteurised milk or contaminated water consumption (Blaser, 1997). Point source infections are more common, and are often a result of ingestion of contaminated food sources, in particular poultry products. In fact, 50-70% (Epps et al., 2013) of point source infections are from contaminated poultry. Seasonal patterns are often seen in *Campylobacter* infections. The summer months generally come with higher infection rates, because *Campylobacter* has an optimal replication rate at a higher temperature (Dasti et al., 2010). This may also be due to the high volume of outdoor cooking that takes place over the warmer months.

# 5 Laboratory Diagnosis of *Campylobacter*

## 5.1 Sample type

Fresh faecal samples are the most ideal specimen type to isolate *Campylobacter*, the fresher the sample, the higher the chances of isolation. The high number of bacteria found in faeces is what makes it an ideal sample type (On, 2013). If direct faecal samples are unable to be obtained, though not ideal, rectal swabs can be used (Andrews, 1998). This is commonly the case for reptiles and birds, where faecal samples can be difficult to obtain so cloacal swabs are often used.

## 5.2 Media

Isolation media for *Campylobacter* contain selective properties in order to enhance recovery of the organism. Some media for *Campylobacter* isolation, such as Skirrows agar contains blood as a base ingredient, where other media can be blood free, such as *Campylobacter* isolation agar - Charcoal Cefaperazone Deoxycholate Agar (CCDA). CCDA contains ingredients such as sodium pyruvate, ferrous sulphate (Mossel, 1985) and bacteriological charcoal among other ingredients that aids in the isolation and identification of *Campylobacter*. Selective media used for *Campylobacter* isolation also contain various antibiotics that aid recovery of *Campylobacter* while inhibiting other organisms that are commonly found in the gastrointestinal tract.

Antibiotic usage in selecting for *Campylobacter* have become much more refined over the years in their combinations (On, 2013) to allow for greater recovery. Cefaperazone (Tenover & Gebhart, 1988) (Andrews, 1998) is a commonly used antibiotic in *Campylobacter* media because of the organism's resistance to this antibiotic (Andrews, 1998), where many other organisms are susceptible. Some media contain other antibiotics such as Cephalothin and Polymixin B (Andrews, 1998). It has been noted in studies by Tenover et al. (1988) and On (2013), that some *Campylobacter* species, such as *Campylobacter upsaliensis* (On, 2013) may be inhibited by these antibiotics, so care should be taken on selecting antibiotic containing media and in some instances be avoided (Tenover & Gebhart, 1988) (On, 2013). Selective media should meet the requirements of the application at

hand and therefore it is important to select antibiotics that will enhance the target organism and species.

CCDA agar is the most commonly used medium for *Campylobacter* isolation (Gharst et al., 2013), due to the ease at which visual differentiation is achieved. *Campylobacter* colonies grow as distinctive grey, flat swarming colonies (Gharst et al., 2013) against the black CCDA agar, making the primary steps of isolation an easier task.

## 5.3 Isolation

Faecal samples can be streaked directly on to selective media, or enrichment broth, such as Bolton broth may be used before plate inoculation, in order to facilitate growth of *Campylobacter* particularly when only small numbers are present. However, this is a is a more commonly used method when attempting to isolate *Campylobacter* from food samples rather then faecal samples and is not a necessary step (Tenover & Gebhart, 1988). In some instances, using enrichment broth on faecal samples also facilitates the growth of many other organisms present, which may hinder isolation of *Campylobacter* (Gharst et al., 2013). Less fastidious organisms can flourish more rapidly in an enriched environment.

Due to the thermophilic nature of *Campylobacter,* culture plates are incubated at an elevated temperature of 42 °C for 72 hours. This elevated incubation temperature favoured by *Campylobacter* also inhibits growth of some of the competing organisms that are present in faeces (Gharst et al., 2013). Another incubation requirement required for *Campylobacter* isolation is that of an atmosphere of reduced oxygen. These microaerophilic organisms favour an atmosphere of 3-5% $O_2$, 3-10% $CO_2$ and 85% $N_2$ (Andrews, 1998). This environment can be achieved with the use of gas-generating sachets within an anaerobic jar (Tenover & Gebhart, 1988), if the use of a microaerophilic incubator is unavailable.

## 5.4 Identification

### 5.4.1 Conventional Methods

Following the primary isolation and growth of typical (flat, grey, swarming) colonies of suspicious organisms, the next step is their identification.

Conventional identification methods generally consist of a combination of assessment of morphological characterisitcs (both macro- and micro-scopic), biochemical reactions and antibiotic susceptibilities. *Campylobacter* spp. exhibit certain colonial morphological features on selective agar but, this alone is not sufficient evidence to conclude *Campylobacter* spp. (Andrews, 1998).

Based on typical morphology that occurs on selective media such as CCDA agar, suspicious colonies can be stained, with Dilute Carbol Fuchin (DCF) to allow for visualisation of the seagull- shaped rods that are typical of *Campylobacter* spp. This appearance is a result of the joining of daughter cells (Quinn et al., 2011).

Further biochemical testing can be performed to differentiate *Campylobacter* spp., where biochemical reactions that take place can be matched up accordingly to known species reactions (Table 2). For example, *Campylobacter* species can be catalase negative or positive, all are urease negative and oxidase positive. As can be seen in Table 2, only *C. jejuni* is hippurate positive, which is a major identifying feature for this species. Biochemical tests are run in conjunction with the sensitivity profiles to antibiotics Cephalothin (KF) and Nalidixic Acid (NA).

**Table 2**. ***Campylobacter*** **differentiation table** (Markey et al., 2013).

| SPECIES | GROWTH AT | | CATALASE | $H_2S$ PRODUCTION (TSI) | HIPPURATE HYDROLYSIS | SUSCEPTIBILITY TO | |
|---|---|---|---|---|---|---|---|
| | 25° C | 42° C | | | | NALIDIXIC ACID | CEPHALOTHIN |
| *C. fetus* subsp. *fetus* | + | - | + | - | - | R | S |
| *C. fetus* subsp. *venerealis* | + | v | + | - | - | v | S |
| *C. jejuni* subsp. *jejuni* | - | + | + | - | + | S | R |
| *C. jejuni* subsp. *doylei* | - | - | v | - | + | S | S |
| *C. mucosalis* | - | + | - | + | - | R | S |
| *C. hyointestinalis* | v | + | + | v | - | R | S |
| *C. coli* | - | + | + | v | - | S | R |
| *C. lari (C. laridis)* | - | + | + | + | - | v | R |
| *C. upsaliensis* | - | v | Weak / - | - | - | S | v |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. sputorum* biovar *faecalis* | - | + | + | + | - | R | S |
| *C. sputorum* biovar *bubulus* | - | + | - | + | - | v | S |
| *C. sputorum* biovar *sputorum* | - | v | - | + | - | v | S |

TSI: Triple sugar iron, R: Resistant, S: Sensitive, v: Variable, +: Positive, -: Negative.

Growth and biochemical tests aid in identifying different *Campylobacter* spp. Hippurate hydrolysis aids differentiating thermophilic *C. jejuni* from *C. coli.* Antibiotic sensitivity to NA and KF are also important keys to identifying *Campylobacter* spp. (Markey et al., 2013). However, identification using conventional biochemical methods can be difficult and challenging, due to the organisms' fastidious and inert nature and complex taxonomy (On, 2013). Obtaining results is also time consuming, especially with the slow growing nature of *Campylobacter* spp. Often definitive results are unable to be achieved further then a species level. Therefore, the use of other identification techniques can be implemented to aid in further and faster identification.

### 5.4.2 Matrix-Assisted Laser Desorption Ionization Time- of- flight mass spectrometry (MALDI-TOF MS)

Identification of organisms using the MALDI-TOF MS has become common practice in many diagnostic laboratories and has taken over conventional biochemical methods. Identification is achieved through analysing a spectrum of proteins in the organisms and discriminating between the different organisms based on a software database (S. Biswas & Rolain, 2013). Identification via the MALDI-TOF MS is rapid and more cost effective then conventional biochemical methods, allowing for treatment to take place sooner. The MALDI-TOF MS can produce results in a matter of minutes to a species level and in some cases to subspecies level (S. Biswas & Rolain, 2013). It is said, the time taken for one isolate identification, is one minute (Seng et al., 2009), whereas conventional methods require a minimum of overnight incubation for biochemical results. Rapid and accurate identification of organisms is an important factor in many clinical settings, particularly when rapid patient treatment is necessary.

Studies performed by Seng et al. (2009), determined the accuracy and effectiveness of the MALDI-TOF MS, where clinical microbial isolates were investigated. It was found that out of 1660 isolates, 95.4 % were correctly identified using the MALDI-TOF MS, 84.1% of the correct identifications to a species level and 11.3% to a genus level (Seng et al., 2009). Incorrect identifications were said to be due to incorrect database entry. A similar and more recent study comparing MALDI-TOF MS and conventional biochemical techniques, performed by Dingle & Butler-Wu (2013), found that of 980 clinical samples, the MALDI-TOF MS had an identification success rate of 92% versus an 83.1% success rate using biochemical methods (Dingle & Butler-Wu, 2013). Studies like these provide a good indication of the sensitivity of the MALDI-TOF MS. The MALDI-TOF MS is also more cost effective as shown by Seng et al. (2009). They found that the cost for isolate identification by MALDI-TOF MS is 22%-32% lower than conventional methods.

Like conventional biochemical identification methods, primary cultures are required in order to obtain microbial colonies. Once growth has been achieved, single colonies are transferred on to a MALDI-TOF MS target plate for analysis (Mari L. DeMarco & Ford, 2013), which can take approximately 10-15 minutes (Dingle & Butler-Wu, 2013). For more fastidious organisms, such as *Campylobacter* or anaerobes, that usually require longer incubation periods, the rapid identification of the MALDI-TOF MS is ideal for a faster turn around.

A matrix solution along with bacterial colonies are innoculated onto a target plate. This solution desorbs the laser energy (S. Biswas & Rolain, 2013) and can have great effects on the quality of the profile peaks (Mari L. DeMarco & Ford, 2013) obtained during analysis. The target plates are inserted into the MALDI-TOF MS analyser, where through a series of processes, the bacterial cells are ionised via short laser pulses (Dingle & Butler-Wu, 2013). The matrix solution absorbs the laser pulses, where it causes desorption and ionisation of the sample being analysed. The ionised proteins pass through an electrostatic field into the TOF mass analyser where they are quantified to produce a protein profile of the unknown organism. These profiles form a ‘fingerprint’ that is unique to the organism being analysed.

A database within the MALDI-TOF MS analyser acts as a reference to identify the organism based on the protein profile 'fingerprint' obtained during analysis. Profiles within the database were previously obtained and built up from other methods of identification (Lay, 2001), such as molecular techniques. As more identifications and protein profiles are made, the database possesses the ability for organisms to be added to the library for future reference.

### 5.4.3 Molecular identification techniques

Conventional identification methods are historically based on phenotypic characteristics (Barghouthi, 2011), whereas, molecular methods, such as polymerase chain reaction (PCR), or real-time PCR (qPCR) are based on genotypic characteristics. Molecular diagnostic methods are established worldwide and are commonly used as a rapid alternative to conventional identification techniques (Malorny et al., 2003). Identification occurs through the amplification and detection of specific gene sequences that are unique to the bacterium in question, such as 16S rRNA. The use of specific sequence primers is widely used to identify bacteria to a genus or species level (Maurin, 2012) via the use of an oligonucleotide BLAST database.

#### 5.4.3.1 Polymerase chain reaction (PCR)

PCR is a cyclic process that involves heating and cooling of a DNA/primer mixture to amplify target sequences. This occurs over 3 main stages: denaturing, annealing and elongation.

1- Denaturing: The target DNA/primer solution is heated, separating the double stranded DNA (dsDNA) into single strands (Lilit Garibyan & Avashia, 2013).
2- Annealing: The mixture is cooled, during which the single strands 'anneal' with the complimentary primer template (Mark A. Valasek & Repa, 2005).
3- Elongation: By extending the primers, DNA polymerase adds nucleotides to the developing DNA strands (Lilit Garibyan & Avashia, 2013), resulting in a new complete dsDNA strand.

Following the 3 main processes of denaturing, annealing and extension, the cyclic process begins again, where oligonucleotide primers bind to the original DNA mixture as well as the newly synthesised copies (Gibbs, 1990), resulting in exponential amplification of the target DNA sequence. After the amplification

process, PCR product obtained must be analysed. Traditionally, PCR products are stained with a chemical, such as ethidium bromide, then gel electrophoresis is performed, which separates the PCR products based on base sizes and charges (Lilit Garibyan & Avashia, 2013), producing visual bands that can be measured to determine the size of the products (Figure 1), which are compared against known base pair sizes of the organism in question.

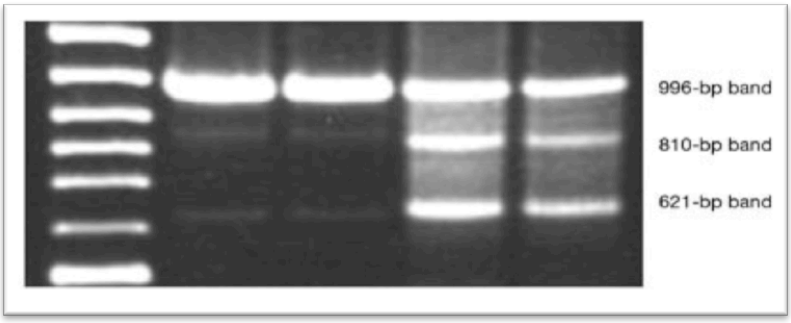


**Figure 1. Separated PCR products after gel electrophoresis.** Resulting bands (right side) are measured to determine their molecular sizes. Known control strains are used and compared against the target DNA. Picture from Garibyan & Avashia (2013).

PCR is a rapid identification method, enabling easy amplification of very small quantities of target DNA, to produce sufficient amounts for manipulation. However, conventional PCR methods require post-PCR quantification analysis that can suffer from laboratory cross-over contamination such as previously amplified material, DNA from the analyst or DNA fragments of the allelic ladder. (Heid, Stevens, Livak, & Williams, 1996; Sarkar & Sommer, 1990; Sundquist & Bessetti, 2005).

#### 5.4.3.2 Real time PCR (qPCR)

Following on from conventional PCR, real time PCR (qPCR) is another popular molecular method that aids in the identification and amplification of target sequences. Real time PCR is based on conventional PCR (Toplak, Kovač, Piskernik, Možina, & Jeršek, 2012), but with the inclusion of fluorescent dyes, allowing real time quantitative measurement and monitoring (Mark A. Valasek & Repa, 2005) of the PCR products after each cycle.

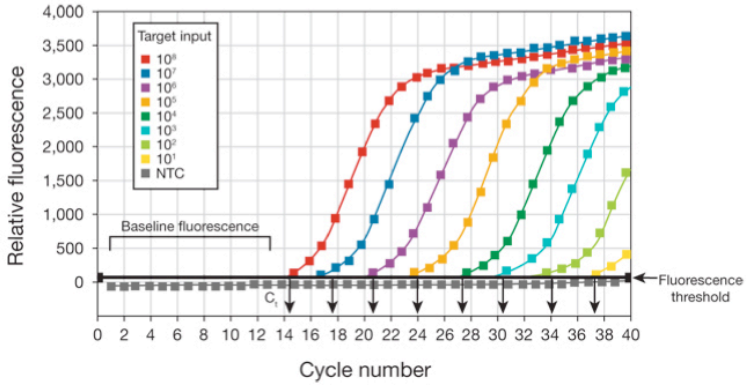


**Figure 2. Real-time PCR amplification fluorescence.** Fluorescence is plotted against each cycle number, representing the accumulation of amplified PCR product (Technologies, 2012).

Via the addition of fluorescent dyes, such as SYBR Green I (the most commonly used fluorescent dye) to the primer mixture (pre-reaction), fluorescence occurs during PCR amplification when target DNA anneals with the fluorescently labelled complimentary primers producing dsDNA (Barghouthi, 2011; Mark A. Valasek & Repa, 2005). Fluorescence is proportional to the PCR product acquired during amplification, the greater the amount of dsDNA present in the reaction, the greater the fluorescence will be (Mark A. Valasek & Repa, 2005).

This PCR method allows real time visualisation of the amplification (Figure 2) through visual graphical plots that are produced within the thermal cycling instrument. This negates the need for post PCR analysis with gel electrophoresis that is required with conventional PCR, and also reduces the risk of laboratory contamination that can occur during analysis stages of conventional PCR (Toplak et al., 2012). Real-time PCR with SYBR Green I also allows for the added analysis of melting curves, which cannot be achieved via conventional PCR methods.

#### 5.4.3.3 Melting curve analysis

Another useful analysis tool available to qPCR is melting curve analysis. Annealing and melting (denaturing) can provide useful information (Maria Erali, Karl V. Voelkerding, & Wittwer, 2008) on PCR products. For example, genotyping, sequence matching (Reed, Kent, & Wittwer, 2007) and discrimination (Von Keyserling, Bergmann, Wiesel, & Kaufmann, 2011).

Like qPCR, melting curve analysis also utilises fluorescence of the PCR/primer product, however, conversely to qPCR, fluorescence measurements are taken of the 'melting temperature' when dsDNA 'melts' into single strands throughout the heating process of the PCR cycle. As the PCR mixture denatures, and the dsDNA 'melts' into ssDNA, fluorescence drops. The Melting temperature is based on the characteristics of the GC content (Reed et al., 2007), the measurements quantify the melting temperature at which 50% of the DNA is dsDNA and 50% is ssDNA (Maria Erali et al., 2008).

Melting curves have allowed rapid analysis; where amplicon sequences can be discriminated due to their unique melting temperature and curve shape (Price, Smith, Huygens, & Giffard, 2007), as well as mutation scanning and sequence mutations (Reed et al., 2007). The melting curve can also be utilised to validate qPCR performance (Von Keyserling et al., 2011).

PCR is considered one of the most accurate and reliable identification methods available (Kozera & Rapacz, 2013). However, often times sample selection, such as faecal samples, can exhibit a vast array of microorganisms which may be problematic due to the presence of bacterial overloading and various inhibitors (Linton, Lawson, Owen, & Stanley, 1997). Thus it is possible to incorporate conventional isolation techniques with molecular identification techniques. Samples may be cultured and isolated following which bacterial growth may then be identified via PCR.

# 6 Aim

The aim of this study is to perform a cross-sectional analysis to determine the prevalence of *Campylobacter* circulating within a captive wildlife population. This will involve culturing faecal samples collected from various animals found within the Auckland Zoo and the identification of strains isolated by using conventional biochemical methods and protein analysis (MALDI-TOF MS). In conjunction with identification of all *Campylobacter* isolated strains, molecular techniques will be utilised to investigate the presence of putative virulence genes that are commonly associated with the pathogenesis of *Campylobacter* infections. Phylogenetic relationships will be made and compared between the strains isolated using genotypic sequences, to help build a molecular data profile of the circulating *Campylobacter* strains in the Auckland Zoo. Genes investigated are selected on the basis of their involvement in motility, adhesion, invasion and toxin production, all of which are required in the multifactoral pathogenic process of *Campylobacter*.

Studies involving *Campylobacter* in wildlife populations are limited, as most of the studies are largely focused on *Campylobacter* within domestic and food source animals, given their vector potential for human infection. A study of a healthy captive wildlife animal population may provide a good insight and further knowledge of *Campylobacter's* ability to persist in such a wide variety of animal hosts. This will provide baseline data on the pathogen circulating among the zoo population and may be utilised for future health screening purposes.

# 7 Experiment Materials and Methods

## 7.1 Materials

### 7.1.1 Media and reagents

*Campylobacter* blood free isolation agar Charcoal Cefaperazone Deoxycholate Agar (CCDA), Mueller Hinton blood agar, 2.5 ml sterile saline (0.85%), 2.5ml sterile water, CRYOBANK cryogenic beads and BBL Taxo Hippurate differentiation discs were purchased from Fort Richard Laboratories, Auckland. Oxoid Cephalothin (KF) sensitivity discs 30 µg, Oxoid Nalidixic acid (NA) sensitivity discs 30 µg, Remel Bactidrop Ninhydrin, Oxoid oxidase strips and KingFisher Duo Magjet Viral nucleic acid kit were all purchased from Thermofisher Scientific, Auckland. Dilute Carbol Fuchsin stain was purchased from Milton Adams, Auckland.

### 7.1.2 Other materials

Mitsubishi Gas Chemical Company AnaeroPack- MicroAero gas sachets were purchased from Ngaio Diagnostics in Nelson. Sterile 10 µl inoculation loops were purchased from Thermofisher Scientific, Auckland.

### 7.1.3 Bacterial strains

*Campylobacter* control strains *C. jejuni* subsp. *jejuni* NCTC lll68, *C. coli* (Doyle 1948) NCTC ll366, *C. lari* NCTC ll352 and *C. upsaliensis* ATCC 43954 were all used to run in conjunction with all molecular testing procedures. These were provided by the Institute of Veterinary, Animal & Biomedical Sciences, Massey University, Palmerston North.

### 7.1.4 Primer sequences

Oligonucleotides were synthesized and obtained from IDT (Integrated DNA Technologies, Singapore).

## 7.2 Methods

### 7.2.1 Sampling

Over a period of 8 months, between 1 November 2013 and 30 August 2014 faecal samples were collected from 202 (Table 3) of the 850+ captive wild animals from within the Auckland Zoo's collection. This included, 89 avians, 55 mammals (other than primates), 23 primates, 31 reptiles, 1 amphibian and 3 insects. Samples were collected on a random basis, for health screening to be performed by the Auckland Zoo veterinary hospital. Samples were collected in 70ml sterile plastic specimen containers (Thermofisher). 5g was collected where possible, however, for some bird, reptile, amphibian and insect samples 5g was impossible to obtain. In this instance 2g or less was collected. Samples were stored at room temperature for no longer then 2 hours before inoculation took place.

**Table 3. Total number of faecal samples tested.** The P denotes pooled sample, S denotes single sample.

| Common name | Scientific name | Number tested | |
|---|---|---|---|
| **Avians** | | | |
| African grey parrot | *Psittacus erithacus* | 1 | P |
| Australian king parrot | *Alisterus scapularis* | 1 | P |
| Antipodes island parrot | *Cyanoramphus unicolor* | 1 | S |
| Banded rail (Moho-pereru) | *Gallirallus philippensis* | 1 | S |
| Bellbird | *Anthornis melanura* | 1 | S |
| Blue duck | *Hymenolaimus malacorhynchos* | 2 | 2xP |
| Brolga | *Grus rubicunda* | 1 | P |
| New Zealand Brown teal | *Anas chlorotis* | 2 | 1xS 1xP |
| Campbell island teal | *Anas nesiotis* | 1 | S |
| Chicken | *Gallus gallus domestic* | 1 | S |
| Sulphur- crested cockatoo | *Cacatua galerita* | 1 | S |
| Common peafowl (Peacock) | *Pavo cristatus* | 2 | S |
| Eclectus parrot | *Eclectus roratus* | 1 | *P* |
| Emu | *Dromaius novaehollandiae* | 1 | P |
| Greater flamingo | *Phoenicopterus roseus* | 6 | 5xs 1xP |
| Grey tailed duck | *Anas superciliosa superciliosa* | 1 | S |
| Guinea fowl | *Numida meleagris* | 1 | P |
| Kakapo | *Strigops habroptilus* | 12 | 10xS 2xP |
| Kea | *Nestor notabilis* | 2 | P |
| Kingfisher | *Halcyon sancta vegans* | 2 | P |
| Little black shag | *Phalacrocorax sulcirostris* | 1 | S |
| Little blue penguin | *Eudyptula minor* | 3 | 2xS 1xP |
| Little spotted kiwi | *Apteryx owenii* | 3 | 2xS 1xP |
| Lovebirds | *Agapornis* | 1 | P |
| Luzon bleeding heart dove | *Gallicolumba luzonica* | 3 | S |
| Major Mitchell's cockatoo | *Lophochroa leadbeateri* | 1 | P |
| Musk lorikeet | *Glossopsitta concinna* | 1 | S |

| | | | |
|---|---|---|---|
| New Zealand brown teal | *Anas chlorotis* | 2 | 1xS 1xP |
| New Zealand dotterel | *Charadrius obscurus* | 1 | P |
| North island brown kiwi | *Apteryx mantelli* | 7 | 3xS 4xP |
| North island kākā | *Nestor meridionalis septentrionalis* | 10 | 10xS 2xP |
| Pheasant | *Glossopsitta concinna* | 2 | 1xS 1xP |
| Pied stilt | *Himantopus himantopus* | 1 | S |
| Red tail black cockatoo | *Calyptorhynchus banksii* | 1 | P |
| Spotted shag | *Phalacrocorax punctatus* | 1 | P |
| Sun conure | *Aratinga solstitialis* | 3 | P |
| Takahē | *Porphyrio (Notornis) hochstetteri* | 2 | 1xS 1xP |
| Weka | *Gallirallus australis* | 5 | 1xS 4xP |
| North American wood duck | *Aix sponsa* | 1 | P |
| | **Total** | | **89** |
| **Mammals** | | | |
| African crested porcupine | *Hystrix africaeaustralis* | 2 | P |
| African lion | *Panthera leo leo* | 3 | 1xS 2xP |
| Agouti | *Dasyprocta leporina* | 1 | P |
| Asian elephant | *Elephas maximus* | 2 | S |
| Asian small- clawed otter | *Aonyx cinerea* | 4 | P |
| Cheetah | *Acinonyx jubatus* | *3* | P |
| Giraffe (Rothschild) | *Giraffa camelopardalis* | 5 | S |
| Guinea pig | *Cavia porcellus* | 1 | S |
| Hippopotamus | *Hippopotamus amphibious* | 1 | P |
| Kune kune pig | *Sus scrofa domesticus* | 1 | P |
| Little red flying fox | *Pteropus scapulatus* | 2 | P |
| Meerkat | *Suricata suricatta* | 2 | P |
| NZ lesser short- haired bat | *Mystacina tuberculata* | 1 | P |
| Norway rat | *Rattus norvegicus* | 3 | 2xS 1xP |
| Red-necked wallaby | *Macropus rufogriseus* | 1 | S |
| Red panda | *Ailurus fulgens* | 4 | 2xS 2xP |
| Serval | *Leptailurus serval* | 3 | 2xS 1xP |
| Sumatran tiger | *Panthera tigris sumatrae* | 8 | 7xS 1xP |
| Tasmanian devil | *Sarcophilus harrisii* | 7 | S |
| Zebra | *Equus burchelli bohmi* | 1 | P |
| | **Total** | | **55** |
| **Primates** | | | |
| Hamadryas baboon | *Papio hamadryas* | 2 | P |
| Bonnet macaque | *Macaca radiata* | 3 | 2xS 1xP |
| Cotton top tamarin | *Saguinus Oedipus* | 1 | S |
| Golden lion tamarin | *Leontopithecus rosalia* | 5 | 4xS 1xP |
| Bornean orangutan | *Pongo pygmaeus* | 3 | 1xS 2xP |
| Ring- tailed Lemur | *Lemur catta* | 1 | P |
| Siamang gibbon | *Hylobates syndactylus* | 1 | *P* |
| Spider monkey | *Ateles geoffroyi* | 2 | 1xS 1xP |
| Squirrel monkey | *Saimiri boliviensis boliviensis* | 5 | 2xS 3xP |
| | **Total** | | **23** |
| **Reptiles** | | | |
| American alligator | *Alligator mississippiensis* | 1 | P |
| Coastal bearded dragon | *Pogona barbata* | 6 | 3xS 3xP |
| Cunningham's skink | *Egernia cunninghami* | 2 | P |
| Eastern blue tongue lizard | *Tiliqua scincoides scincoides* | 2 | 1xS 1xP |
| Eastern water dragon | *Physignathus lesueurii* | 1 | P |
| Fallas skink | *Oligosoma fallai* | 1 | P |
| Galapagos tortoise | *Chelonoidis nigra* | 3 | S |
| Grand skink | *Oligosoma grande* | 2 | S |

| | | | | |
|---|---|---|---|---|
| Jeweled gecko | *Naultinus gemmeus* | 1 | S | |
| Moko skink | *Oligosoma moco* | 1 | P | |
| Otago skink | *Oligosoma otagense* | 8 | 6xS 2xP | |
| Rough gecko | *Naultinus rudis* | 1 | P | |
| Scheltopusik | *Ophisaurus apodus* | 2 | S | |
| | **Total** | | **31** | |
| **Amphibians** | | | | |
| Golden bell frog | *Litoria aurea* | 1 | P | |
| | **Total** | | **1** | |
| **Inverterbrates** | | | | |
| Weta punga | *Deinacrida heteracantha* | | 3 | 3 |
| | **Total** | | **3** | |

### 7.2.2 Isolation techniques

All experimental testing was performed in a controlled environment within the New Zealand Veterinary Pathology Laboratories (NZVP), situated in the New Zealand Centre for Conservation Medicine (NZCCM), Auckland Zoo. This is a PC2 laboratory, with all plating procedures being performed aseptically within an ESCO 2, Class 2 BSC laminar flow cabinet.

Faecal samples were plated onto *Campylobacter* blood free isolation agar (Fort Richard), using five phase streaking methods (with 10 µl sterile disposable loops). Inoculated plates were incubated in a micro-aerophilic and capnoaerophilic (5% $O_2$, 10% $CO_2$ and 85% N) environment, for a period of 72 hours at 42°C. The micro-aerophilic environment was achieved using sealed 2.5L containers with a micro-aerophilic gas generating sachet (Mitsubishi Gas Chemical Company). All inoculated plates were run in conjunction with control plate containing *C. jejuni* (positive control) and *E. coli* (negative control) as a quality control measure for both media and incubation conditions.

### 7.2.3 Presumptive identification

After the incubation period, cultures were observed for growth morphology that is typical of *Campylobacter* spp. These appear as small flat runny and spreading colonies on the *Campylobacter* isolation media. Those that had the typical appearance were checked for their oxidase activity (all *Campylobacter* species are oxidase positive), to rule out members of the *Enterobacteriaceae* family that are commonly present in faecal samples. A colony of the suspected *Campylobacter*

spp. was smeared onto a strip of Oxoid oxidase paper (Thermofisher). A deep blue colour indicated positive reaction.

Oxidase positive colonies were smeared on a glass slide, heat fixed and then stained with Dilute Carbol Fuchsin (DCF) for no less then 3 minutes, in order to visualise the organism under the microscope at 1000x magnification. The organisms take up the DCF stain allowing better confirmation of the tiny 'seagull winged' shaped organisms (Figure 3). The seagull appearance is a result of the joining of daughter cells (Quinn et al., 2011).

Once the characteristic microscopic morphology was obtained, a Hippurate hydrolysis test was performed. 100 µl of sterile water was pipetted into a tube and inoculated with the presumptively identified *Campylobacter* colony. A BBL Taxo Hippurate differentiation disc was then added to the suspension. This suspension was then incubated aerobically at 37°C for 2 hours. Following incubation, 3 drops of Ninhydrin (Remel) were added to suspension and incubated for another 15 minutes. After this additional incubation, the suspension is checked for the colour change from clear to purple (Figure 4).

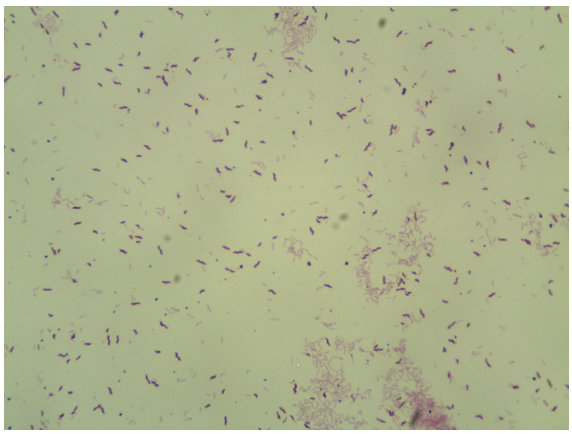

**Figure 3. DCF stain of *Campylobacter* species**. Taken under the microscope at 1000x magnification. Positive sample isolated from the Red-necked wallaby.

*Campylobacter jejuni* is the only *Campylobacter* that hydrolises hippurate into glycine and benzoic acid forming a purple colour. This test also provided any evidence of hippurate negative *Campylobacter* species that were present in this study.

For the further differentiation of *Campylobacter* spp. the Nalidixic Acid and Cephalothin (Oxoid) susceptibility test by disk diffusion method was performed on Mueller-Hinton agar (Fort Richard Laboratories Ltd, Auckland, New Zealand) under microaerobic conditions. The zone diameter breakpoints were determined according to the CLSI-established guidelines.

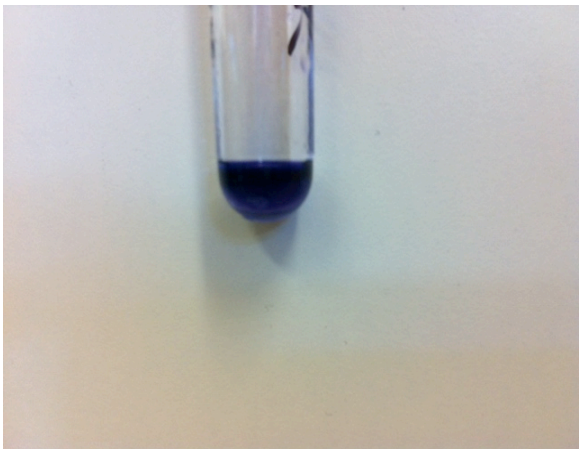

**Figure 4. Positive hippurate hydrolysis**.

### 7.2.4 Confirmatory identification

The confirmatory identification was performed on each presumptively identified *Campylobacter* spp. in the Microbiology Department of Waitemata DHB Laboratory Services, North Shore Hospital, Auckland. For rapid identification of the *Campylobacter* strains isolated, the Bruker MALDI-TOF MS was utilised. A colony of each isolate was individually innoculated onto a well within a stainless steel target plate with a small sterile wooden stick. Once the colony has dried, 1 µl of matrix solution (Bruker MALDI matrix HCCA; HCCA = α-Cyano-4-hydroxycinnamic acid) was added. The bacterial/matrix mix was then air dried and then placed into the MALDI-TOF MS, where mass spectrometry of the proteins was assessed to provide a fast and efficient identification of the isolated *Campylobacter* species.

MALDI-TOF MS was performed with a MicroFlex LT system (Bruker Daltonics) tabletop mass spectrometer using the manufacturer's suggested settings. Briefly, ions generated with a 337-nm nitrogen laser were captured in the positive linear mode in a mass range of 1960 to 20,200 mass-to-charge (m/z). Captured spectra were analysed using MALDI Biotyper automation control and Bruker Biotyper

3.1 software (Bruker Daltonics, Bremen, Germany). The MALDI Biotyper database contains 5,627 spectra from 3,995 species. Parameter settings (ion source 1 [IS1], 20 kV; IS2, 18 kV; lens, 6.0 kV; detector gain, 2877 V;) had been optimized for the mass range 1960 to 20,200 mass-to-charge.

### 7.2.5 DNA preparation for real-time polymerase chain reaction (qPCR)

DNA was extracted using the KingFisher Duo Magjet Viral nucleic acid kit ThermoFisher), without the addition of carrier RNA. Using a DNA/RNA free transfer pipette, 1 bacterial colony was suspended in 250 µl of KingFisher buffer (50 µl of Proteinase K, 200 µl of lysis buffer). After a vigorous 30-second vortex, DNA was then extracted from the suspension using the KingFisher Duo Magjet Viral nucleic acid kit. Extracted DNA was then diluted in 50 µl of PCR grade water, and stored at -20°C.

### 7.2.6 Primer design

All standard primers (Table 4) from this study were selected from methods used in previous experiments, checked and compared via the Genebank database with the primer BLAST program, to ensure primer specificity for *Campylobacter*.

**Table 4. PCR primer sequences**

| Primer | Sequence (5'- 3') | bp | Reference |
|---|---|---|---|
| F2 16S rRNA | ATC TAA TGG CTT AAC CAT TAA AC | 681 | |
| Cam Rev | AAT ACT AAA CTA GTT ACC GTC | | (Marwan Abu-Halaweh, J. Bates, & Patel, 2005) |
| F3-gyrA-cj-cc | GTA CTT TTG GTG TGA TTA TG | 500 | |
| R4-gyrA-cj-cc | TAA TCT CTT TTA ATT CAT CGC G | | (Menard, Dachet, Prouzet-Mauleon, Oleastro, & Megraud, 2005) |
| flaA F | ATG GAA TTT CGT ATT ACC AC | 465 | |
| flaA R | ACC YAA AGC ATC RTT ACC ATT | | (Lick et al., 2007) |
| cadF U | TTG AAG GTA ATT TAG ATA TG | 455 | |
| cadF R | CTA ATA CCT AAA GTT GAA AC | | (Nayak et al., 2005) |
| cdtA F | CTA TTA CTC CTA TTA CCC CAC | 712 | |
| cdtA R | AAT TTG AAC CGC TGT ATT GCT C | | (Martínez et al., 2006) |
| cdtB F | AGG AAC TTT ACC AAC AGC C | 628 | |
| cdtB R | GGT GGA GTA GTT TGT TGT C | | (Martínez et al., 2006) |

| | | | |
|---|---|---|---|
| cdtC F | ACT CCT ACT GGA GAT TTG AAA G | 546 | |
| cdtC R | CAC AGC TGA TGT TGT TGG C | | (Martínez et al., 2006) |
| CL55 | ATG CAA GTC GAA CGA TGA AGC GAC | 579 | |
| CL632 | CCA CTC TAG ATT ACC AGT TTC CC | | (Oyarzabal, Wesley, Barbaree, Lauerman, & Conner, 1997) |

### 7.2.7 Real time PCR conditions for gene targeting in the Lightcycler™

All real time PCR reactions were performed in glass capillary tubes, with total reagent mixtures equating to 15 µl. Each capillary contained 7 µl of sterile PCR grade $H_2O$, 2 µl of each target primer (1mM), 4 µl of Roche LightCycler Faststart DNA Master$^{PLUS}$ SYBR green mixture (Faststart enzyme + Faststart reaction mixture), 5 µl of DNA was then added to the primer masters mix to a total of 20 µl. The negative control consisted of 15 µl of primer master mixture with 5 µl of PCR sterile $H_2O$. Addition of $MgCl_2$ was not required in this mixture due to the reaction mix contained in the Faststart package. All real time PCR reactions were performed in the Roche LightCycler™ 2.0.

### 7.2.8 16S rRNA

Based on modified conditions from Marwan et al. (2004), 16S rRNA/cam Rev primers had an activation of 95°C for 5 minutes, denaturing temperature of 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 28 seconds. The melting curve fluorescence reactions were set at 95°C for 0 seconds, 65°C for 15 seconds with a continuous 95°C ramp rate of $0.1°C.s^{-1}$. Each procedure was performed over 55 cycles of real time PCR.

*C. lari* control strain required different 16S rRNA PCR conditions, which was based on modified conditions from Oyarzabal et al. (1997). The *C. lari* 16S rRNA primers (CL55 and CL632) had an activation of 95°C for 5 minutes, denaturing temperature of 95°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 24 seconds. The melting curve fluorescence reactions were set at 95°C for 0 seconds, 65°C for 15 seconds with a continuous 95°C ramp rate of $0.1°C.s^{-1}$. Reaction cooling was at 40°C for 30 seconds. Each procedure was performed over 35 cycles of real time PCR.

### 7.2.9 Gyrase A (*gyrA*)

The *gyrA*-cj-cc real time PCR conditions were also modified, from Menard et al. (2005). The activation was also at 95°C for 5 minutes. Denaturation was 95°C for 6 seconds, annealing at 54°C for 12 seconds, and primer extension at 72°C for 25 seconds. The melting curve analysis was performed at 95°C for 0 seconds, 45°C for 15 seconds and 95°C with a continuous ramp rate of $0.1°C.s^{-1}$.

### 7.2.10 Flagella A (*flaA*)

Based on modified conditions of Lick et al. (2007), *flaA* real time PCR conditions were based over 40 cycles. The PCR profile included an activation temperature of 95°C for 5 minutes, denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds, extension at 72°C for 8 seconds. The melting curve fluorescence was set to 95°C for 0 seconds, 65°C for 15 seconds and 95°C continuous ramp rate of $0.1°C.s^{-1}$.

### 7.2.11 *Campylobacter* adhesion factor (*cadF*)

*cadF* real time PCR conditions were modified from Nayak et al. (2005), over 45 cycles of PCR reactions. Activation temperature was 95°C for 5 minutes, denaturation at 95°C for 15 seconds, annealing 50°C for 15 seconds, extension 72°C for 20 seconds. The melting curve fluorescence was set to 95°C for 0 seconds, 65°C for 15 seconds and 95°C continuous ramp rate of $0.1°C.s^{-1}$.

### 7.2.12 Cytolethal distending toxin A, B and C (*cdtA*, *cdtB* and *cdtC*)

All real time PCR conditions for *cdtA*, *cdtB* and *cdtC* were modified from Martinez et al. (2006), however conditions were modified to compliment the expected DNA product size for the respective protein products.

*cdtA* PCR was performed over 30 cycles with an activation of 95°C for 5 minutes, denaturation at 94°C for 15 seconds, annealing at 57°C for 15 seconds, extensionat 72°C for 13 seconds. The melting curve fluorescence was set to 95°C for 0 seconds, 65°C for 15 seconds and 95°C continuous ramp rate or $0.1°C.s^{-1}$. *cdtB* PCR was performed over 30 cycles, with an activation of 95°C for 5 minutes, denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds, extension at 72°C for 21 seconds. The melting curve fluorescence was set to 95°C for 0 seconds, 65°C for 15 seconds and 95°C continuous ramp rate or $0.1°C.s^{-1}$. *cdtC*

PCR was performed over 40 cycles had an activation of 95°C for 5 minutes, denaturation at 94°C for 15 seconds, annealing at 57°C for 15 seconds, extension at 72°C for 13 seconds. The melting curve fluorescence was set to 95°C for 0 seconds, 65°C for 15 seconds and 95°C continuous ramp rate or 0.1°C.$s^{-1}$.

## 7.3 Amplification of PCR products with the Lightcycler 96

Following qPCR analysis to determine the presence of the selected putative virulence genes, the respective positive DNA/primer samples were reamplified with the LightCycler 96 to get PCR products for sequencing in a 96-well plate format.

### 7.3.1 Amplification procedures

All real time PCR reactions were performed in 96-well plates, with total reagent mixtures equating to 15 µl. Each well contained 3 µl of sterile PCR grade $H_2O$, 1 µl of each target primer (1 mM), 10 µl of Roche Faststart DNA Probes Master mixture (Faststart reaction mixture- Taq DNA Polymerase), 5 µl of DNA was then added to the primer masters mix to a total of 20 µl. PCR reactions were performed using the Roche LightCycler™ 96.
PCR conditions for each primer were the same conditions as those used for the qPCR amplifications with the LightCycler 2.0.

### 7.3.2 Analysis of amplified PCR products for sequencing

For analysis of amplified PCR products obtained for sequencing, the Agilent 2100 Bioanalyser was utilised in place of gel electrophoresis. This method provides digital assays of electrophoresis. Following the instruction protocols from the standard Agilent DNA 1000 Assay kit, reagents and amplified PCR products were added to the 12-well DNA chips used for the assay.
The chips were then analysed via the 2100 Bioanalyser, which determined the amplified PCR products along with the size, producing a digital visualisation of the amplified products.

### 7.3.3 Sequence alignment and phylogenetic analysis

Genetic sequencing of isolated genes were performed by Macrogen (Republic of Korea). Multiple Sequence Alignment (MSA) was performed and generated using MSA program Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Following alignment, Neighbor-Joining phylogenetic trees were generated using MEGA 6.0 (Molecular Evolutionary Genetics Analysis) software. The MEGA 6.0 sequence files were uploaded to Geneious 9 bioinformatics software in order to highlight the regions of sequence diversity. Settings within Geneious 9 also allowed to run a BLAST of these nucleotide sequences to confirm their presence in *Campylobacter*.

# 8 Results

## 8.1 *Campylobacter* species isolated

Of the 202 samples tested, *Campylobacter* species were isolated from 17 (8.9%) the animal species investigated (Table 5). *C. jejuni* was the most frequently isolated species (52.9%), followed by *C. upsaliensis* (35.3%). Both *C. coli* and *C. lari* were isolated least frequently (5.9%). Birds yielded the highest *Campylobacter* isolation rates, 52.9% of the positive samples, followed by non-primate mammals (29.4%) and primates (17.6%). Reptiles, amphibians and insects did not yield *Campylobacter* strains.

**Table 5. *Campylobacter* positive isolates. (**S denotes single sample, P denotes Pooled sample).

| Common name | Scientific name | Number | Species isolated |
|---|---|---|---|
| **Avian** | | | |
| Brolga | *Grus rubicunda* | 1xP | *C. jejuni* |
| Greater flamingo | *Phoenicopterus roseus* | 1xS | *C. jejuni* |
| Little blue penguin | *Eudyptula minor* | 1xP | *C. lari* |
| New Zealand dotterel | *Charadrius obscurus* | 1xP | *C. jejuni* |
| Pied stilt | *Himantopus himantopus* | 1xS | *C. coli* |
| Takahē | *Porphyrio (Notornis) hochstetteri* | 1xS 1xP | *C. jejuni* |
| Weka | *Gallirallus australis* | 1xS 1xP | *C. jejuni* |
| | | Total | 9 |
| **Mammals** | | | |
| Cheetah | *Acinonyx jubatus* | 1xP | *C. upsaliensis* |
| Meerkat | *Suricata suricatta* | 2xP | *C. upsaliensis* |
| Red-necked wallaby | *Macropus rufogriseus* | 1xS | *C. jejuni* |
| Serval | *Leptailurus serval* | 1xS | *C. upsaliensis* |
| | | Total | 5 |
| **Primates** | | | |
| Bonnet macaque | *Macaca radiata* | 1xS | *C. jejuni* |
| Golden lion tamarin | *Leontopithecus rosalia* | 2xS | *C. upsaliensis* |
| | | Total | 3 |

Among the 89 bird faecal samples, 10.1% yielded *Campylobacter* species. *C. jejuni* (77.78% ) was the most frequently isolated species in birds followed by *C. coli* and *C. lari* (11.1%). All but two of the avian species found to harbour *Campylobacter* are New Zealand natives.

*Campylobacter* isolated from non- primate mammalian species were isolated in 9.1% of the 55 faecal samples tested. Only two different strains were isolated *C. upsaliensis* (80%) and *C. jejuni* (20%). *Campylobacter* was isolated in 13% of the 23 primate faecal samples investigated. *C. upsaliensis* (66.7%) and *C. jejuni* (33.3%).

### 8.1.1 Monthly *Campylobacter* spp. isolation peaks

Isolation of *Campylobacter* spp. in this study had 3 peak months (Figure 5), December 2013, May 2014 and June 2014. May and June exhibited the highest isolation rates, both 29.4% of the *Campylobacter* species isolated, where December exhibited the third highest isolation rate of 23.5%. January 2014 and July 2014 exhibited 11.8% and 5.9% isolation rates respectively. The months February 2014 to April 2014 and August 2014 did not yield any isolates of *Campylobacter*.

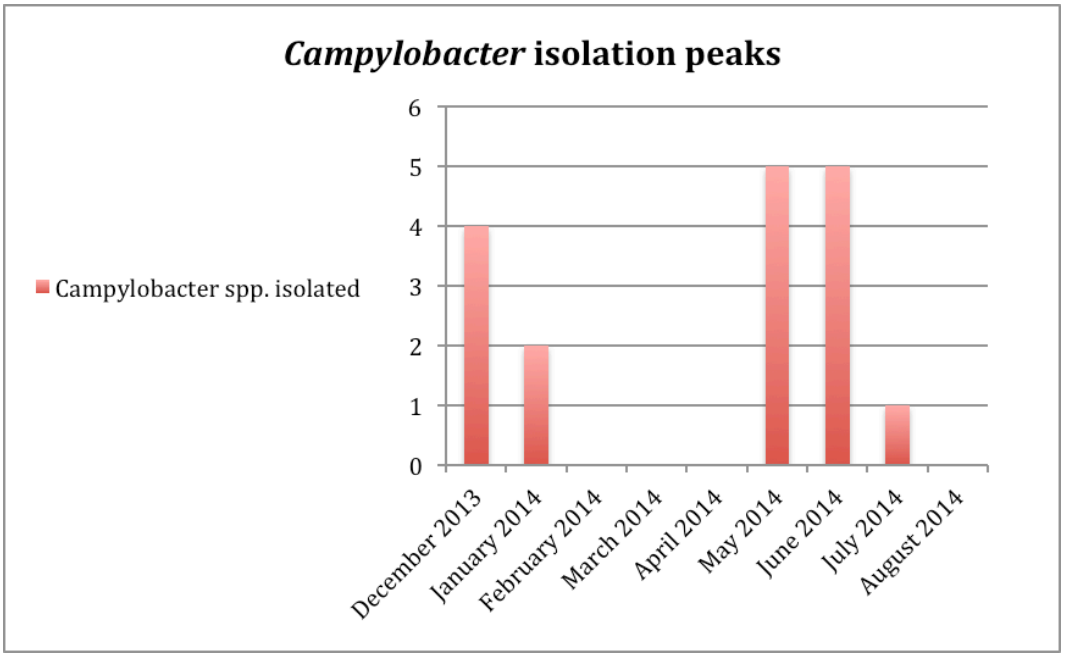


**Figure 5. Isolation peaks of *Campylobacter* between December 2013- August 2014.**

## 8.2 Real- time PCR gene investigation

Among the putative virulence genes investigated (Table 6), *flaA* (Figure 6) was detected in 100% of the *Campylobacter* isolates. *gyrA* gene was found at a rate of 70.6%, *cadF* in 58.8%, *cdtA* in 70.6%, *cdtB* in 47.1% and *cdtC* in 35.3% of the total number of positive *Campylobacter* isolates.

**Table 6. Amplification of virulence genes.** The isolation rate of putative virulence genes investigated in the *Campylobacter* strains isolated from the animal species in this study.

| Gene | Birds (n= 9) | Mammals other than primates (n= 5) | Primates (n= 3) | Melting Temperature Average |
|---|---|---|---|---|
| *gyrA* | 100% | 40% | 33.3% | 78.9°C ± 1.1°C |
| *flaA* | 100% | 100% | 100% | 82.3°C ± 0.8°C |
| *cadF* | 66.7% | 60% | 33.3% | 79.7°C ± 0.7°C |
| *cdtA* | 77.8% | 60% | 66.7% | 80.8°C ± 1.7°C |
| *cdtB* | 44.4% | 60% | 33.3% | 81.7°C ± 0.5°C |
| *cdtC* | 33.3% | 60% | 33.3% | 78.1°C ± 0.4°C |

Genes investigated exhibited high degrees of conservation among the animal species, with birds exhibiting the highest gene amplification rates of the animals tested. This may suggest higher conservation of these genes among *Campylobacter* spp. isolated from bird species.

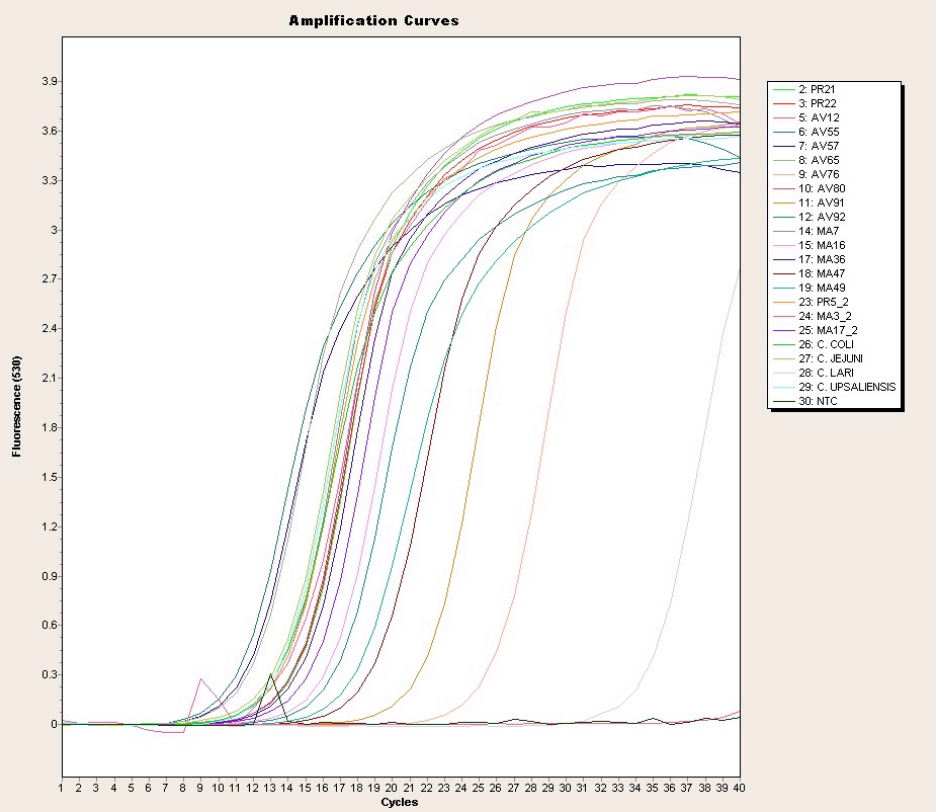


**Figure 6. *flaA* Real-time PCR amplification**.

## 8.3 Melting temperature

The average melting temperatures (Table 6) seen among *flaA* (Figure 7) exhibited the highest average. Followed by *cdtB*, *cdtA*, *cadF*, *gyrA*, and *cdtC* respectively.

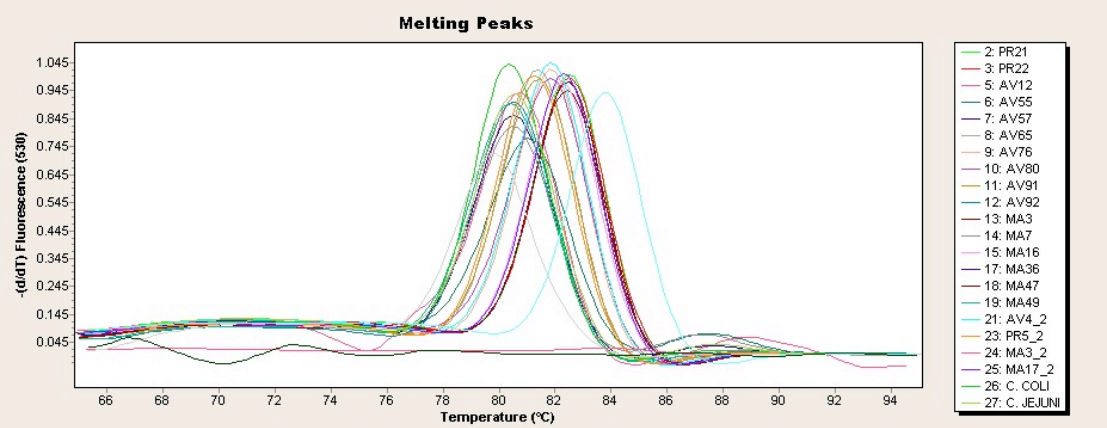


**Figure 7. *flaA* Melting temperatures.**

## 8.4 Gene sequencing alignment and phylogenetic analysis

Following sequencing of the 6 genes investigated in this study, sequence alignments and phylogenetic analysis were performed to generate dendrograms and visualise relationships between the *Campylobacter* strains isolated. Phylogenetic analysis revealed *flaA* to exhibit a high degree of diversity between the different isolates. Two main clusters of *flaA* (Figure 8) were generated following sequence alignments. Interestingly, these sequence clusters are species specific.

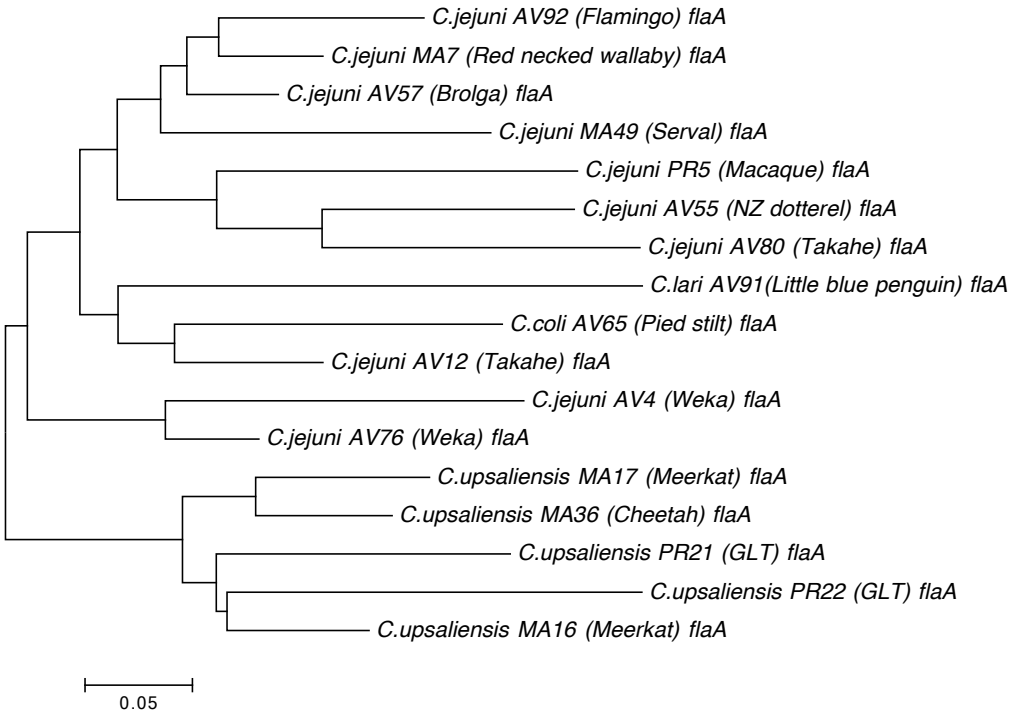


**Figure 8: *flaA* gene phylogenetic tree.** GLT; Golden lion tamarin.

The smaller cluster group of *flaA* consisted of only *C. upsaliensis* strains, while the larger cluster group, which also exhibited 3 minor clusters, were found to consist of *C. jejuni* with the exception of the single *C. coli* and *C. lari* isolates (which were found to have a close phylogenetic relationship within the same minor cluster). Another interesting pattern discovered following phylogenetic analysis of *flaA* was that clusters were found to harbour those with melting temperature similarities. Sequence alignment analysis of *flaA* revealed higher degrees of conserved sequence regions and similarities in base pair (bp) regions 65-180bp, with nucleotide differences exhibiting some patterns of species specificity. Within the *flaA* sequence alignments, base pair regions of 180bp and above exhibited higher degrees of sequence heterogeneity.

Phylogenetic analysis of *gyrA* gene sequences (Figure 9) among the *Campylobacter* strains in this study revealed 2 major cluster groups. Surprisingly, one cluster of *gyrA* reveals close ancestral relationships among the 4 *Campylobacter* strains that exhibited all 6 genes investigated in this study. The second major cluster exhibits *Campylobacter* strains that are solely from birds

species, in particular NZ natives. Interestingly *gyrA* amplified from the 2 takahe isolates exhibited moderate sequence variation with each other.

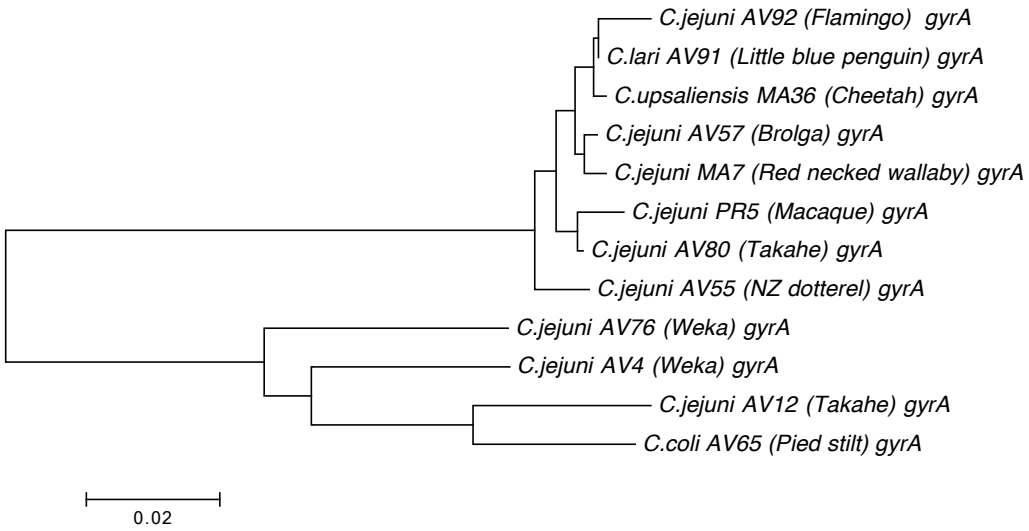


**Figure 9: *gyrA* gene phylogenetic tree**

Phylogenetic analysis of *cadF* (Figure 10) also showed 2 major clusters. The first and largest cluster exhibited high sequence homology with highly conserved nucleotide sequences seen between 30-300 bp regions. In fact, *cadF* exhibited the highest degree of sequence similarities among all of the genes investigated in this study, suggesting high conservation of this gene irrespective of the animal host. The second major cluster exhibited more frequent nucleotide differences than what was seen in the first cluster. Three of the strains found among the second cluster are from animals housed within the same enclosure, revealing a close relationship between the strains, which again indicates the conserved nature of *cadF*.

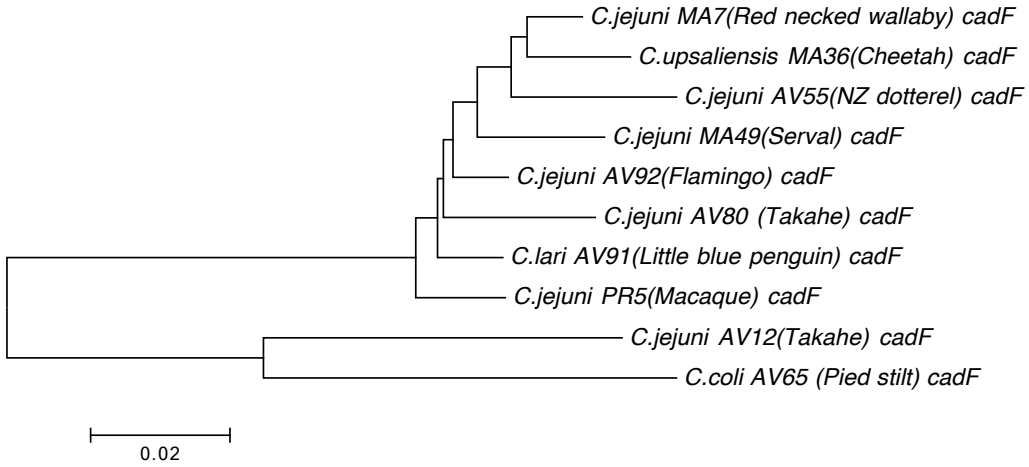


**Figure 10: *cadF* gene phylogenetic tree**

One third of the tripratite holotoxin gene *cdtA* revealed 2 major cluster groups with one simplicifolious clade (Figure 11). Sequence alignment revealed varying degrees of conserved sequence regions. The first major cluster exhibits high degrees of sequence conservation as well as numerous sequence deletions, which were interestingly found within the same regions. The high degrees of sequence homology seen in this cluster suggests high sequence conservation among this gene. Again the 2 takahe isolates exhibited high degrees of variation, indicating high levels of genetic shift in *cdtA* found in these animals.

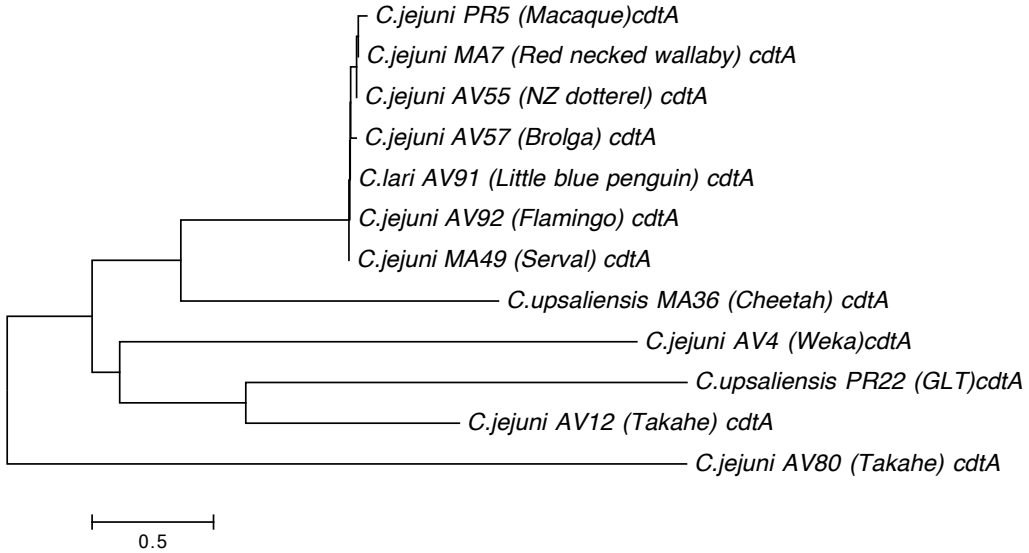


**Figure 11: *cdtA* gene phylogenetic tree**

The toxin producing *cdtB* gene also exhibited 2 major cluster groups, one consisting solely of *C. jejuni* isolates and the other of *C. jejuni* and a single *C. upsaliensis* (Figure 12). Sequence analysis revealed high sequence homology

between isolates with only small small degrees of nucleotide point mutation exhibited. Again higher degrees of homology were seen among those *Campylobacter* isolates that possessed all 6 genes investigated in this study.

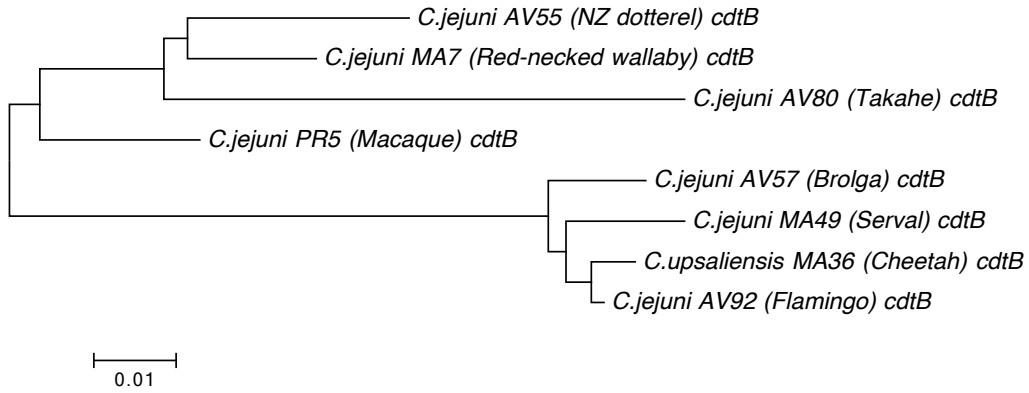


**Figure 12: *cdtB* gene phylogenetic tree**

Analysis of *cdtC* revealed one simplicifolious clade and one major cluster (Figure 13). While close relationships can be seen in the dendrogram, high degrees of sequence heterogeneity were detected through sequence analysis. Especially in two outlying species, New Zealand dotterel and macaque, where numerous single nucleotide differences were demonstrated. Interestingly, many of the nucleotide differences were the reverse complement to those found in the major cluster. Greater sequence homology was seen within the major clade in base regions of 60-290 bp, which may be due to high levels of conservation of *cdtC* within these regions regardless of the source of *Campylobacter*.

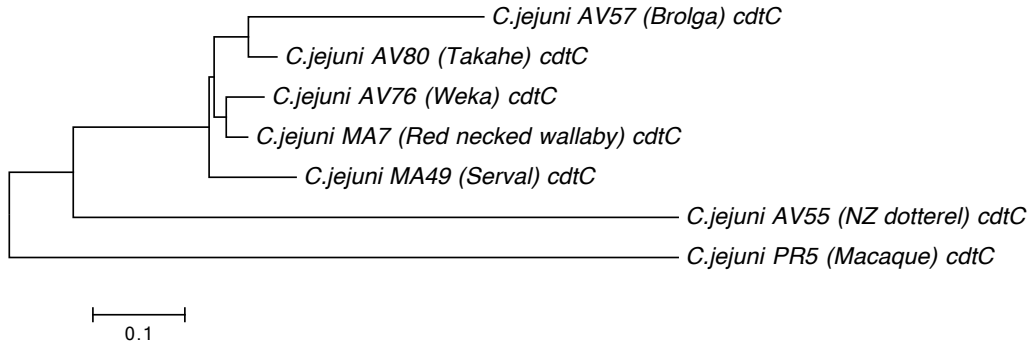


**Figure 13: *cdtC* gene phylogenetic tree**

# 9 Discussion

Little is known about *Campylobacter* circulating in wildlife populations, as previous studies are commonly focused on the prevalence of the pathogen in food source (poultry, cattle, pigs) and domestic animals, due to their implication in human health as reservoirs of infection. By sampling faecal samples taken from a healthy captive animal population within the Auckland Zoo, this study set out to determine the prevalence of *Campylobacter* species found within the captive wildlife population.

## 9.1 Bacterial isolation

### 9.1.1 Bacterial isolation and identification

During the course of this study we successfully utilised multiple methods. Some are traditional (conventional identification of culture from faecal samples by biochemical means), or modern (MALDI-TOF MS), others are state of the art such as the molecular methods (real-time PCR, sequencing). All combined they allowed us to isolate and identify *Campylobacter* spp. present in a captive wild life animal population and build phylogenetic trees showing the inferred relationships among isolates circulating at Auckland Zoo.

Due to multiple bacterial species often present in faecal samples, PCR alone may have provided skewed results. Therefore, traditional culture techniques were used for *Campylobacter* isolation. However, due to the fastidious nature of the organisms, identification by biochemical methods is very limited and can often not be made further than genus level. Thus the MALDI-TOF MS was utilised for rapid and efficient identification of the isolates in this study, successfully identifying all isolates to the species level. Following identification, molecular techniques were used to amplify and investigate the 6 putative pathogenic genes selected in this study.

### 9.1.2 Seasonal patterns

Isolation of *Campylobacter* spp. in this study had 3 peak months, December 2013, May 2014 and June 2014, with May and June exhibiting the highest isolation rates. The first month of the New Zealand summer, December 2013, saw an average

temperature of 19.6°C with a total of 222 hours of sunshine (Turner, 2013a). While the average temperature was within the average temperature range for December, the sunshine hours were elevated (112%) which is commonly seen in December. The autumn month of May in 2014, exhibited a monthly temperature average of 14.4 °C which was near the average temperature (Brandolino, 2014b) that is usually exhibited in Auckland during May. The month of May 2014 experienced a higher than average (127%) in sunlight hours (Brandolino, 2014b). While the month of June 2014 saw an unusually warm month for the start of the New Zealand winter. Auckland had a month average of 15.6°C with a total of 123 sunshine hours (Brandolino, 2014a).

*Campylobacter* has the ability to cause infection on an all year round basis, however there are usually seasonal patterns with higher incidences often seen in summer and autumn (Blaser, 1997), where the days are often warmer, longer or coming off the tail end of warmer months, respectively. Wallace et al. (1997) found seasonal peaks of *Campylobacter* in chicken populations, demonstrating season peaks during spring and autumn. These findings are similar to this study, in which the season peaks were found during very early summer, autumn and the very beginning of winter.

Although *Campylobacter* are thermophilic in nature, they also are sensitive to high temperatures (Park, 2002), exhibiting a narrow optimal growth temperature. As the temperatures increased coming out of the winter months into the midst of the New Zealand summer (December-March), the higher temperatures may have reduced *Campylobacter* metabolism over the hotter periods, resulting in a lag in growth of the micro-organisms. Subsequently higher isolation rates during the first month of autumn were recorded as the temperature cooled. November post 2013/2014 summer was seasonally high for the Auckland average temperature (17.8°C) (Turner, 2013b) that is usually seen in November.

### 9.1.3 *Campylobacter* isolation

Birds are one of the biggest reservoirs of *Campylobacter* spp. As an example, *C. jejuni* is frequently isolated in birds. The favourable body temperature often seen in birds (42°C), is optimal for growth of *C. jejuni,* hence the high isolation rates.

In this study, *Campylobacter* was isolated at slightly lower rates than in previous studies, where isolation rates varied from 20% to 100% (Ludovico Dipineto et al., 2014; Mohan, 2015; Saleha et al., 1998). The differences in isolation rates may be influenced by feeding habits, birds species, (Abdollahpour, Zendehbad, Alipour, & Khayatzadeh, 2015) and even husbandry practices that are incorporated into the daily zoo keepers routines.

With the exception of the little blue penguin (who has a diet of small fish) and the flamingo (bottom feeder), all of the avian species found to harbour *Campylobacter* in this study, are foraging birds with a diet of mainly invertebrates and insects. Host and feeding habits may be factors in explaining the presence of *Campylobacter* in some hosts over others (J. Waldenström et al., 2010). Similar to this study, Waldenström et al. (2002) also found foraging birds feeding on both invertebrates and insects to exhibit higher rates of *Campylobacter* isolation (11% and 20.3% respectively) compared to species who feed on grains or plants.

The little blue penguin differs in eating habits, in that its main diet is small fish, much like all sea dwelling birds. Interestingly, *C. lari* has commonly been associated with other sea dwelling birds, especially seagulls as well as water and shellfish (Matsuda & Moore, 2011). This may suggest that the penguin acquired *C. lari* from either the environment or from the food source. However we cannot rule out the possiblity of cross contamination from the many seagulls that are frequently found within the zoo grounds.

Enclosure habitat found at the Auckland Zoo, may also provide an explanation for the lower isolation rates of *Campylobacter* seen in bird species in this study. For example, in broiler farms, a whole chicken flock can rapidly become infected with *Campylobacter* within 3 days (Newell, 2002) due to overcrowding that is so often seen in farms. Though unlike broiler farms, avian housing at the Auckland Zoo are less crowded, where some species are housed in pairs, some may require a larger space due to the territorial nature of the bird , and some may be housed in slightly larger numbers depending on species. Lower numbers housed in the enclosures may reduce time and ability for the spread of *Campylobacter* that is so often seen in broiler houses. As well as reduced crowding of enclosures, increased

husbandry practices at the zoo also play a role in reducing *Campylobacter* numbers. Unlike broiler farms, the zoo enclosures are cleaned every day by the keepers, therefore reducing potential environmental transmission of the organism.

Mammals comprise a variety of animals species and like birds, studies commonly focus on those that are in close contact with humans (domestic animals) or those used for food source (farm animals). *Campylobacter* has long been known to commensally inhabit the intestinal tracts of a number of mammals, which has been a common source of infection for humans. *Campylobacter* species isolated from non-primate mammalian species in this study varies between domestic and production animals. Isolation rates were similar to those found in domestic animals, 11 to 92% (Horrocks et al., 2009), but lower to what is commonly found in production animals, 50 to 70% (Blaser, 1997). Animal species, housing, environmental sources and highly carnivorous eating habits may play a role in the differences of isolation rates.

Similar to their domestic counterparts, the big cat species in this study had an isolation rate of only 13%. The only species isolated was *C. upsaliensis,* which is commonly found in both healthy cats, but is also known to be problematic in kittens (Shen et al., 2001). *C. upsaliensis* has been associated, albeit less frequently, with human gastrointestinal illness. Shen et al. (2001) isolated *C. upsaliensis* from 47% of 47 *Campylobacter* isolates from healthy laboratory reared cats, in which there was no association between the presence of the organism and disease (Shen et al., 2001). Hald et al. (1997) also isolated only *C. upsaliensis* from healthy cats, however, they found much lower isolation rates (5%). These findings suggest that *Campylobacter* is conserved among felines species, regardless of their domestic status.

The presence of *C. jejuni* isolated from the Red-necked wallaby may also suggest the ability to carry the organism without ill-effect, as the animal showed no clinical signs of gastroenteritis. At present there appears to be no data suggesting this is either a commensal or pathogen for this animal. Investigating the bacterial diversity of the anal and urogenital tracts of marsupial counterparts, the tammar wallabies (*Macropus eugenii*), Chhour et al. (2008), found a variety of organisms

present in both tracts investigated. This included the likes of clostridia and *Lactobacillus* spp. (most frequently isolated organisms), *Streptococcus dysgalactiae* subsp. *dysgalactiae*, Bacteroidetes and Actinobacteria (Chhour, Hinds, Deane, & Jacques, 2008). Among these organisms, *C. helveticus* was also isolated from the genital tract. While also investigating the presence of gastrointestinal pathogens in a captive animal population, Stirling et al. (2008), isolated *C. jejuni* in 20% of faecal samples from red kangaroo's tested, but did not isolate *Campylobacter* species from any of the 7 parma wallabies tested.

Further studies by Chhour et al. (2010), revealed no *Campylobacter* isolation from the gastrointestinal tract of young tammar wallabies. Wallabies have a shorter than normal gestation period (35 days), after which, the less developed neonate takes up residence in the pouch of the mother for further development (Lentle et al., 2006). Therefore, wallaby neonates rely on maternal defences during the developmental period in the pouch. At birth young animals are susceptible to numerous organisms, though are protected against potentially harmful pathogens by maternal defences (Chhour, Hinds, Jacques, & Deane, 2010). The mother wallaby possess the ability to manipulate the microbial environment of her pouch, reducing bacterial load during times when a neonate is present (Lentle et al., 2006), protecting them from susceptibility of infection that may readily occur in the immunocompromised neonate.

Thus, the absence of *Campylobacter* in young wallabies, yet the presence of the organism in older wallabies may be suggestive of the animal acquiring *Campylobacter* after birth, from an environmental source found within their habitat. With little data, we can only speculate that *Campylobacter* isolated from these marsupial animals may either be found as a commensal organism or acquired from an environmental source, yet remain clinically insignificant for the animal.

Humans have been known to harbour around 400 different species of intestinal organisms (McKenna et al., 2008). Similarly, non-human primates also harbour an abundance of enteric organisms and parasites, such as tapeworms, *Giardia* and *Campylobacter* (Nunn, 2012). Both *C. jejuni, C. hyointestinalis* have both been

identified in healthy individuals. In severe cases of illness *C. fetus* has been associated with *Campylobacter*-induced foetal death in macaque species (Baze & Bernacky, 2002). Naoaki et al. (2000) isolated *C. jejuni* from chimpanzees, *C. hyointestinalis* from macaques and simians, and at the same time simultaneously isolated *C. jejuni* and *C. hyointestinalis* from 2 chimpanzees. It was also noted that *C. hyointestinalis* had previously been isolated from macaques in earlier studies (Misawa et al., 2000).

On a larger scale, Stirling et al. (2012) investigated 155 primates from a wide range of species, such as golden lion tamarins, chimpanzees, lemurs, marmosets and macaques among many others. It was found that only *C. jejuni* was isolated with an overall isolation rate was 8.4%. Tamarin species exhibited the highest isolation rates of all the primates tested. The golden lion tamarin had the highest *C. jejuni* isolation of 44.4% followed by the emperor tamarin (33.3%), black top tamarin (22.2%) and the cotton top tamarin (12.5%).

In this study *Campylobacter* was isolated in 15.8% of primate faecal samples, which fits inline with previous published studies. In this study *C. upsaliensis* was recovered from the golden lion tamarin, where previous studies have recovered only *C. jejuni* from tamarin species. At present there appears to be no data on *C. upsaliensis* isolation from tamarin species, and very little on *C. upsaliensis* in primate species in general. However, Anonymous (2011) performed *Campylobacter* analysis on faecal samples from 2 groups of chimpanzees. Group 1 was living in close proximity to humans while Group 2 was situated in a non-human habituated area. It was found that Group 1 was the only group to have had *Campylobacter* recovered (34%) from the samples investigated. After 16s RNA PCR procedures were performed, it was revealed that all the *Campylobacter* spp. most closely resembled *C. upsaliensis* (95.5%) and *C. helveticus* (94.7%) (Anonymous, 2011).

Not only is *Campylobacter* exhibited in a wide range of non-human primates, but various species of the organism can be found too. This suggests that non-human primates can act as a reservoir for *Campylobacter* (Misawa et al., 2000), in

particular, *C. jejuni* and *C. hyointestinalis*. Differences in species may be determined by environmental conditions, primate species and dietary habits.

## 9.2 Virulence gene sequencing

Few studies exist that have provided information on the presence of putative virulence genes isolated in *Campylobacter* species from such a variety of strains isolated from a captive wild animal population. The determination of specific unique genes and their sequences may provide invaluable information for detection and identification of *Campylobacter* in future diagnostic settings, and provide indications on the virulence potential.

One aspect of this study set out to investigate selected virulence genes that are commonly associated with the pathogenesis of *Campylobacter*. Genes selected for this study were based on their ability for motility, adhesion factors, toxin production and DNA negative supercoiling; *flaA*, *cadF*, *cdtA*, *cdtB* and *cdtC* and *gyrA*, respectively. These genes have been well studied and are considered highly conserved among *Campylobacter*.

Gyrase is a 2 subunit protein (GyrA and GyrB) that is highly conserved among bacteria (Maxwell, 1997) and is involved in catalysing negative supercoiling of DNA which facilitates DNA replication (Ruiz, Pons, & Gomes, 2012). Gyrase is also a common target used for therapeutic treatment for *Campylobacter*, where quinolones such as Nalidixic acid (NA) (D. E. Taylor, Ng, & Lior, 1985), are ideal therapeutic treatment option for the Nalidixic sensitive *C. jejuni* and *C. coli*. Fluoroquinolones target the quinolone-binding site of the *gyrA* gene (Marja-Liisa Hänninen & Hannula, 2007), preventing DNA replication and transcription. In fact, all isolates (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*) in this study that exhibited *gyrA* gene are isolates that are sensitive to NA. Interestingly, *gyrA* was amplified in all *Campylobacter* species isolated from avians, which may suggest *gyrA* is more highly conserved among *Campylobacter* isolates from avian species. In fact, *gyrA* is a common target for treatment procedures in poultry farms, where fluoroquinolones are often used in the poultry industry to reduce *Campylobacter* numbers among broiler flocks (M C M. de Jong, K T. Veldman, R M. van Boven,

& Mevius, 2003). The ultimate aim is to reduce the density of *Campylobacter* in carcasses, reducing poultry induced campylobacteriosis.

As expected, *flaA* was amplified at a high incidence of the *Campylobacter* isolates in this study, which correlates with previous studies, where *flaA* was also isolated in such high frequencies. Datta et al. (2003) detected *flaA* from 100% of 111 *C. jejuni* isolated from a variety of sources (humans, poultry, bovine). Similarly, Andrzejewska et al (2011) also detected *flaA* from all *C. jejuni* and *C. coli* isolates from cats, dogs and children. These high incidence rates found in this study and previous studies, suggests that *flaA* is conserved among all *Campylobacter* species, regardless of sources and reservoirs. In fact, Fisher et al. (1991) have stated that the amino acid sequence in the N-terminal region of the flagellum is homologous in all *C. jejuni* strains (Fischer & Nachamkin, 1991).

The *flaA* gene is required for the expression of flagella, which is an important and primary mechanism for pathogenesis. *Campylobacter* must be flagellated to achieve motility in order to enter and cross the mucus layer covering epithelial cells (T.M. Wassenaar & Blaser, 1999). Without this ability *Campylobacter* is unable to cross the mucus layer and invade host cells. Studies have shown that mutant flaA$^-$ *C. jejuni* strains have non-functional flagella and exhibit reduced colonisation abilities (Nuijten et al., 2000). The highly conserved nature of *flaA* in *Campylobacter,* make this gene an ideal candidate for rapid *Campylobacter* species identification and epidemiological studies.

Adhesion associated protein CadF is found highly conserved among *Campylobacter* species. Studies have amplified *cadF* gene in *Campylobacter* species from humans, domestic animals, cattle and birds in 88.3%- 100% (Datta et al., 2003; M E. Konkel et al., 1999; M. Andrzejewska et al., 2011; Rizal et al., 2010). This was demonstrated in this study where *cadF* was amplified in 58.8% of *Campylobacter* species across avian, mammal and primate species. These findings are slightly lower than reported in previous studies but still indicating that *cadF* gene is highly conserved in *Campylobacter* isolates regardless of source.

The corresponding protein is a 220kDa glycoprotein found in regions of bacteria-host cell interaction contact with the gastrointestinal epithelium (Konkel et al., 2005). This attachment ability to the intestinal tract is considered an important primary step to colonisation of *Campylobacter* (M E. Konkel et al., 1999). Without this mechanism, *Campylobacter* are limited in colonisation, therefore limited in pathogenic abilities. *Campylobacter cadF* mutant strains exhibiting cadF$^-$ have shown marked reduced ability for adhesive properties, as high as 50% (Marshall R. Monteville, Julie E. Yoon, & Konkel, 2003), suggesting an important role played by *cadF* in the multifactorial pathogenesis process.

Cytolethal Distending Toxin (CDT) is one of the most studied virulence mechanism found in *Campylobacter*. The CDT holotoxin is composed of a tripartite genes *cdtA*, *cdtB* and *cdtC*. Although *cdtB* is considered to be the toxin-producing component, all 3 are required for complete cytotoxic function. In this study, *cdtA*, *cdtB* and *cdtC* were amplified at varying rates (70.6%, 47.1%, 35.3% respectively). These findings are not uncommon, as previous studies have often found the presence of 1 or 2 of the genes where the other is absent. Only 6 of the 17 *Campylobacter* strains isolated in this study exhibited all 3 of the CDT genes, and all 6 of these strains are *C. jejuni*, 50% of which are from avian origin. No other *Campylobacter* species isolated in this study exhibited all 3 CDT genes, indicating the cytotoxic potential the *C. jejuni* strains present. Though it has been said that of the *C. jejuni* strains that do make CDT, there are strain to strain variations which may result in the varyiable virulence levels for different *C. jejuni* isolates (Pickett et al., 1996).

The pathogenic process of *Campylobacter* is a multifactorial process that requires various intracellular genetic mechanisms that are involved in motility, adhesion, colonisation and toxin production. The absence of one of these processes can limit and reduce the organism's pathogenic and virulence potential.

## 9.3 Sequence analysis

Utilising sequence analysis and phylogenetic dendrograms, relationships were generated and demonstrated for 6 different genes (*flaA, cadF, gyrA, cdtA, cdtB* and *cdtC*) investigated in this study. These genes are considered to be conserved

among *Campylobacter* species, and each play their own role in the virulence process. While conservation of these genes is well defined, we have demonstrated heterogeneity within the respective gene sequences. While surprising, this is not a rare occurrence among *Campylobacter*. Frequent genomic rearrangements and interstrain genetic exchange are often drivers of genetic diversity in *Campylobacter* (Carrillo et al., 2012).

The phylogenetic dendrogam and sequence analysis of *flaA* revealed high diversity among this gene. This gene is highly conserved among *Campylobacter* strains and is a common target for epidemiological studies, yet the heterogeneity found among this gene may make these studies a challenging task. The variation seen in this study fits in line with previous studies who have also demonstrated high genetic diversity among *flaA* genes. For instance, Nachamkin et al. (1993) observed 18 distinct *flaA* RFLP patterns among 54 *C. jejuni* isolates investigated and Rahem et al. (2015) found 11 different genotypes among 90 *C. jejuni* isolates. Variation in sequences found in *Campylobacter* has been previously observed and may be explained by host age, geographical diversity (Khoshbakht, Tabatabaei, Hosseinzadeh, Aski, & Seifi, 2015) and in this instance, housing isolation of animal species.

A cluster of *C. upsaliensis* isolates revealed close relationships between these strains, however, it was found that isolates from 2 meerkats and 2 golden lion tamarins (GLT) that were housed in the same respective enclosures, showed sequence variations. This was an interesting finding in this study where sequence heterogeneity among *Campylobacter* species was evidenced from the same animals species found within the same enclosures. This was demonstrated in the 2 golden lion tamarin *C. upsaliensis* isolates, 2 meerkat *C. upsaliensis* isolates as well as the 2 takahe and 2 weka *C. jejuni* isolates. The takahe and weka are actually housed in the same enclosure, so we would have expected to find higher levels of sequence homology among isolates from these animals. Phylogenetic analysis of all investigated virulence genes from the takahe revealed only minor relationships between the 2 isolates, while the weka demonstrated a much closer relationship with each other. We would have expected such sequence

hetereogeneity among different animals species housed in different enclosures, not those housed together.

Studies have in fact revealed this is a common occurrence in *Campylobacter*, where animals housed together are often colonised with more than one genotype of *Campylobacter*. Hosny et al. (2013) demonstrated genetic variation among a turkey flock while investigating *flaA* typing from *Campylobacter* isolated within the flock. Of the 14 *C. jejuni* strains isolated, *flaA* typing revealed 4 different genotypes, demonstrating that a single flock may in fact be colonised with more that one genotype (El-Adawy et al., 2013). The ability for *Campylobacter* to have strain to strain variation through mechanisms such as horizontal gene transfer, intra and inter- genomic recombination (El-Adawy et al., 2013; Jeon, Muraoka, & Zhang, 2010) have resulted in high genetic variablity among conserved genes.

The subunit gyrase A (*gyrA*) is involved in the DNA replication and transcription processes as well as being a target for antibiotic treatments. In this study all 12 gyrA genes were successfully sequenced, with slight variation of sequence lengths (approximately 400 to 500bp). All *Campylobacter* species isolated from birds were found to harbour this gene, suggesting high conservation among birds irrespective of species. The highly conserved nature of *gyrA* found in birds is the reasoning behind the heavy usage of antibiotics in poultry farms to reduce *Campylobacter* numbers found in commercial carcasses.

Cluster analysis revealed 2 major clusters, interestingly one shows close relationships with bird species two weka and one takahe that are housed in the same enclosure. While slight sequence differences were observed, major sequence homologies also existed. This was a similar occurrence seen with the second major cluster where major sequence homologies were also observed. While some variations were observed, homologies seen suggest that a high conservation of *gyrA* exists, particularly among *C. jejuni* isolates, that made up over 80% of isolates harbouring *gyrA*. The relationship differences seen between the two clusters may be a consequence of environmental differences and/or genetic recombination in isolates that are observed in those that were housed together compared to those found in different enclosures.

In this study, high sequence homology seen in *gyrA* may also suggest reduced stress conditions that their animal hosts are faced with. For example, unlike poultry flocks, these animals species are faced with little to no antibiotic pressure. Thus there is no need for high genetic variability for survival in the face of this pressure. It is well known that bacteria are able to increase mutation capabilities during times of high stress induced conditions (Marja-Liisa Hänninen & Hannula, 2007), without these stressors (such as antibiotics) the necessity for mutations of *gyrA* is low.

Investigations of the phylogenetic dendrogram of *cadF* demonstrated close relationships between the *Campylobacter* strains irrespective of species type. While sequences exhibited slight variation in lengths ranging from approximately 370 to 455bp, sequence homology was seen among all isolates. Interestingly, the *C. jejuni* isolates that exhibited all 6 genes in this study showed high levels of homolgy in both sequence size and alignments. This suggests high conservation of *cadF* in *Campylobacter* species and little genetic recombination, making this an ideal target for epidemiological studies.

Very little data exist on *cadF* sequence heterogeneity/homology seen among *Campylobacter* species. However one such study performed by Hirayama et al. (2009) demonstrated high levels of homology (89.4 to 100%) in 17 *C. lari* isolates investigated, exhibiting conservation of *cadF* sequences across a number of hosts. Given the low levels of sequence diversity seen in the *C. lari* isolate compared to *C. jejuni*, *C. upsaliensis* and *C. coli* seen in this study, we can deduce that these results fit inline with the high levels of homology demonstrated by Hirayama et al. (2009). In this study, geographical isolation of animals species within the Auckland Zoo did not allow major sequence variations among this gene. This result is also similar to what Hirayama et al. reported in 2009. By investigating *Campylobacter* strains retreived from various sources and locations, they found high homology in sequences generated, indicating the high levels of conservation of the gene sequence of *cadF*.

The 3 cytolethal distending toxin genes *cdtA*, *cdtB* and *cdtC* are well established and ubiquitously distributed in *Campylobacter*. In this study, the 3 genes were amplified in varying degrees exhibiting varying degrees of sequence variation. Each of the 3 genes showed varying sequence sizes 542 to 720bp for *cdtA*, 508 to 628 bp for *cdtB* and 297 to 546 bp for *cdtC*. The phylogenetic dendrogram of *cdtA* showed close relationships between the isolates, yet sequence analysis revealed *cdtA* to exhibit the highest sequence diversity, having shown the least amount of identical sites among the 12 *cdtA* gene sequences. This was also a similar occurrence with the *cdtC* gene which also exhibited high sequence diversity across the 7 sequences obtained. *cdtB* had the least amount of sequence variation among the *cdt* genes, suggesting that *cdtB* may be more highly conserved among the *cdt* genes. Similarly Masahiro et al. (2007) also found sequence homologies to be higher in *cdtB* compared to *cdtA* and *cdtC* gene sequences. It was found that *cdtA* and *cdtC* amplified from *C. jejuni*, *C. coli* and *C. fetus* showed 34 to 48% homology, where cdtB showed 57 to 67% homology.

Only 5 isolates exhibited all 3 *cdt* genes showing close phylogenetic relationships with each other among the *cdtA cdtB* and *cdtC* genes. The presence of all 3 genes is required for toxin production. Without it the holotoxin is unable to exert its cytotoxic effects. However, there were some deletions identified in the neuleotide sequences of all 3 genes (*cdtA* exhibting the highest number of deletions). These deletions may be a cause for a reduction in toxin production (Masahiro Asakura et al., 2007) in these strains, subsequently permitting residence of these organisms in the gastrointestinal tract of these animals without showing ill effect.

Another interesting cluster pattern demonstrated in this study is the close phylogenetic relationships seen among the genes from the only 4 *Campylobacter* isolates that exhibited all 6 genes investigated. Sequence similarities in these isolates may suggest higher conservation among those with higher pathogenic potential. While we did not investigate pathogenesis of the isolates in this study, we can speculate that virulence potential may be high due to the demonstration of the genes required for virulence of *Campylobacter*.

Investigating these 6 genes we have demonstrated varying degrees of sequence variation and homology of the *Campylobacter* isolates circulating at the Auckland Zoo. Some patterns were seen among both animal and *Campylobacter* species, while some exhibited very little. However over all, the high homology seen among the isolates may indicate low variation of *Campylobacter* that is circulating around the zoo irrespective of animal species. The sequence variation that was seen among these isolates may be a result of genetic shift, recombination, point mutations and even deletions/insertions. It is a known phenomenon that *Campylobacter* are sensitive to genomic evolution, resulting in extensive genomic and phenotypic diversity (Carrillo et al., 2012). Subsequently, altering genotypes and in severe cases expression of the corresponding genes may result in reduced pathogenic potential.

# 10 Summary

In this study, we successfully determined the presence of *Campylobacter* spp. circulating among the captive animal population at the Auckland Zoo. Isolation rates were slightly lower than what previous studies have reported. We can put this down to a number of reasons: (i) husbandry practices seen at the zoo ensure enclosures are cleaned on a daily basis, resulting in reduced numbers of environmental organisms, leading to reduced *Campylobacter* spp. transmission between animals; (ii) eating habits of animals species may also play a role in diversity of bacterial flora that is found in the intestinal tract, (iii) animals tested in this study were healthy individuals exhibiting no symtoms of illness, where previous studies tend to predominantly investigate those who exhibited signs of gastroenteritis. As expected, *C. jejuni* exhibited the highest incidence of *Campylobacter* in this study. This organism is found ubiquitously in the environment, warm blooded animals and humans. Its presence in animals is considered to be one of the major sources of infection in humans.

Through the investigation of putative virlulence genes and their sequences we have demonstrated relationships between the circulating *Campylobacter* spp. found at the Auckland Zoo. We saw conservation in both the presence and sequences of many of the genes investigated. While some regions in each gene (*flaA, cadF, gyrA, cdtA, cdtB* and *cdtC*) came with high homology, nucleotide differences were also found between species. Genetic variation in *Campylobacter* is a common occurrence due to the organism's sensitivity to genetic shift. Variation in this study may be due to many reasons, such as the introduction of *Campylobacter* from an outside source (wild birds), being an open circuit environmental factors may permit the ability of *Campylobacter* to be introduced. Age of the animal may also play a role in variation, as well as geographical location, point mutations, insertions/deletions and interstrain genetic exchange.

Of the 17 *Campylobacter* isolates, only 4 (all *C. jejuni*) exhibited all 6 genes investigated in this study. While we did not investigate the pathogenicity of the isolates in this study, we can speculate on their virulence potential due to the presence of the genes required for virulence of *Campylobacter*. The presence of

all 6 genes found in these 4 *C. jejuni* isolates provides the mechanisms required for pathogenesis of *Campylobacter* infection. We may also speculate that there is a reduced pathogenic potential of the remaining isolates due to the absence of some required genes involved in pathogenic mechanisms. This may provide a reasoning as to how the organism is able to reside in the intestinal tract of these animals and other animals without causing ill effect.

This study helps highlight the ability of zoos and other animal dense environments, to harbour potential pathogenic enteric organisms that could have the potential to cross-contaminate between animals and even between animals and humans (zoonosis), with the potential to spread diseases.

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