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Identification and Genotyping of *Campylobacter* spp. Strains Isolated from a Population of Captive Wild Animals

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Abstract

In this study, we investigated the prevalence, phenotypic and molecular typing features of *Campylobacter* isolates in a population of captive wild animals at Auckland Zoological Park in New Zealand. Animal species included mammals, avians and reptiles with a mixture of exotic and native species. Over a 10-month period, 202 faecal samples were collected for evaluation of the presence of *Campylobacter* spp. (isolation rate 8.4%). Presumptive identification by conventional microbiological methods and confirmatory identification by MALDI-TOF MS of the isolates were performed. *Campylobacter jejuni* was the most frequently isolated species (58.8%) followed by *C. upsaliensis* (29.4%), *C. coli* (5.9%) and *C. lari* (5.9%). Using real-time PCR and sequencing methods, isolates were then investigated for the presence of genes that are associated with virulence mechanisms involved in *Campylobacter* infections. Genes of interest were selected on the basis of their involvement in motility (*flaA*), adhesion (*cadF*), antibiotic resistance (*gyrA*) and toxin production (*cdtA*, *cdtB* and *cdtC*). Of the six genes investigated, the mean carrying rates were 100% for flagellin gene *flaA*, 58.8% for *Campylobacter* adhesion factor gene *cadF*, 70.6% for gyrase A gene *gyrA* and 70.6%, 47.1% and 41.2% for cytotoxins A *cdtA*, B *cdtB* and C *cdtC* genes, respectively. Sequencing results revealed both homology and heterogeneity of the virulence genes within isolated *Campylobacter* species, demonstrating both genetic conservation and variation respectively. These results highlight the possibility of zoological parks and other animal dense environments to harbour potential pathogenic enteric organisms that could cross-contaminate between animals and potentially infect humans.

Keywords:*Campylobacter,*Genotyping, Virulence, Sequencing, Zoonosis

1. Introduction

Campylobacter is a genus of gram-negative, motile, spiral-shaped, non-spore forming bacteria belonging to the *Campylobacteraceae* family. *Campylobacter* spp. are ubiquitous in the environment and form long term associations with animal hosts [1]. *Campylobacter* spp. can be found as both commensals in the intestinal tracts of many warm-blooded animals and humans, as well as pathogenic organisms which can lead to illness in the host [2]. In humans, *Campylobacter* spp. are known to cause infections such as gastroenteritis, periodontal disease, septicemia, abortion and in severe cases, post infectious immune-mediated disorder Guillain-Barré syndrome [2, 3, 4]. *C. jejuni* and *C. coli* are among the *Campylobacter* spp. that are most associated with gastroenteritis [4]. Less frequently, *C. upsaliensis* has also been reported as human pathogen [2]. Similarly, those that hold importance

in a veterinary setting include *C. jejuni*, *C. coli* and *C. upsaliensis* [5].

The ability of *Campylobacter* to colonise both humans and animals is not fully understood [2]. To colonise its host, *Campylobacter* must overcome both mechanical and immunological barriers that are found within the gastrointestinal tract [1]. In order to establish and maintain colonisation, *Campylobacter* spp. use various mechanisms such as motility, adhesion, invasion and toxin production. These mechanisms are controlled by a set of virulence genes that are commonly associated with *Campylobacter* spp. [4, 6, 7]. Studying these genes may provide us a means to better understand the genetic diversity and population structure of *Campylobacter* spp. ultimately preventing and minimizing the impact of disease [8].

Molecular and genetic analysis has become a useful tool in the epidemiology of *Campylobacter*, where molecular methods such as multilocus sequence typing (MLST), restriction fragment length polymorphism (RFLP) and microarrays have been successfully used [9, 10]. These tools aid in the characterization of genes and their sequences in order to investigate mutation rates, synonymous and non-synonymous polymorphisms [11], genetic diversity [12] and population structure of *Campylobacter* spp. [13]. The application of next-generation sequencing to molecular diagnostics has improved testing technologies, 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 [14]. 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.

Little is known about the epidemiology of *Campylobacter* in captive wild animals (e.g. zoological animals) as previous and current studies focus on domestic and food producing animals, as well as *Campylobacter* in medical settings. Based on comparison of partial DNA sequences from six virulence genes (*flaA*, *cadF*, *cdtA*, *cdtB* and *cdtC* and *gyrA*) found in *Campylobacter* spp., the work presented in this paper was aimed at understanding microbial dynamics in captive wild animals by studying the diversity of *Campylobacter* spp. based on the circulation of different *Campylobacter* species and the presence of these virulence genes. To our knowledge, no studies exist that have provided information on the presence of virulence genes in *Campylobacter* strains isolated from a captive wild animal population.

2. Materials and methods

2.1. Sample collection and preparation

Over a period of 10 months, between 1 November 2013 and 30 August 2014, 202 faecal samples were randomly collected from 850 captive wild animals at the Auckland Zoo (Table 1). This included 89 avians, 55 mammals other than primates, 23 primates, 31 reptiles, 1 amphibian and 3 insects. Samples from healthy individuals were randomly collected in 70 ml sterile plastic specimen containers (Thermo Fisher Scientific, New Zealand). Five grams were collected where possible, however, for some bird, reptile, amphibian and insect samples 5 g were impossible to obtain. In this instance 2 g or less were collected. Samples were stored at room temperature for no longer than 2 hours before inoculation took place.

2.2. Media and incubation conditions

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. Faecal samples were plated onto *Campylobacter* blood free isolation agar (Fort Richard Laboratories Ltd, Auckland, New Zealand), using the five phase streaking method (with 10 µl sterile disposable loops). Inoculated plates were incubated in micro-aerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) for a period of 72 hours at 42°C. The micro-aerobic environment was achieved using sealed AnaeroPack rectangular 2.5 L jars (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. All the strains were isolated using the same batch of culturing media, in order to reduce any potential unwanted phenotypic variation.

2.3. Presumptive identification

For each sample one colony was used for phenotypic tests, MALDI-TOF and DNA isolation. After the incubation period, cultures were observed and colonies (one per sample) with morphology typical of *Campylobacter* spp. were checked for their oxidase activity (all *Campylobacter* spp. are oxidase

positive), to rule out members of the *Enterobacteriaceae* that are commonly present in faecal samples. Suspected *Campylobacter* spp. were smeared onto a strip of Oxoid oxidase paper (Thermo Fisher Scientific, New Zealand). A dark blue colour indicated positive reaction. Oxydase positive colonies were then smeared on a glass slide, heat fixed and stained with Dilute Carbol Fuchsin (Sigma Aldrich, New Zealand) to visualise the typical 'seagull winged' shaped organisms under the microscope at 1000x magnification. Once the characteristic microscopic morphology was confirmed the sub-culture of the suspected colony was performed on a non-selective blood agar medium (Fort Richard Laboratories Ltd, Auckland, New Zealand). Plates were incubated in a micro-aerobic environment for up to 72 hours at 37°C before performing the hippurate hydrolysis test. Presumptively identified *Campylobacter* colonies were suspended in 100 µl of sterile water and a BBL Taxo hippurate differentiation disc was added. Suspensions were incubated aerobically at 37°C for 2 hours before adding the ninhydrin reagent (Thermo Fisher Scientific, New Zealand). After a second incubation at 37°C for 15 minutes, the suspensions were checked for the appearance of a purple colour. *C. jejuni* is the only *Campylobacter* species that hydrolyses hippurate into glycine and benzoic acid. For the further differentiation of *Campylobacter* spp. the Nalidixic Acid and Cephalothin (Thermo Fisher Scientific, New Zealand) susceptibility test by disk diffusion method was performed on Mueller-Hinton agar (Fort Richard Laboratories Ltd, Auckland, New Zealand) under micro-aerobic conditions. The zone diameter breakpoints were determined according to the CLSI-established guidelines. All further methods including MALDI-TOF and DNA isolation were performed from sub-cultures.

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, a Bruker MicroFlex LT tabletop mass spectrometer (Bruker Daltonics, Bremen, Germany) was employed following the manufacturer's suggested settings. A colony of each isolate from a sub-culture was individually spotted onto a well of a MALDI-TOF-MS stainless steel plate with a small sterile wooden stick and air dried for 60 minutes at ambient temperature. Then, 1 µl of Bruker MALDI matrix solution containing HCCA (α-Cyano-4-hydroxycinnamic acid) was added to each well. The bacterial/matrix mix was then air dried and placed into the MicroFlex LT system. 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). Three analytical replicates were collected from each strain.

2.5. Preparation of DNA samples

For each positive faecal sample, one *Campylobacter* colony collected from a sub-culture plate was suspended in 250 µl of KingFisher buffer made of 50 µl of proteinase K and 200 µl of lysis buffer (Thermo Fisher Scientific, New Zealand) using DNA/RNA free transfer pipette. After a vigorous 30-second vortex, genomic DNA was extracted using the KingFisher Duo Magjet Viral nucleic acid kit (Thermo Fisher Scientific, New Zealand), without the addition of carrier RNA. Extracted DNA was then diluted in 50 µl of PCR grade water, and stored at -20°C until further analysis. These reference strains were used in our study: *C. jejuni* subsp. *jejuni* NCTC 11168, *C. coli* (Doyle) NCTC 11366, *C. lari* NCTC 11352 and *C. upsaliensis* ATCC 43954.

2.6. Primer sequences

All primers used in this study (Table 2) were selected from methods used in previous experiments, checked and compared with the primer BLAST program to ensure primer specificity for *Campylobacter* spp. Oligonucleotides were synthesized and obtained from IDT (Integrated DNA Technologies, Singapore).

2.7. Real-time PCR conditions

Qualitative real time PCR experiments (presence or absence of targeted virulence genes, melting curve analysis) were run on a LightCycler 2.0 using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Applied Science, New Zealand). Amplifications were performed in glass capillaries (Roche Applied Science, New Zealand) in a 20 µl total volume containing 7 µl of sterile PCR grade water, 2 µl of each target primer (final concentration 1 mM), 4 µl of reconstituted FastStart DNA Master mix and 5 µl of previously purified DNA. PCR conditions for all target genes are summarised in Table 3. The same melting curve analysis was used for all genes at a linear temperature transition rate of 0.1°C/s from 65 to

95°C with continuous fluorescence acquisition. The amplification products were directly analyzed using the LightCycler 2.0 software (version 4.1.1.21).

Following real-time PCR analysis with the LightCycler 2.0 to determine the presence of the selected virulence genes, the positive DNA samples were amplified on the LightCycler 96 (Roche Applied Science, New Zealand) in a 96-well plate format using the FastStart Essential DNA Probes Master kit (Roche Applied Science, New Zealand). PCR conditions were the same as described above. This kit does not contain SYBR Green I making the PCR products compatible with automated sequencing procedures. Each well (total volume 20 µl) contained 3 µl of sterile PCR grade water, 1µl of each target primer (final concentration 1 mM), 10 µl of FastStart Essential DNA Probes Master mix and 5 µl of previously purified DNA. The amplification products were directly analyzed by on-chip electrophoresis using an Agilent 2100 Bioanalyser combined with a DNA 1000 kit (both from Agilent Technologies, Waldbronn, Germany).

2.8. Sequencing and phylogenetic analyses

DNA sequencing of amplified PCR products was performed by Macrogen Inc. (Seoul, Republic of Korea). Sequencing results obtained were checked for quality to eliminate sequences containing any ambiguous nucleotides that may be present. Sequences were further trimmed to only include nucleotides within the 2 priming sites for each pair of primers. Multiple Sequence Alignment (MSA) was performed and generated using MSA program Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).
Following alignment, phylogenetic analysis on nucleotide sequences was conducted by neighbor-joining method using the p-distance method with bootstrap (1,000 replicates) in MEGA (Molecular Evolutionary Genetics Analysis) version 6.0 software. The MEGA 6.0 sequence files were uploaded to Geneious 9 bioinformatics software to highlight the regions of sequence diversity. A BLAST (Basic Local Alignment Search Tool) analysis was run to confirm the presence of these sequences in *Campylobacter* and compare them against the *Campylobacter* genomes that are publicly available in GenBank database. Phylogenetic analysis aimed to classify the various genotypes by constructing phylogenetic trees based on the concatenated amplicon sequences from *flaA*, *gyrA*, *cadF*, *cdtA*, *cdtB* and *cdtC* genes. Sequences were aligned to a FASTA format using the online multi sequence alignment tool Clustal (http://www.ebi.ac.uk/Tools/msa/clustalo/).
Aligned sequences were then deposited in the publicly accessible database GenBank, through the web submission tool Bankit (http://www.ncbi.nlm.nih.gov/WebSub/index.cgi?tool=genbank).

3. Results

3.1. *Campylobacter* species isolation and determination

Oxidase activity for *Campylobacter* spp. isolated in this study was positive, indicated by a dark blue colour on the oxidase strips. DCF stain performed on presumptive colonies revealed spiral shaped rods typical of *Campylobacter* spp. Hippurate hydrolysis test was positive for 9 *C. jejuni* isolates while other isolates (*C. upsaliensis*, *C. coli* and *C. lari*) were hippurate negative. *C. jejuni* and *C. coli* were sensitive to Nalidixic acid and resistant to Cephalothin. *C. upsaliensis* and *C. lari* were resistant to Nalidixic acid and sensitive to Cephalothin.

Of the 202 samples tested, 17 (8.4%) yielded *Campylobacter* spp. across all animal species (95% CI [0.04, 0.12]). As shown in Table 4, *C. jejuni* was the most frequently isolated species (58.8%) followed by *C. upsaliensis* (29.4%). Both *C. coli* and *C. lari* were isolated least frequently (5.9%). Birds harboured the highest *Campylobacter* isolation rates with 10.1% of the positive samples (9 positives from 89 samples, 95% CI [0.04, 0.16]) followed by primates with 13.0% of the positive samples (3 positives from 23 samples, 95% CI [0.01, 0.27]) and mammals other than primates with 9.1% of the positive samples (5 positives from 55 samples, 95% CI [0.03, 0.15]). Reptiles (31 samples), amphibians (1 sample) and insects (3 samples) were tested but did not yield *Campylobacter* spp. All positives were retrieved from 9 individual faecal samples (from greater flamingo, pied stilt, takahe, weka, red-neck wallaby, serval, bonnet macaque and golden lion tamarin) and 8 pooled faecal samples (from brolga, little blue penguin, New Zealand dotterel, cheetah and meerkat).

Among the 89 bird faecal samples, 10.1% yielded *Campylobacter* spp. *C. jejuni* (77.8%) 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 was isolated from non-primate mammals in 9.1% of the 55 faecal samples tested. Only two different species were isolated, *C. upsaliensis* (60%) and *C. jejuni* (40%). *Campylobacter* was isolated in 13% of the 23 primate faecal samples investigated. *C. upsaliensis*

(66.7%) and *C. jejuni* (33.3%) were the only two species recovered.

3.2. Amplification of *Campylobacter* virulence genes

Among the virulence genes investigated (Table 5), *flaA* was the most detected gene in 100% of the *Campylobacter* spp. isolates (10 *C. jejuni*, 5 *C. upsaliensis*, 1 *C. coli* and 1 *C. lari*). *gyrA* gene was found at a rate of 70.6% (9 *C. jejuni,* 1 *C. upsaliensis*, 1 *C. coli* and 1 *C. lari*), *cadF* in 58.8% (7 *C. jejuni,* 1 *C. upsaliensis*, 1 *C. coli* and 1 *C. lari*), *cdtA* in 70.6% (9 *C. jejuni,* 2 *C. upsaliensis* and 1 *C. lari*), *cdtB* in 47.1% (7 *C. jejuni* and 1 *C. upsaliensis*) and *cdtC* in 41.2% (7 *C. jejuni*) of the total number of *Campylobacter* isolates. Our results showed variable degrees of conservation among the animal species, with birds exhibiting the highest mean carrying rate for *flaA*, *gyrA*, *cadF* and *cdtA*.

3.3. Gene sequence alignment and phylogenetic analysis

3.3.1. flaA phylogenetic tree

Phylogenetic analysis of *flaA* gene sequences revealed a high degree of diversity between the different isolates (Figure 1) with two clusters made of all *C. upsaliensis* strains for the minor one and the other species for the major one (mostly *C. jejuni* then *C. lari* and *C. coli*). *C. coli* and *C. lari* isolates were found to have a close phylogenetic relationship within the same minor cluster. Another interesting pattern was that clusters were found to have *flaA* sequences with similar melting temperatures (82.4°C ± 0.1°C; 81.2°C ± 0.6°C). Sequence alignment analysis of *flaA* revealed higher degrees of conserved sequence regions and similarities in the lower base pair (bp) regions (65-180 bp), with nucleotide differences showing patterns of species specificity. Conversely, higher degrees of sequence heterogeneity were found above 180 bp.

3.3.2. gyrA phylogenetic tree

Phylogenetic analysis of *gyrA* gene sequences (Figure 2) revealed 2 major clusters. Surprisingly, one cluster of *gyrA* showed close ancestral relationships among the 4 *Campylobacter* strains (isolated from a macaque, NZ dotterel, red necked wallaby and takahe) that had all 6 genes investigated in this study. The second major cluster exhibited *Campylobacter* strains that are solely from birds species, in particular NZ natives.

3.3.3. cadF phylogenetic tree

Phylogenetic analysis of *cadF* (Figure 3) also showed 2 major clusters. The largest one comprised highly conserved nucleotide sequences in the 30 to 300 bp region. 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. 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*.

3.3.4. cdtA phylogenetic tree

Comparison of *cdtA* sequences revealed 2 major cluster groups (Figure 4) with one simplicifolious clade. Sequence alignment revealed varying degrees of conserved sequence regions. The first major cluster mostly made of *C. jejuni* strains, showed high degrees of sequence conservation as well as numerous sequence deletions, which were interestingly found within the same regions. The high degree of sequence homology seen in this cluster suggests the conserved nature of *cdtA*. Again the 2 takahe isolates exhibited high degrees of variation, indicating high levels of genetic shift in *cdtA* found in these animals.

3.3.5. cdtB phylogenetic tree

Analysis of *cdtB* gene sequences also exhibited 2 major cluster groups, one consisting solely of *C. jejuni* isolates and the other of *C. jejuni* and a single *C. upsaliensis* (data not shown). 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 between *Campylobacter* isolates that possessed all 6 genes investigated in this study.

3.3.6. cdtC phylogenetic tree

Comparison of *cdtC* sequences revealed one simplicifolious clade and one major cluster (data not shown). High degrees of sequence heterogeneity were detected in two outlying animal 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 regions of 60 to 290 bp, which may be due to high levels of conservation of *cdtC* within these regions regardless of the source of *Campylobacter*.

4. Discussion

In this study, a genotyping method based on sequence analysis and phylogenetic dendrograms is described. It exploits the genetic variation present in six virulence loci (*flaA*, *cadF*, *gyrA*, *cdtA*, *cdtB* and *cdtC*) in *Campylobacter* spp. to determine the genetic relationships among strains isolated from a population of captive wild animals. Consistent isolate identification schemes are essential for population genetic and epidemiological studies of *Campylobacter* infections. MLST typing schemes using a defined set of housekeeping genes represent a reproducible and simple way to determine nucleotide sequence variability [15]. Since the first description in 2001 of a MLST scheme for *C. jejuni* [16], additional MLST methods for other *Campylobacter* species have been published [13, 17]. Because of its portability and international use, MLST is considered as the 'gold standard' for species and sub-species identification as it provides consistent information about the global epidemiology and population structure of *Campylobacter* [9]. However, when it comes to short-term studies its discriminative power becomes less reliable [17]. Investigating more variable genes in conjunction with MLST may increase the discriminatory power of sequence-based typing MLST methods to make them available for short-term epidemiological investigations of outbreaks of *Campylobacter*. As an example, the most frequently used gene for this purpose is *flaA* [17]. We agree that this addition of genetic variation at other genes loci is a change compared to the regular housekeeping genes and is therefore not analogous to "true" MLST, especially in the context of internationally standardized approaches and the use of publicly available typing tools. However, interlaboratory comparisons of subtyping methods based on the sequencing of multiple loci (not solely of housekeeping genes) are now easier to achieve thanks to the increasing availability of DNA sequencing methods. As an example, Korczak et al. [13] published an optimized MLST scheme with primer sets for 7 housekeeping genes and additional primers for sequence determination of *flaA*, *flaB*, *rpoB* and *gyrA*. This interesting multiplex strategy for MLST and antimicrobial resistance provided a more comprehensive approach for *C. jejuni* and *C. coli* isolates.

In our study, *Campylobacter* was isolated from birds with a rate of 10.1%. However, the prevalence of *Campylobacter* differs among different species of birds. For example, other studies have shown that isolation rates of *Campylobacter* in birds varied from 20% to 100% [18, 19, 20, 21, 22]. *Campylobacter* was isolated in 15.8% of primate faecal samples as reported in previous studies. As an example, Stirling et al. [23] investigated 155 individuals from a wide range of primate species. It was found that only *C. jejuni* was isolated with an overall isolation rate of 8.4%. Similarly, the authors also found that the golden lion tamarin had the highest *Campylobacter* isolation rate.

Gyrase A is a common target used for therapeutic treatment for *Campylobacter*, where quinolones such as Nalidixic acid (NA) [24] are an ideal choice for Nalidixic sensitive *C. jejuni* and *C. coli* strains. In this study, *gyrA* was detected and amplified in all *Campylobacter* species isolated from avians suggesting a highly conserved gene. In fact, gyrase A is a common target for treatment procedures in poultry farms. Fluoroquinolones are often used in the poultry industry to reduce poultry induced campylobacteriosis [25]. Cluster analysis of *gyrA* sequences revealed 2 major clusters, one showing close relationships with bird species, 2 weka and 1 takahe that are housed in the same enclosure. While slight sequence differences were observed, major sequence homologies also existed suggesting a low genetic variability particularly among *C. jejuni* isolates. The differences seen between the two clusters may be a consequence of environmental factors 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 antibiotic pressure. Compared to commercial settings, antibiotics are not given to the animals of Auckland Zoo on a daily basis, thus considerably reducing antibiotic pressure. It is well known that *Campylobacter* are able to increase mutation rates when submitted to stressors such as antibiotics [26].

As expected, *flaA* was detected in all of the isolates. Similarly, Datta et al. [8] detected *flaA* from 100% of 111 *C. jejuni* isolated from various sources (humans, poultry, bovine). Andrzejewska et al. [27] also detected *flaA* from all *C. jejuni* and *C. coli* strains isolated from cats, dogs and children. All these studies and our results show that *flaA* is strongly conserved among all *Campylobacter* species, making this gene an ideal candidate for rapid *Campylobacter* species identification and epidemiologic studies. The high genetic diversity seen for *flaA* has been demonstrated in previous studies. For instance, Nachamkin et al. [3] observed 18 distinct *flaA* RFLP patterns among 54 *C. jejuni* isolates investigated and Khoshbakht et al. [28]

found 11 different genotypes among 90 *C. jejuni* isolates. Such variation in sequences may be explained by host age, geographical diversity [28]. We found that *C. upsaliensis* isolates from 2 meerkats and 2 golden lion tamarins (GLT) that were housed in the same respective enclosures, showed sequence variations. The same sequence heterogeneity was evidenced with the 2 takahe and 2 weka *C. jejuni* isolates. This is a common occurrence in *Campylobacter*, where animals housed together are often colonised with more than one genotype. El-Adawy et al. [29] evidenced genetic variation among a turkey 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 than one genotype [29]. Mechanisms such as horizontal gene transfer, intra and inter- genomic recombination have been described to explain this high genetic variablity among conserved genes [29, 30].

Adhesion associated protein CadF is found highly conserved among *Campylobacter* species. Different studies have detected *cadF* in *Campylobacter* species from humans, domestic animals, cattle and birds [8, 27, 31, 32]. In our study, c*adF* was amplified in 58.8% of *Campylobacter* isolates retrieved from avians, non primate mammals and primates. Attachment ability to the intestinal tract is considered an important primary step to colonisation of *Campylobacter* [31] implying the central role played by *cadF* in the multifactorial pathogenetic process. Investigation of the phylogenetic dendrogram of *cadF* sequences demonstrated close relationships between the *Campylobacter* strains irrespective of species type. *C. jejuni* isolates that exhibited all 6 genes in this study showed high levels of homology in both sequence size and alignments. This suggests high conservation of *cadF* and little genetic recombination, making this gene an ideal target for epidemiologic studies.

Similarly, a study performed by Hirayama et al. [33] on *cadF* in *Campylobacter* demonstrated high levels of homology (89.4 to 100%) in 17 *C. lari* isolates. Given the low levels of sequence diversity in the *C. lari* isolate compared to *C. jejuni*, *C. upsaliensis* and *C. coli* seen in this study, our results suggest high levels of homology as reported by Hirayama et al. [33].

In this study, the cytolethal distending toxin genes *cdtA*, *cdtB* and *cdtC* were amplified at varying rates (70.6%, 47.1%, 41.2% respectively) and exhibited varying degrees of sequence variation. These findings are not uncommon, as previous studies have often found the presence of only 1 or 2 genes. Only 6 of the 17 *Campylobacter* strains isolated in this study exhibited all 3 of the CDT genes, and all 6 of these strains were *C. jejuni*, indicating the cytotoxic potential of these *C. jejuni* strains. 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 Asakura et al. [34] 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.

The comparison of partial DNA sequences from six virulence genes presented in this study provides a means for the investigation of *Campylobacter* outbreaks in a local context such as Auckland Zoo where a population of captive wild animals is maintained. This study provides an insight on *Campylobacter* circulating in a captive wild animal population along with genotypic diversity among putative virulence genes commonly associated with the pathogenesis of *Campylobacter*. However, there are limitations to this study, which should be noted. Time constraints of the study have limited the ability to increase sample numbers for investigation, in order to obtain more potential positive isolates. This has resulted in the inability to obtain duplicate samples, which may have yielded more *Campylobacter* isolates due to intermittent shedding. Seasonal variation of isolation was unable to be concluded, again due to the time constraints this study possessed.

Wild animals have been maintained in captive environments for many years, during which management and husbandry processes have improved, with a strong emphasis on the impact of the spread of infectious diseases [35]. The differences in *Campylobacter* isolation seen across the animals in this study may be influenced by different factors including animal species, housing, geographical location of enclosures, environmental sources, eating habits [36] and husbandry practices that are incorporated into the daily zoo keepers routines. For instance, at Auckland Zoo enclosures are cleaned every day by the keepers, therefore reducing potential environmental transmission of the organism. Reduced crowding of enclosures may also reduce time and ability for *Campylobacter* to spread around and cross-contaminate other animal species. Zookeepers visually observe animals for symptoms of malaise on a daily basis and any irregularities are

communicated to veterinary staff. Health screens are routinely performed in order to detect potential infections. The veterinary hospital provides housing quarantine for animals that exhibit severe disease symptoms to not only prevent spread of disease, but to also provide careful monitoring of the animals during times of illness. On a different scale, in backyard poultry, low biosecurity and management measures, poor cleaning and disinfection, high presence of faeces and presence of ill animals represent risk factors of exposure as they may increase the spread of *Campylobacter* species [21]. The same biosecurity and management rules apply to zoo enclosures where captive wild animals are present. These rules should be easy to implement, cost-effective and traceable with an efficient monitoring system. Increasing awareness of staff and management teams in captive wild animal populations may help to prevent animal-to-animal transmission and zoonotic problems.

Our approach successfully yielded new information on types and antibiotic resistances of *Campylobacter* strains isolated at Auckland Zoo. We still need to match the data obtained from our study with case-control information to prove the involvement of *Campylobacter* species identified at Auckland Zoo with potential cases of human disease and identify transmission pathways. Molecular typing of *Campylobacter* has been extensively used in the clinical setting and the food production chain [9]. Studies still need to be performed on captive populations of wild animals to assess the overall population structure and epidemiology of *Campylobacter* spp. With conventional genotyping methods and the increasing popularity of whole genome sequencing (WGS), new molecular typing methodologies are going to bring a new approach that could benefit these out of the ordinary environments.

5. Conclusions

Using sequence analysis and phylogenetic dendrograms, relationships were generated and demonstrated for the 6 target genes investigated in this study. While conservation of these genes is well defined, we have demonstrated heterogeneity within the nucleotide sequences. This is not a rare occurrence among *Campylobacter* as frequent genomic rearrangements and interstrain genetic exchange are often drivers of genetic diversity in *Campylobacter*. It is a known phenomenon that *Campylobacter* are sensitive to genomic evolution, resulting in extensive genomic and phenotypic diversity.

The genetic diversity of *Campylobacter* strains isolated at the Auckland Zoo suggests the possibility of sporadic infections (with or without clinical manifestations) with very diverse strains. Hence the necessity of having rapid and robust molecular typing tools to analyse this diversity in terms of molecular epidemiology, tracking of outbreak strains and emergence of resistance to antibiotics. More genes could be investigated to get a more effective molecular typing tool and analyse epidemiologic relationships among isolates.

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Table 1

Total number of faecal samples tested. “P” denotes pooled samples; “S” denotes a single sample.

Common names	Scientific names	Numbers tested	
Avians			
African grey parrot	*Psittacus erithacus*	1	P
Australian king parrot	*Alisterus scapularis*	1	P
Antipodes island parrot	*Cyanoramphus unicolor*	1	1
Banded rail (Moho-pereru)	*Gallirallus philippensis*	1	S
Bellbird	*Anthornis melanura*	1	S
Bleeding heart dove	*Gallicolumba luzonica*	3	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 mitchells 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*	*8xS 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

Mammals (not including primates)

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*

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*

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*

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Otago skink	*Oligosoma otagense*	8	6xS 2xP
Rough gecko	*Naultinus rudis*	1	P
Scheltopusik	*Ophisaurus apodus*	2	S
Amphibians			
Golden bell frog	*Litoria aurea*	1	P
Invertebrates			
Weta punga	*Deinacrida heteracantha*	3	3

Table 2

PCR primer sequences

Primers	Sequences (5'- 3')	Target gene	bp	References
F3-gyrA	GTACTTTTGGTGTGATTATG	*gyrA*	500	[39]
R4-gyrA	TAATCTCTTTTAATTCATCGCG			
flaA F	ATGGAATTTCGTATTACCAC	*flaA*	465	[37]
flaA R	ACCYAAAGCATCRTTACCATT			
cadF U	TTGAAGGTAATTTAGATATG	*cadF*	455	[40]
cadF R	CTAATACCTAAAGTTGAAAC			
cdtA F	CTATTACTCCTATTACCCCAC	*cdtA*	712	[38]
cdtA R	AATTTGAACCGCTGTATTGCTC			
cdtB F	AGGAACTTTACCAACAGCC	*cdtB*	628	[38]
cdtB R	GGTGGAGTAGTTTGTTGTC			
cdtC F	ACTCCTACTGGAGATTTGAAAG	*cdtC*	546	[38]
cdtC R	CACAGCTGATGTTGTTGGC			

Table 3

Real time PCR conditions for both LightCycler 2.0 and 96 experiments

Target gene	Activation step*	Denaturation	Annealing	Polymerisation	Cycles
gyrA	95°C for 5 min	95°C for 6 s	54°C for 12 s	72°C for 25 s	40
flaA	95°C for 5 min	95°C for 15 s	60°C for 10 s	72°C for 8 s	40
cadF	95°C for 5 min	95°C for 15 s	50°C for 15 s	72°C for 20 s	45
cdtA	95°C for 5 min	94°C for 15 s	57°C for 15 s	72°C for 13 s	30
cdtB	95°C for 5 min	95°C for 15 s	57°C for 15 s	72°C for 21 s	30
cdtC	95°C for 5 min	94°C for 15 s	57°C for 15 s	72°C for 13 s	40

* required for FastStart enzyme activation

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Table 4

Distribution of *Campylobacter* strains isolated from various animal species

Common name	Scientific name	*Campylobacter* species	Strain
Avians			
Brolga	*Grus rubicunda*	*C. jejuni*	AV57
Greater flamingo	*Phoenicopterus roseus*	*C. jejuni*	AV92
New Zealand dotterel	*Charadrius obscurus*	*C. jejuni*	AV55
Takahē	*Porphyrio (Notornis) hochstetteri*	*C. jejuni*	AV12
Weka	*Gallirallus australis*	*C. jejuni*	AV80 AV4 AV76
Little blue penguin	*Eudyptula minor*	*C. lari*	AV91
Pied stilt	*Himantopus himantopus*	*C. coli*	AV65
Mammals (not including primates)			
Cheetah	*Acinonyx jubatus*	*C. upsaliensis*	*MA36*
Meerkat	*Suricata suricatta*	*C. upsaliensis*	*MA16* *MA17*
Red-necked wallaby	*Macropus rufogriseus*	*C. jejuni*	MA7
Serval	*Leptailurus serval*	*C. jejuni*	MA49
Primates			
Bonnet macaque	*Macaca radiata*	*C. jejuni*	PR5
Golden lion tamarin (GLT)	*Leontopithecus rosalia*	*C. upsaliensis*	*PR21* *PR22*

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Table 5

Frequency of detection of selected virulence genes among 17 *Campylobacter* strains isolated from various animal species.

Target gene	Birds (n= 9)	Mammals other than primates (n= 5)	Primates (n= 3)
gyrA	100%	40%	33.3%
flaA	100%	100%	100%
cadF	66.7%	60%	33.3%
cdtA	77.8%	60%	66.7%
cdtB	44.4%	60%	33.3%
cdtC	33.3%	60%	33.3%

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Figures

Figure 1

Neighbour-joining phylogenetic tree of 17 *Campylobacter* isolates (all from faecal samples) based on *flaA* gene sequences. The bootstrap consensus tree was inferred from 1,000 replicates. Bar, 0.05% divergence. Phylogenetic analyses were conducted in MEGA 6.0.

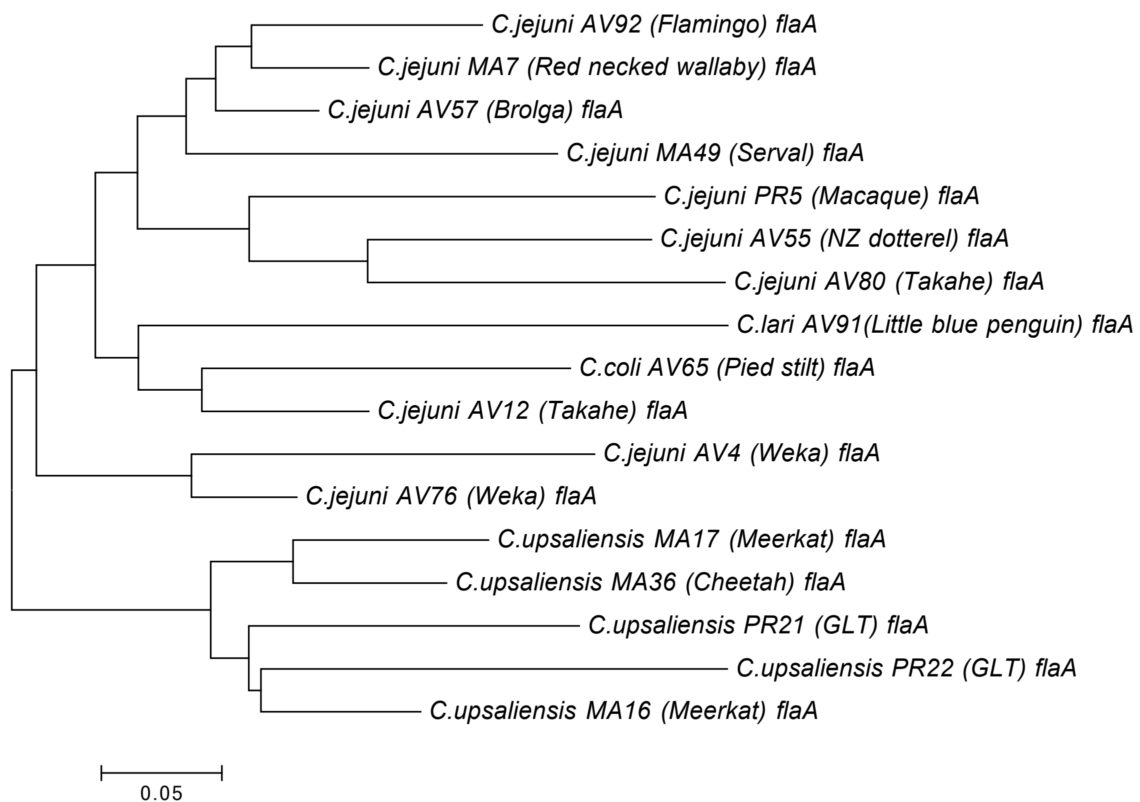

Figure 2

Neighbour-joining phylogenetic tree of 12 *Campylobacter* isolates (all from faecal samples) based on *gyrA* gene sequences. The bootstrap consensus tree was inferred from 1,000 replicates. Bar, 0.02% divergence. Phylogenetic analyses were conducted in MEGA 6.0.

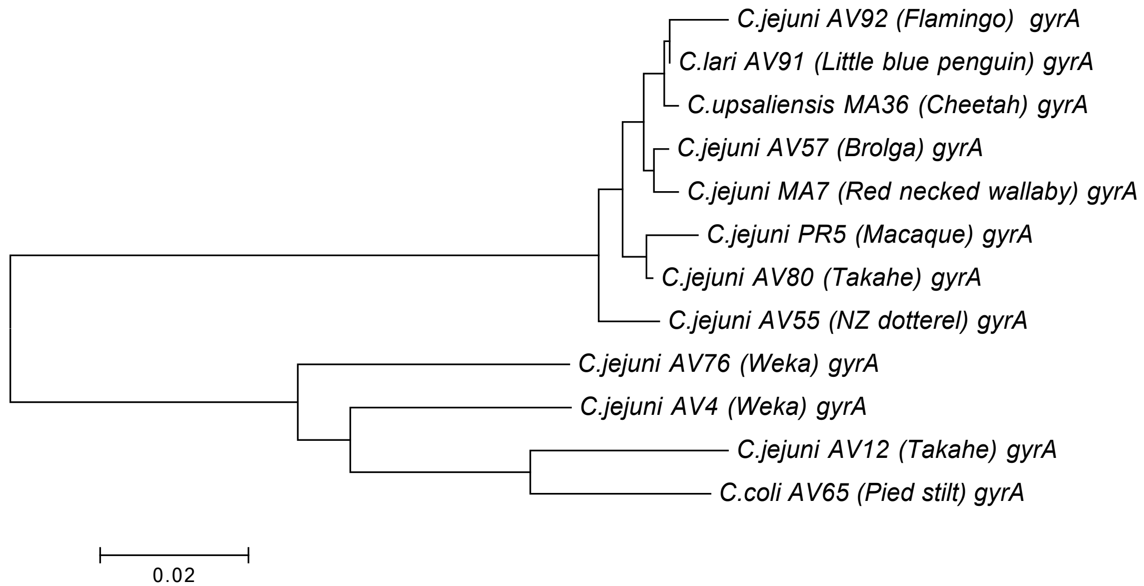

Figure 3

Neighbour-joining phylogenetic tree of 10 *Campylobacter* isolates (all from faecal samples) based on *cadF* gene sequences. The bootstrap consensus tree was inferred from 1,000 replicates. Bar, 0.02% divergence. Phylogenetic analyses were conducted in MEGA 6.0.

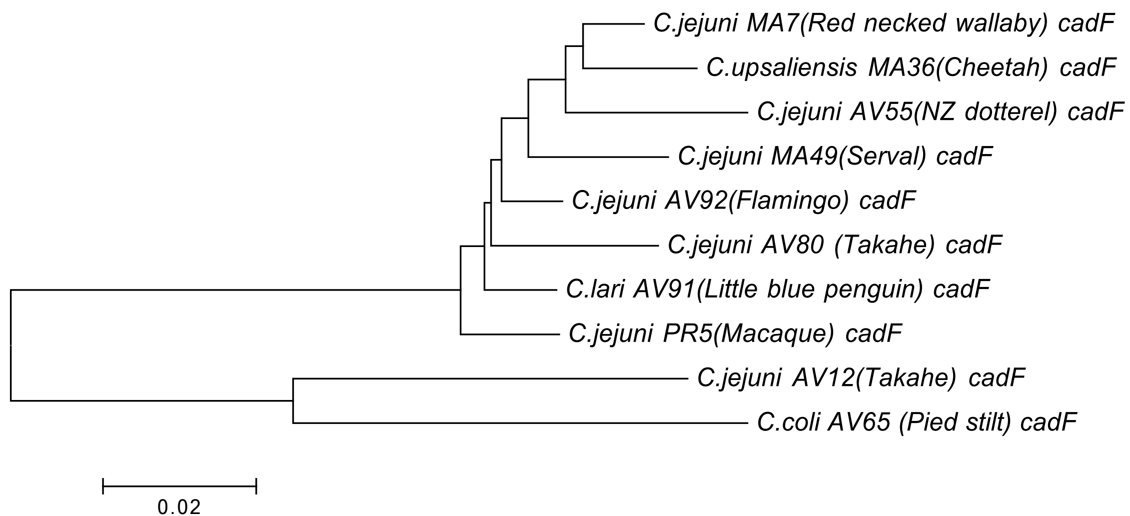

Figure 4

Neighbour-joining phylogenetic tree of 12 *Campylobacter* isolates (all from faecal samples) based on *cdtA* gene sequences. The bootstrap consensus tree was inferred from 1,000 replicates. Bar, 0.5% divergence. Phylogenetic analyses were conducted in MEGA 6.0.