Epidemiol. Infect. (2015), **143**, 3498–3509. © Cambridge University Press 2015
doi:10.1017/S0950268815000886

Comparison of epidemiologically linked *Campylobacter jejuni* isolated from human and poultry sources

S. A. LAJHAR[1,2,3], A. V. JENNISON[4], B. PATEL[2] AND L. L. DUFFY[1]*

[1] *CSIRO Animal, Food and Health Sciences, Coopers Plains, Brisbane, QLD, Australia*
[2] *School of Physical and Biomolecular Science, Griffith University, Brisbane, QLD, Australia*
[3] *Department of Laboratory Medicine; Faculty of Medical Technology, Derna, Libya*
[4] *Public Health Microbiology, Communicable Disease, Queensland Health, Forensic and Scientific Services, Brisbane, QLD, Australia*

Received 22 December 2014; Final revision 7 April 2015; Accepted 8 April 2015; first published online 4 May 2015

SUMMARY

Campylobacter jejuni is responsible for most foodborne bacterial infections worldwide including Australia. The aim of this study was to investigate a combination of typing methods in the characterization of *C. jejuni* isolated from clinical diarrhoeal samples ($n = 20$) and chicken meat ($n = 26$) in order to identify the source of infection and rank isolates based on their relative risk to humans. Sequencing of the *flaA* short variable region demonstrated that 86% of clinical isolates had genotypes that were also found in chicken meat. A polymerase chain reaction binary typing system identified 27 different codes based on the presence or absence of genes that have been reported to be associated with various aspects of *C. jejuni* pathogenicity, indicating that not all isolates may be of equal risk to human health. The lipooligosaccharide (LOS) of the *C. jejuni* isolates was classified into six classes (A, B, C, E, F, H) with 10·4% remaining unclassified. The majority (72·7%) of clinical isolates possessed sialylated LOS classes. Sialylated LOS classes were also detected in chicken isolates (80·7%). Antimicrobial tests indicated a low level of resistance, with no phenotypic resistance found to most antibiotics tested. A combination of typing approaches was useful to assign isolates to a source of infection and assess their risk to humans.

Key words: *Campylobacter*, clinical microbiology, poultry.

INTRODUCTION

Campylobacter spp. are recognized as the leading cause of foodborne bacterial enteritis in Australia with 90% of campylobacteriosis caused by *C. jejuni* [1, 2]. Point-source infection has been associated with exposure to various vehicles including red meat, poultry, water, milk and eggs [2, 3]. In Australia, campylobacteriosis is generally sporadic in nature and outbreaks have rarely been reported [2]. Despite the self-limiting nature of infection, antibiotic-resistant *Campylobacter* strains have been implicated in severe diarrhoea [4]. Fluoroquinolones and macrolides are used commonly for treatment of campylobacteriosis [5]. The increasing prevalence of fluoroquinolone-resistant *Campylobacter* isolates has been associated with the use of fluoroquinolones in food-producing animals particularly in poultry, suggesting transmission of resistance through the food chain to humans [6].

Furthermore, *C. jejuni* produces a cell surface structure termed lipooligosaccharide (LOS) and exhibits variation in the LOS outer core [7]. LOS plays an essential role in invasion of epithelial cells, evading immune

* Author for correspondence: Mrs L. L. Duffy, CSIRO Food and Nutrition Flagship, 39 Kessels Road, Coopers Plains, Australia 4108.
(Email: Lesley.Duffy@csiro.au)

response, and triggering Guillain–Barré syndrome (GBS) and Miller Fisher syndrome (MFS) [7]. Structural mimicry between sialylated LOS in the cell wall of *C. jejuni* and the human gangliosides in peripheral nerve tissue is thought to be essential in triggering the production of autoantibodies against human gangliosides [7]. Nineteen LOS loci classes have been identified [7]. Sialylated LOS classes (A, B, C) are ganglioside mimics [7]. In a polymerase chain reaction (PCR)-based study, Parker *et al.* [8] found that all GBS-associated *C. jejuni* and 64% of clinical (36 isolates) and environmental (13 chickens, 1 goat, 4 turkeys, 2 cows, 1 lamb) isolates belonged to sialylated LOS classes. Additionally, Godschalk *et al.* [9] showed that 62% of 21 isolates associated with enteritis-related *C. jejuni* possessed LOS classes A, B or C. To the best of our knowledge, the distribution of LOS classes from clinical and chicken-derived *Campylobacter* in Australia is unknown. In addition, the variability in sialylated LOS loci and its correlation with clinically associated strains has not been explored.

Several case-control studies have identified consumption of poultry contaminated with *C. jejuni* as the major cause of gastroenteritis in humans in Western countries including Australia [2, 6, 10]. LOS class variation in *C. jejuni* plays an important role as a major risk factor in human campylobacteriosis [11]. A plethora of methods have been utilized to discriminate between isolates, determine the prevalence of antimicrobial resistance (AMR), assess the risk of the strain to human health and infer the potential LOS structure associated with human illness. Each method has advantages and disadvantages depending on the purpose of the study [12].

In this study we characterized *Campylobacter* isolates from an epidemiologically linked cluster derived from clinical and chicken sources using PCR binary typing (P-BIT), flagellin A short variable region (*flaA*-SVR), AMR, and LOS. These isolates were then compared to non-epidemiologically linked isolates derived from chicken sources. Such information, will further our understanding of the population structure, genes implicated in pathogenicity, and AMR of Australian *C. jejuni*.

MATERIALS AND METHODS

Isolate collection

A total of 48 *C. jejuni* isolates from clinical and poultry origin, previously characterized using *flaA*-restriction fragment length polymorphism (RFLP) by the Queensland Public Health Microbiology Laboratory, Department of Health during a public health investigation, were included in this study. Of these 48 isolates, a total of 22 were isolated from different patients who suffered diarrhoea with or without blood and/or abdominal pain and/or fever (age range 2–78 years). The stool samples were routinely collected and sampled for *Campylobacter* by Public Health Microbiology laboratories between May and October 2011. Cases were spatially and temporally related and case-series investigations could not identify any common risk factors or exposures other than history of chicken consumption during the week before onset of illness. Isolates from two regions in Australia were included in the study. Region 1 is defined as the area with the ongoing (2011–2012) high incidence of campylobacteriosis cases causing concern to public health authorities, hence termed the outbreak region [approximately 180% above the 5-year mean (A. V. Jennison, personal communication)] and identified as having a single major chicken supplier. Region 2, geographically distinct from region 1, was not associated with any increase in campylobacteriosis prevalence and was supplied by numerous other poultry processors. Clinical isolates were obtained only from the outbreak region (region 1). Twenty-six isolates (15 isolates from outbreak region 1 and 11 isolates from the non-outbreak region) were isolated from raw chicken meat collected between June and November 2011 (during the outbreak period in region 1) by staff at Queensland Public Health from suppliers and retailers within the two regions in Australia.

Growth conditions and DNA extraction

Isolates were subcultured on charcoal cefoperazone deoxycholate agar (CCDA) (without antibiotics) (Oxoid, UK) at 42 °C for 48 h under 5% CO_2. The isolates were stored at -80 °C in Protect bacterial preservers (Technical Service Consultants Ltd, UK).

For DNA extraction, a loopful of bacteria was resuspended in 10 ml Nutrient Broth No. 2 (Oxoid, UK) and incubated at 42 °C for 48 h under 5% CO_2. *Campylobacter* cultures were transferred to 10 ml of Nutrient Broth No. 2 in a 10 ml centrifuge tube and incubated at 42 °C for 48 h under 5% CO_2. A 1 ml aliquot was centrifuged at 13 000 ***g*** for 3 min, resuspended in 200 μl TE buffer (10 mM Tris–HCl and 1 mM EDTA; pH.8), boiled for 10 min in a dry block heater (Thermo Fisher Scientific, Australia) before centrifuging at 13 000 ***g*** for 5 min. The supernatant was used as the DNA template. For all LOS analysis DNA was extracted using a genomic DNA purification kit

(Promega, USA) following the manufacturer's instructions and the DNA stored at 4 °C until required. Confirmation of genus and species was determined by a previously published PCR for the 16S rRNA gene [13], and species-specific *mapA* and *ceuE* genes.

P-BIT of putative virulence genes

P-BIT was performed as previously described by Cornelius *et al*. [14]. Briefly, the PCR reaction was performed under the following conditions and parameters: denaturation for 5 min at 94 °C, followed by 40 cycles of amplification, denaturation for 1 min at 94 °C, annealing for 1 min at 59 °C, extension for 1 min at 74 °C and a final extension at 74 °C for 8 min using Applied Biosystems Veriti™ Thermal Cycler (USA). The amplification mixture consisted of 2 μl template DNA (0·5 or 20 ng), 1× Dream Taq™ buffer (Thermo Fisher Scientific), 250 mM dNTPs (Thermo Fisher Scientific), 0·02 mg/ml bovine serum albumin (Sigma-Aldrich, USA), 12·5 pmol forward and reverse primers of epidemicity gene markers (GeneWorks; www.geneworks.com.au), and 1·25 U *Taq* DNA polymerase (GeneWorks). The PCR products were resolved on 2% agarose gel for 40 min at 100 V and presence of bands of the anticipated size for each target gene indicates a positive result. *Cj0008*, 245 bp; *Cj0122*, 250 bp; *Cj0265*, 175 bp; *panB* (*Cj0298c*), 150 bp; *Cj0423*, 148 bp; *cfrA* (*Cj0755*), 203 bp; *Cj1135*, 303 bp; *Cj1136*, 252 bp; *wlaN* (*Cj1139*), 252 bp; *CJE1500*, 545 bp; *Cj1321*, 249 bp; *maf5*/*pseE* (*Cj1337*) 146 bp; *gmhA2* (*Cj1424*) 450 bp; *flgE2* (*Cj1729c*), 555 bp; *CJE1733*, 447 bp. For further data analysis the results were loaded into Bionumerics version 6.5 (Applied Maths, Belgium) as binary data (0 for a PCR-negative result and 1 for a PCR-positive result). A similarity dendrogram was produced using simple matching coefficient and unweighted paired-group method with arithmetic mean values (UPGMA). The six figure P-BIT code was generated as previously described [14].

Due to multiple bands being generated in the PCR for two of the target genes, additional primers were designed using Primer 3 [15] and analysed using the same PCR conditions in P-BIT. Primer pairs targeting the arsenic-resistant gene *CJE1733* were designed from the genomic sequence of *C. jejuni* (Cam1733 F primer: CATTTTCCCCAATATCGCTTT and Cam1733 R: AAATCGCTCCATACCACCAC). Another primer pair was designed to target the *Cj1136* gene. The *Cj1136* gene in NCTC 11 168 was amplified using the forward primer Cam1136 F: TGGTGGTTTAA GTAGTGCTAGAAATG and the reverse primer Cam1136 R: TACCACCCCAGCTAAACGAG.

flaA-SVR sequencing

PCR for *flaA*-SVR sequencing was performed on the isolates according to the method of Meinersmann *et al*. [16]. Amplified products (25 μl) were electrophoresed before purifying using the Promega SV (Promega, USA) gel and PCR clean-up system in preparation for sequencing. Each sequencing mix contained 10–25 ng purified PCR product as measured spectrophotometrically using a Nanodrop 1000 (Thermo Fisher Scientific, Australia). Primers used for amplification were diluted to 5 pmol and added to 5 μl of purified DNA before submission to Macrogen (Korea) for sequencing. Based on the *Campylobacter flaA*-SVR database (http://pubmlst.org/campylobacter/flaA), the *flaA*-SVR allele number and peptide number were assigned to each isolate.

LOS classification and sequencing.

Previously published PCR primers were used to assign *C. jejuni* isolates into LOS classes [8, 17]. In this study, isolates were assigned to specific LOS classes based on the presence or absence of the 16 LOS biosynthesis genes and on the size of the LOS locus, as demonstrated by others [8, 17].

AMR

For *Campylobacter* spp. from both clinical and animal sources, the criteria for antimicrobial susceptibility testing and the assessments of the cut-off values are not standardized internationally, so in this study, breakpoints established by Clinical and Laboratory Standards Institute (CLSI) were used when available [18]. Otherwise National Antimicrobial Resistance Monitoring System (NARMS) criteria were utilized [19].

To determine the minimum inhibitory concentration (MIC) of each antimicrobial agent, all isolates were subcultured onto blood agar and tested for susceptibility to nine antimicrobials using the standard *Campylobacter* test plate from Sensititre™ (Thermo Fisher Scientific, UK) according to the manufacturer's protocol. The antimicrobials included were gentamicin, azithromycin, telithromycin, erythromycin, ciprofloxacin, nalidixic acid, tetracycline, florfenicol and clindamycin. The breakpoint listed for florfenicol is the susceptible breakpoint. Isolates that exceeded the MIC value of the

susceptible breakpoint were reported as non-susceptible [19]. *Campylobacter* strain ATCC 33560 was used as a control strain to verify the accuracy of the result [20].

Statistical analysis

The χ^2 test was used to compare the level of carriage of each gene and to compare the number of isolates of each LOS class within specific groups. Comparisons were made between poultry and human, outbreak- and non-outbreak-associated strains, and between geographical regions. All analysis was conducted using Minitab15 (Minitab Inc., USA) or Graphpad (http://graphpad.com) software. χ^2 test, with differences between isolates were considered significant at $P < 0{\cdot}05$.

RESULTS

Campylobacter identification

All isolates recovered on CCDA were confirmed to be *Campylobacter* spp. with expected amplicon sizes of 857 bp using PCR targeting the 16S rRNA gene. Species-specific *mapA* and *ceuE* genes indicated that the 48 isolates were *C. jejuni* (589 bp)

P-BIT of putative virulence genes

The relationships between *C. jejuni* were assessed by generating a cluster dendrogram of P-BIT data. The P-BIT data generated a total of five clusters (Fig. 1) at the 74% level. Clinical and chicken isolates were distributed in all of the dendrogram clusters except cluster V which contained only a single poultry isolate. Isolates from regions 1 and 2 were found in clusters I, II and III. Cluster III contained a single isolate from region 2. Only isolates from region 1 were found in clusters IV and V.

The prevalence of putative virulence genes in clinical and chicken isolates across both regions is demonstrated in Table 1. No significant difference ($P > 0{\cdot}05$) in the carriage of any gene was noted between clinical and chicken isolates; however, the carriage of some genes varied between regions. Isolates from region 1 had a significantly higher prevalence ($P < 0{\cdot}05$) of *Cj0265* and *tet(O)* compared to region 2 which had a significantly higher representation ($P < 0{\cdot}01$) of *Cj0008, gmhA2* and *CJE1733*. Interestingly, *flgE2* and *virB8* were not detected in any isolates regardless of source or region.

flaA-SVR

A total of 14 *flaA*-SVR nucleotide types were identified (Table 2). In the outbreak region (region 1), 10 genotypes were identified in clinical isolates and four in chicken while eight genotypes were detected in the non-outbreak region (region 2). The most commonly detected genotypes in region 1 were genotypes 9 and 67 which were identified in 53·3% and 33·3% of chicken isolates, respectively, whereas these genotypes were identified in 27·3% of clinical isolates.

Comparisons of *flaA*-SVR from human and chicken isolates indicated that a total of 19 (86·36%) out of 22 clinical isolates had *flaA-SVR* types indistinguishable from the chicken isolates from the two regions. Of these, three genotypes (67, 9, 21) were detected in chicken isolates from region 1 and four genotypes (18, 36, 52, 320) were detected in chicken isolates from region 2 (Table 2). Some genotypes were unique to a region or a source. For example, genotypes 67, 9 and 21, were unique to clinical and chicken isolates whereas genotypes 162, 222 and 16 were unique to chicken from region 2. Genotypes 146, 57 and 26 were identified within the outbreak region and were unique to human isolates.

Assignment of LOS into classes

Based on gene content 89·6% (43/48) of *C. jejuni* isolates were assigned into six classes (A, B, C, E, F, H) (Table 3). The remaining isolates (10·4%, 5/48) could not be assigned into a known LOS locus class based on the primers used in this study. *C. jejuni* of LOS classes A, E and H were significantly ($P < 0{\cdot}0001$) underrepresented compared to other classes. Of the total number of the tested isolates, prevalence of class C in region 1, 67·5% (25/37), was significantly ($P < 0{\cdot}0011$) higher than the prevalence of class C in region 2 (9·1%). Region 2 had a significantly ($P < 0{\cdot}0023$) higher prevalence of isolates with LOS class B than region 1. The representation of LOS classes in both clinical and chicken isolates was slightly different; however, there was no significant association between the source of the isolate and LOS locus class ($P > 0{\cdot}05$).

LOS classes *vs. flaA*-SVR

Sialylated LOS classes were distributed throughout the dendrogram and showed concordance with

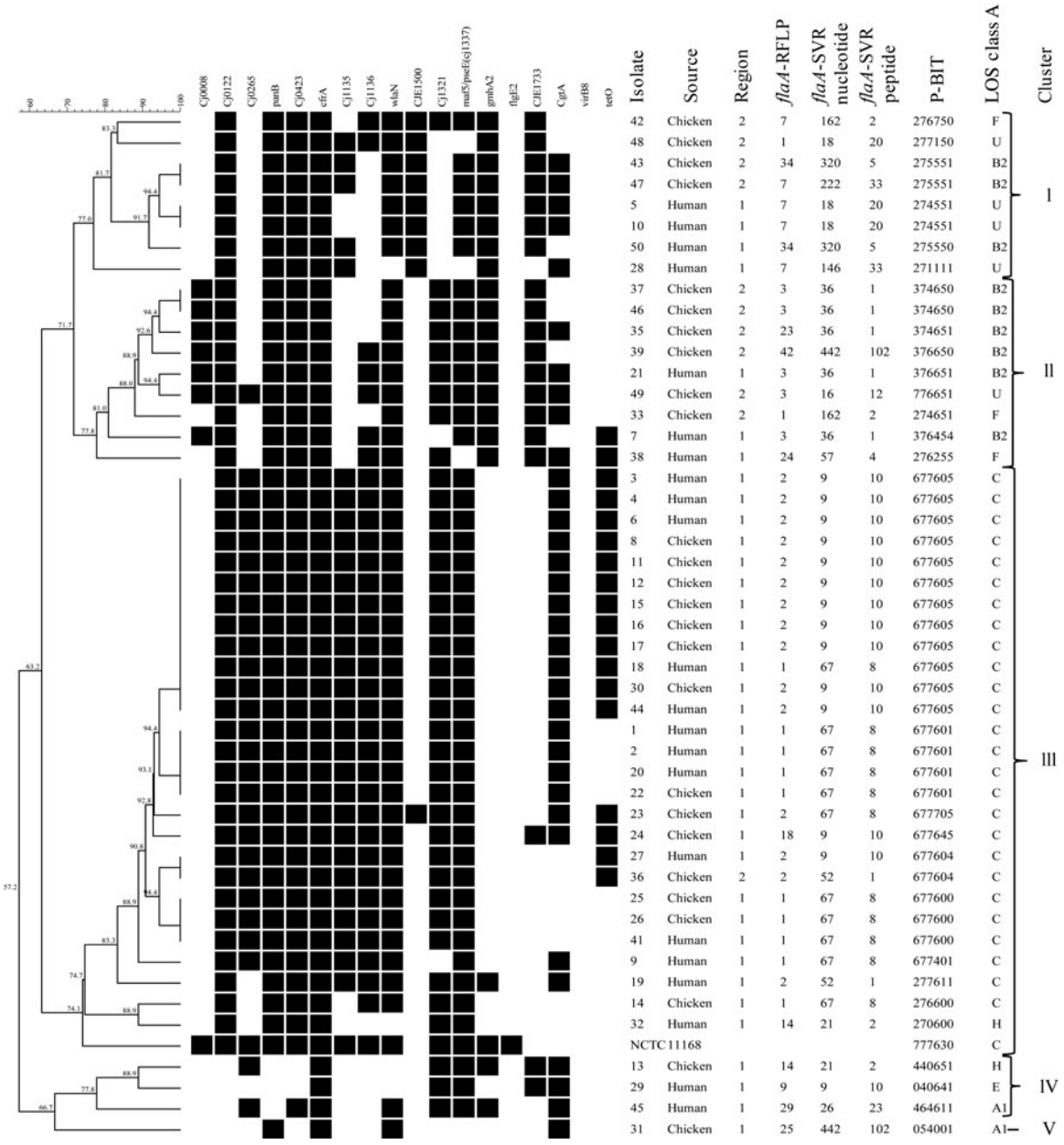

Fig. 1. Dendrogram generated using Bionumerics v. 6·5 (bionumerics.software.informer.com/6·5/) with simple matching coefficient and unweighted paired-group method with arithmetic mean values on the basis of P-BIT data. Columns following the P-BIT binary data are: 1, isolate number; 2, source; 3, region; 4, original *flaA*-RFLP; 5, *flaA*-SVR nucleotide; 6, *flaA*-SVR peptide; 7, P-BIT code; 8, LOS class A (U, unknown LOS classes); 9, cluster. Numbers on the branches of the dendrogram indicate the similarity level between isolates. P-BIT, PCR binary typing; RFLP, restriction fragment length polymorphism; LOS, lipooligosaccharide.

certain *flaA*-SVR type (Fig. 1). The majority of isolates (92·8 %, 13/14) possessing LOS class C were *flaA*-SVR 9 and 100% of isolates of *flaA*-SVR type 67 were LOS class C. LOS class H isolates were distributed into two clusters, both isolates were *flaA*-SVR type 21.

AMR

The AMR of the isolates is listed in Table 4. The overall prevalence of resistance to any antimicrobial was 6·3% (3/48). Most of the *C. jejuni* isolates (89·6%, 43/48) were susceptible to all antibiotics tested with the exception of five isolates; two unrelated

Table 1. *Association between epidemicity gene targets with* C. jejuni *isolates from human and chicken origin compared and isolates from regions 1 and 2 compared*

	Prevalence (%)			Region (%)		
Gene	Human ($N = 22$) *n* (%)	Chicken ($N = 26$) *n* (%)	*P* value	1 ($N = 37$) *n* (%)	2 ($N = 11$) *n* (%)	*P* value
Cj008	2 (9)	5 (19)	n.s.	2 (5·4)	5 (45)	0·0044
Cj0122	20 (90·9)	24 (92·3)	n.s.	33 (89)	11 (100)	n.s.
Cj0265	12 (54)	15 (57·6)	n.s.	25 (67·5)	2 (18)	0·005
panB	20 (90·9)	25 (96·1)	n.s.	34 (91·8)	11 (100)	n.s.
Cj0423	21 (95·4)	24 (92·3)	n.s.	34 (91·8)	11 (100)	n.s.
cfrA	22 (100)	26 (100)	n.s.	37 (100)	11(100)	n.s.
Cj1135	14 (63)	16 (61·5)	n.s.	26 (70)	4 (36·3)	n.s.
Cj1136	15 (68·1)	18 (69·2)	n.s.	28 (75·6)	5 (45)	n.s.
wlaN	19 (86·3)	25 (96·1)	n.s.	33 (89·1)	11 (100)	n.s.
CJE1500	4 (4)	5 (19·2)	n.s.	5 (13·5)	4 (36·3)	n.s.
Cj1321	16 (72·7)	22 (84·6)	n.s.	30 (81)	8 (72·7)	n.s.
Maf5/pseE	20 (90·9)	24 (92·3)	n.s.	34 (91·8)	10 (90·9)	n.s.
gmhA2	9 (40·9)	11 (42·3)	n.s.	10 (27)	10 (90·9)	0·0002
FlgE2	0	0	n.s.	0	0	n.s.
CJE1733	7 (31·8)	12 (46)	n.s.	9 (24)	10 (90·9)	0·0001
cgtA	17 (77)	17 (56·3)	n.s.	29 (78)	5 (45)	n.s.
virB8/comB	0	0	n.s.	0	0	n.s.
tetO	8 (36)	10 (38·4)	n.s.	17 (46)	1 (9)	0·0352

n.s., Not statistically significant.

Table 2. flaA-*SVR genotypes in region 1 (human and chicken) and region 2 (chicken only)*

	Prevalence (%)			Regions		
*fla*A-SVR	Human ($N = 22$) *n* (%)	Chicken ($N = 15$) *n* (%)	*P* value	1 ($N = 37$) *n* (%)	2 ($N = 11$) *n* (%)	*P* value
67	6* (27·3)	5 (33·3)	n.s.	11 (29·7)	0	0·0478
9	6 (27·3)	8 (53·3)	n.s.	14 (37·8)	0	0·0206
18	2 (9·1)	0	n.s.	2 (5·4)	1 (9·1)	n.s.
36	2 (9·1)	0	n.s.	2 (5·4)	3 (27·3)	n.s.
21	1 (4·5)	1 (6·6)	n.s.	2 (5·4)	0	n.s.
52	1 (4·5)	0	n.s.	1 (2·7)	1 (9·1)	n.s.
146	1 (4·5)	0	n.s.	1 (2·7)	0	n.s.
162	0	0	n.s.	0	2 (18·2)	n.s.
57	1 (4·5)	0	n.s.	1 (2·7)	0	n.s.
442	0	1 (6·6)	n.s.	1 (2·7)	1 (9·1)	n.s.
320	1 (4·5)	0	n.s.	1 (2·7)	1 (9·1)	n.s.
26	1 (4·5)	0	n.s.	1 (2·7)	0	n.s.
222	0	0	n.s.	0	1 (9·1)	n.s.
16	0	0	n.s.	0	1 (9·1)	n.s.

n.s., Not statistically significant.
* Number of isolates belonging to a particular genotype.

Table 3. *Comparisons of the distribution of lipooligosaccharide (LOS) classes from humans and chickens in regions 1 and 2 with expected LOS size*

		Prevalence (%)			Prevalence (%)		
LOS class	LOS size (kb)	Human ($N = 22$) n (%)	Chicken ($N = 26$) n (%)	P value	Region 1 ($N = 37$) n (%)	Region 2 ($N = 11$) n (%)	P value
C	13·49	12 (54·5)	14 (53·8)	n.s.	25 (67·6)	1 (9·1)	<0·0011
B2	12·46	3 (13·6)	6 (23·1)	n.s.	3 (8·1)	6 (54·6)	<0·0023
H	14	1 (4·5)	1 (3·8)	n.s.	2 (5·4)	0	n.s.
E	15·2	1 (4·5)	0	n.s.	1 (2·7)	0	n.s.
A1	11·47	1 (4·5)	1 (3·8)	n.s.	2 (5·4)	0	n.s.
F	7·8	1 (4·5)	2 (7·7)	n.s.	1 (2·7)	2 (18·2)	n.s.
Unknown 5, 10	13	2 (9·1)	0	n.s.	2 (5·4)	0	n.s.
Unknown 28	6·03	1 (4·5)	0	n.s.	1 (2·7)	0	n.s.
Unknown 48	12·7	0	1 (3·8)	n.s.	0	1 (9·1)	n.s.
Unknown 49	9·05	0	1 (3·8)	n.s.	0	1 (9·1)	n.s.

n.s., Not statistically significant.

isolates of chicken origin were non-susceptible to florfenicol, and two other chicken isolates and a single clinical isolate, were resistant to tetracycline.

DISCUSSION

P-BIT

P-BIT depends on the presence or absence of genes amplified by PCR analysis. The genes used in this study have been reported to be associated with various aspects of *C. jejuni* pathogenicity [14]. The P-BIT scheme examines the virulence gene profile of isolates, and may predict and classify the potential of a strain to cause serious disease. The P-BIT in the current study identified 27 different codes with three isolates from clinical and chicken samples from the outbreak region producing the P-BIT code 677600. This P-BIT code has been previously identified in the highly pathogenic *C. jejuni* strain SVS1425 suggesting that the isolates in the current study with P-BIT code 677600 may be capable of producing serious illness [14]. In a molecular risk assessment study for typing of *C. jejuni* using a P-BIT system, it was observed that analysis of the virulence profile of a strain compared to other isolates known to be associated with outbreak or severe illness could identify strains that have greater potential to cause serious illness [14]. NCTC 11168 was used as a positive control in this study and it was previously described as a high-risk *C. jejuni* strain [14, 21]. In this study, the strain NCTC 11168 was positive for 89·5% of the virulence genes, produced a P-BIT code 777630 and is located in cluster III. Although no single isolate in this study produced a P-BIT code identical to NCTC 11168, isolates located in the same cluster III may be more likely to cause severe illness than isolates in other clusters. Although the set of clinical isolates used in this study were isolated from patients who have diarrhoea with or without blood and/or abdominal pain and/or fever, no further information about the severity of the gastrointestinal symptoms or complications that may have developed later is available. It is therefore not possible to evaluate the prediction that isolates within cluster III will lead to more severe disease than isolates in other clusters.

The P-BIT system is based on the presence or absence of genes, as tested by PCR analysis, which have been implicated as markers of epidemicity. As such the system is dependent on factors such as primer design. Primers are designed to amplify specific sequence orientation of a target gene, so variation in the target gene sequence at the primer annealing site could affect the reliability of the PCR result [14]. For instance, the hook protein-encoding gene *flgE2* was not detected in any of the *C. jejuni* isolates. One explanation is the high variability in *flgE2* sequence seen in *C. jejuni* of the same strains and even serotype, as reported previously, could have affected the PCR result [22]. In addition, the published PCR primers for the *CJE1733* gene, which is responsible for conferring resistance to arsenic compounds that are given to

Table 4. *Minimum inhibitory concentration (MIC) distribution of antimicrobial agents for* Campylobacter *spp. isolated from human and poultry*

Class*	Antimicrobial, breakpoints (μg/ml)	Range of tested antimicrobials with number and percentage of isolates inhibited by MIC (μg/ml)												
Rank I†														
Amino glycosides	Gentamicin, GEN			$\leqslant$0·12	0·25	0·5	1	2	4	8	16	32	>32	
	$\geqslant$8			0	5 (10·4)	37 (77·1)	5 (10·4)	1 (2·1)	0	0	0	0	0	
Macrolides and ketolides	Azithromycin, AZI	$\leqslant$0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	32	64	>64
	$\geqslant$8	12 (25)	24 (50)	6 (12·5)	3 (6·3)	2 (4·2)	1 (2·1)	0	0	0	0	0	0	0
	Telithromycin, TEL	$\leqslant$0·03	0·06	0·12	0·25	0·5	1	2	4	8	>8			
	$\geqslant$8	0	1 (2·1)	1 (2·1)	11 (22·9)	19 (39·6)	11(22·9)	3 (6·3)	2 (4·2)	0	0			
	Erythromycin, ERY	$\leqslant$0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	32	64	>64
	$\geqslant$32	1 (2·1)	0	6 (12·5)	20 (417)	14 (29·2)	6 (12·5)	1 (2·1)	0	0	0	0	0	0
Quinolones	Ciprofloxacin, CIP	$\leqslant$0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	32	64	>64
	$\geqslant$4	12 (25)	17 (35·4)	15 (31·3)	4 (8·3)	0	0	0	0	0	0	0	0	0
	Nalidixic acid, NAL								$\leqslant$4	8	16	32	64	>64
	$\geqslant$64								38 (79·2)	9 (18·8)	1 (2·1)	0	0	0
Rank II														
Tetracyclines	Tetracycline, TET		$\leqslant$0·06	0·12	0·25	0·5	1	2	4	8	16	32	64	>64
	$\geqslant$16		1 (2·1)	16 (33·3)	22 (45·8)	3 (6·3)	3 (6·3)	0	0	0	0	1 (2·1)	2 (4·2)	0
Phenicols	Florfenicol‡, FFN	$\leqslant$0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	32	64	>64
	$\geqslant$8	0	0	0	1 (2·1)	5 (10·4)	30 (62·5)	8 (16·7)	2 (4·2)	1 (2·1)	1 (2·1)	0	0	0
Rank III														
Lincosamides	Clindamycin, CLI	$\leqslant$0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	>16		
	$\geqslant$8	1 (2·1)	8 (16·7)	21 (43·8)	13 (27·1)	34 (8·3)	1 (2·1)	0	0	0	0	0		

Vertical lines indicate breakpoints for resistance.

* CLSI subclass; CLSI M100 document.

† Rank: WHO categorization of critical antimicrobials in human health (rank I, critically important; rank II, highly important; rank III, important).

‡ For florfenicol, only a susceptible breakpoint ($\leqslant$4 μg/ml) has been established. In this study, isolates with an MIC $\geqslant$8 μg/ml are categorized as non-susceptible.

chickens as a growth promoter and antiparasitic medication, produced non-specific PCR products. The plasmid-encoded *virB8* [23] was not detected in any isolates suggesting that in addition to the primer designation, pVir plasmid may be absent in all the isolates included in this study.

flaA-SVR

flaA-SVR revealed the genetic diversity of *C. jejuni* isolates included in this study. Variation in *flaA*-SVR could be due to mutations or recombination events [12, 24]. *flaA*-SVR demonstrated that 86·4% (19/22) of the clinical isolates had genotypes that were also found in chicken from both regions, which suggests that, while the sample size can be considered small, clinical isolates in this study were related to chicken which supports the importance of poultry as a potential source of campylobacteriosis. It was also observed that six chicken isolates epidemiologically unrelated to the outbreak region had *flaA*-SVR types indistinguishable from that of outbreak clinical isolates. These genotypes were 18, 36, 52 and 320, of which 36 and 52 were combined in a single peptide type 1 due to the redundancy of the genetic code [25]. It is possible that some of the indistinguishable genotypes across both regions represent a common genotype found in chickens. This suggestion could be further confirmed by more extensive testing of poultry *Campylobacter* isolates epidemiologically unrelated to the outbreak region and examination of other sources such as cattle and wild birds. Similar findings were demonstrated by Clark *et al.* [12] who found that several *Campylobacter* strains had a *Sma*I pulsed-field gel electrophoresis (PFGE) pattern, *Kpn*I PFGE pattern, *fla*-RFLP pattern and *flaA*-SVR types that were indistinguishable from the Walkerton waterborne outbreak types although they were epidemiologically, temporally and geographically unrelated to the outbreak.

Assignment of LOS into classes

C. jejuni is characterized by high variability in the LOS structure with sialylated LOS classes (A, B or C) of many *C. jejuni* having structural mimicry with human gangliosides in peripheral nerve tissue [8, 9]. This is thought to be essential in triggering the production of autoantibodies against human gangliosides and the development of post-infectious complications [8, 9]. In this study, characterization of *C. jejuni* isolates utilizing 16 published PCR primers to target the cell surface structure LOS confirmed the diversity of the *C. jejuni* population. Based on gene content and locus size, six classes (A, B, C, E, F, H) were identified in 89·6 % (43/48) of the *C. jejuni* isolates with 77% (37/48) of *C. jejuni* isolates capable of producing ganglioside mimics (classes A, B, C). The other loci identified in our study (E, F, H) lack genes necessary to synthesize sialylated LOS. In this study, the prevalence of sialylated LOS class B (18·7%, 9/48) and class C (54·2 %, 26/48) was higher than other classes. The representation of sialylated LOS classes compared to the non-sialylated LOS classes was highly significant (P = 0·0002) in clinically associated strains. When comparing chicken and clinical isolates, there was no difference in the prevalence of strains capable of producing ganglioside mimics (Table 3). The prevalence of sialylated LOS isolates from clinical and chicken origin is slightly higher (77%) than that found by Parker *et al.* [8] who demonstrated that the prevalence of sialylated LOS in non-GBS-associated isolates was 64% (35/55). A US study of *Campylobacter* from broilers also found approximately 60% carried LOS classes A, B or C [26]. Although is not clear why the incidence of ganglioside-mimicking LOS classes producing strains is high, results suggest that that the production of sialylated LOS classes alone is not sufficient to proceed to further complication such as GBS and MFS; clearly, other host and/or bacterial factors are required [8]. In addition, 10·4% (5/48) of isolates could not be classified into a known LOS class by PCR screening of the 16 genes or in combination with the length of the LOS loci through the LOS XL PCR. The genetic composition of the LOS loci of two isolates with unknown LOS class was predicted to be class J based on the published primers and the length of the LOS loci (13 kb). However, similarity in the gene content and order between the sequence for class J and other classes was reported, despite differences in the size of the XL PCR products. Class J is related to class F but it is divergent from class F in that *orf16* is deleted and replaced by *orf5* [7]. Therefore, additional genes in the two isolates could be targeted by designing additional primers before assigning these isolates into class J.

Relationship between P-BIT, LOS, *flaA*-SVR

In this study, it is not known if any epidemiologically related isolate from patients afflicted with enteritis went on to develop complications such as GBS or MFS. Genes that are overrepresented in genotypes

associated with human illness were previously chosen to develop the P-BIT typing system that has potential for strain risk ranking [14]. Cluster III contained isolates that produced P-BIT codes previously produced in virulent strains such as P- BIT code 677600 and 777630. At the same time, 96·3% (26/27) of *C. jejuni* isolates located in cluster III harbour sialylated LOS class C. It is not clear why all isolates expressing LOS class C were located in cluster III. There may be a relationship between genes included in the P-BIT system and expression of LOS class C, although a single strain of LOS class H was also in cluster III. The isolate possessing non-sialylated LOS class H shares the presence of a number of genes with isolates expressing sialylated LOS class C except genes involved in sialylation of LOS biosynthesis loci such as *Cj1136* and *wlaN*.

In addition, in 91·6% of isolates, *flaA*-SVR typing showed concordance with LOS classes. For example, 100% of isolates belonging to the *flaA*-SVR 36 and *flaA*-SVR 320 genotype possessed LOS class B and 100% of isolates of *flaA*-SVR genotypes 67 or 9 expressed LOS class C and 100% of isolates of *flaA*-SVR genotypes 21 have LOS class H. This suggests that *flaA*-SVR may be able to predict LOS classes although the sample size is small and a larger number of isolates should be investigated.

AMR

In general, the prevalence of AMR in *C. jejuni* isolates from clinical and poultry origin was low (6·3%, 3/48). The absence of resistance to gentamicin, azithromycin, telithromycin, erythromycin, ciprofloxacin, nalidixic acid and clindamycin in all of the clinical and poultry isolates may reflect the restriction on the use of antimicrobials in food-producing animals and the infrequent use of antimicrobials in humans for treatment of *Campylobacter* infection in Australia. One of the Australian studies that utilized the microdilution method using Sensititre reported that of the total 105 *C. jejuni* from poultry origin, 1·7% were resistant to clindamycin, 3·3% to erythromycin, 3·3% telithromycin and 1·7% to tetracycline and no resistance was observed to ciprofloxacin, florfenicol, gentamicin or nalidixic acid [27]. Another recent study reported that of 20 *Campylobacter* isolates, only a single strain was resistant to nalidixic acid and another one resistant to tetracycline [28]. This and other Australian studies increase the acceptance that AMR in *C. jejuni* in Australia is low.

In the current study, overall phenotypic resistance to tetracycline was 6·3 % (two chicken isolates from region 2 and one clinical isolate from region 1). However, a total of 18/48 (37·5%) isolates carried the plasmid-encoded tet(O) gene [14]. While a positive result was recorded for the PCR analysis, the gene may not be functional or alternatively only a small portion of the gene may be present that may align with the utilized primers.

Non-susceptibility to florfenicol which is a synthetic, fluorinated analogue of chloramphenicol [29] was detected in two chicken isolates, one from region 1 and another from region 2 (6·7% and 9·1%, respectively). This drug is not licensed for use in chickens in Australia and is only approved for use in production of cattle and swine. However, molecular testing is required to further explain the result. In an American study, utilizing the disc diffusion method, resistance to florfenicol was detected in 4% of *Escherichia coli* isolated from sick chickens although this drug was not used in poultry [29]. Molecular typing revealed that the florfenicol resistance gene, *flo*, was independently acquired and is plasmid encoded [29]. However, comparison of the current results with others is difficult since the sample collection, bacterial isolates, and the chosen susceptibility method is different and the Australian data on the level of antibiotic resistance of *Campylobacter* is limited [30, 31].

CONCLUSION

Despite the small sample size, a combination of typing methods, *flaA*-SVR and P-BIT support that contact with raw or consumption of undercooked chicken is one of the important sources of campylobacteriosis and evaluated the risk of strains to humans. The present study is the first study in Australia to examine the LOS class diversity in *C. jejuni* isolated from poultry and clinical samples. There was a high incidence of sialylated LOS classes in clinical diarrhoeal samples compared to the non-sialylated LOS classes with no significant differences found in the distribution of sialylated LOS classes in clinical and chicken samples. This could be considered as further evidence and a warning signal for the importance of poultry as potential vehicles of campylobacteriosis and a risk factor in campylobacteriosis preceding neuropathy. Furthermore, results presented here suggest that *flaA*-SVR could predict LOS classes, although further studies should be conducted to determine the epidemiological relevance of this finding with a larger

sample size. Additional characterization using AMR tests identified a low-level of AMR consistent with other published data.

ACKNOWLEDGEMENTS

This project was funded by CSIRO. S. A. Lajhar was funded by the National Board of Technical and Vocational Education, Libya/Department of Laboratory Medicine Derna, Libya (grant no. 2008-628-1491). The authors acknowledge the Queensland Public Health Units, Queensland Department of Health Communicable Disease Branch and Queensland OzFoodNet for the outbreak surveillance data, analysis and case-series investigations. The authors thank all private and pathology laboratories in Queensland who forwarded isolates to the Public Health Microbiology Laboratory, Department of Health during the investigation.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Tenkate T, Stafford R, McCall B.** A five year review of *Campylobacter* infection in Queensland. *Communicable Disease Intelligence* 1996; **20**: 478–482.
2. **O'Reilly LC, Inglis TJJ, Unicomb L.** Australian multicentre comparison of subtyping methods for the investigation of *Campylobacter* infection. *Epidemiology and Infection* 2006; **134**: 768–779.
3. **Nadeau E, Messier S, Quessy S.** Comparison of *Campylobacter* isolates from poultry and humans: association between in vitro virulence properties, biotypes, and pulsed-field gel electrophoresis clusters. *Applied and Environmental Microbiology* 2003; **69**: 6316–6320.
4. **Nelson JM, *et al.*** Prolonged diarrhea due to ciprofloxacin-resistant *Campylobacter* infection. *Journal of Infectious Diseases* 2004; **190**: 1150–1157.
5. **Alfredson DA, Korolik V.** Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiology Letters* 2007; **277**: 123–132.
6. **Gupta A, *et al.*** Antimicrobial resistance among *Campylobacter* strains, United States, 1997–2001. *Emerging Infectious Diseases* 2004; **10**: 1102–1109.
7. **Parker CT, *et al.*** Characterization of lipooligosaccharide-biosynthetic loci of *Campylobacter jejuni* reveals new lipooligosaccharide classes: evidence of mosaic organizations. *Journal of Bacteriology* 2008; **190**: 5681–5689.
8. **Parker CT, *et al.*** Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from a variety of sources. *Journal of Clinical Microbiology* 2005; **43**: 2771–2781.
9. **Godschalk PCR, *et al.*** The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain–Barre syndrome. *Journal of Clinical Investigation* 2004; **114**: 1659–1665.
10. **Stafford RJ, *et al.*** Population-attributable risk estimates for risk factors associated with *Campylobacter* infection, Australia. *Emerging Infectious Diseases* 2008; **14**: 895–901.
11. **Habib I, *et al.*** Correlation between genotypic diversity, lipooligosaccharide gene locus class variation, and caco-2 cell invasion potential of *Campylobacter jejuni* isolates from chicken meat and humans: contribution to virulotyping. *Applied and Environmental Microbiology* 2009; **75**: 4277–4288.
12. **Clark CG, *et al.*** Use of the oxford multilocus sequence typing protocol and sequencing of the flagellin short variable region to characterize isolates from a large outbreak of waterborne *Campylobacter sp*. strains in Walkerton, Ontario, Canada. *Journal of Clinical Microbiology* 2005; **43**: 2080–2091.
13. **Denis M, *et al.*** Development of a m-PCR assay for the simultaneous identification of *Campylobacter jejuni* and *C*. coli. *Letters in Applied Microbiology* 1999; **29**: 406–410.
14. **Cornelius AJ, *et al.*** Comparison of PCR binary typing (P-BIT), a new approach to epidemiological subtyping of *Campylobacter jejuni*, with serotyping, Pulsed-field gel electrophoresis, and multilocus sequence typing methods. *Applied and Environmental Microbiology* 2010; **76**: 1533–1544.
15. **Rozen S, Skaletsky H.** Primer3 on the www for general users and for biologist programmers. In: Misener S, Krawetz S, eds. *Bioinformatics Methods and Protocols*: Humana Press, 1999, pp. 365–386 (Methods in Molecular Biology.) New Jersey, USA: Humana Press Inc.
16. **Meinersmann RJ, *et al.*** Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. *Journal of Clinical Microbiology* 1997; **35**: 2810–2814.
17. **Hotter GS, Li IH, French NP.** Binary genomotyping using lipooligosaccharide biosynthesis genes distinguishes between *Campylobacter jejuni* isolates within poultry-associated multilocus sequence types. *Epidemiology and Infection* 2010; **138**: 992–1003.
18. **CLSI.** Performance Standards for Antimicrobial Susceptibility Testing; Twenty-first Informational Supplement. CLSI document M100-S21: Wayne, PA.; 2011.
19. **USDA.** National Antimicrobial Resistance Monitoring System – enteric bacteria. NARMS 2011 annual report. Athens, GA: USA, 2011.
20. **Bywater R, *et al.*** A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. *Journal of Antimicrobial Chemotherapy* 2004; **54**: 744–754.
21. **Gaynor EC, *et al.*** The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *Journal of Bacteriology* 2004; **186**: 503–517.

22. **Luneberg E, *et al.*** The central, surface-exposed region of the flagellar hook protein FlgE of *Campylobacter jejuni* shows hypervariability among strains. *Journal of Bacteriology* 1998; **180**: 3711–3714.
23. **Bacon DJ, *et al.*** Involvement of a plasmid in virulence of *Campylobacter jejuni* 81–176. *Infection and Immunity* 2000; **68**: 4384–4390.
24. **Sails AD, Swaminathan B, Fields PI.** Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by *Campylobacter jejuni*. *Journal of Clinical Microbiology* 2003; **41**: 4733–4739.
25. **Wassenaar TM, *et al.*** Comparison of *Campylobacter fla*-SVR genotypes isolated from humans and poultry in three European regions. *Letters in Applied Microbiology* 2009; **49**: 388–395.
26. **Hardy CG, *et al.*** Prevalence of potentially neuropathic *Campylobacter jejuni* strains on commercial broiler chicken products. *International Journal of Food Microbiology* 2011; **145**: 395–399.
27. **Barlow R, Gobius KG.** Pilot survey for antimicrobial resistant (AMR) bacteria in Australian food. Australian Government Department of Health and Ageing, 2008.
28. **Wieczorek K, *et al.*** Antimicrobial resistance and genetic characterization of *Campylobacter* spp. from three countries. *Food Control* 2013; **34**: 84–91.
29. **Keyes K, *et al.*** Detection of florfenicol resistance genes in *Escherichia coli* isolated from sick chickens. *Antimicrobial Agents and Chemotherapy* 2000; **44**: 421–424.
30. **Unicomb LE, *et al.*** Low-level fluoroquinolone resistance among *Campylobacter jejuni* isolates in Australia. *Clinical and Infectious Diseases* 2006; **42**: 1368–1374.
31. **Mattheus W, *et al.*** Trend analysis of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from Belgian pork and poultry meat products using surveillance data of 2004–2009. *Foodborne Pathogens and Disease* 2012; **9**: 465–472.