Virulence properties of *Campylobacter jejuni* isolates of poultry and human origin

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Campylobacter jejuni is one of the leading causes of food-borne gastroenteritis. Because of the high prevalence of *C. jejuni* in poultry, poultry meat is considered a major source of *C. jejuni* infections for humans. However, it is not known whether all poultry-associated *C. jejuni* strains are capable of causing disease in humans. Four different virulence properties of *C. jejuni* strains were compared between 20 poultry isolates and 24 human isolates. Strains were chosen based on their PFGE pattern to represent a heterogeneous population. The isolates were compared for their ability to invade and induce interleukin-8 (IL-8) production in T84 cells, their production of functional cytolethal distending toxin (CDT) using HEp-2 cells, and their sodium deoxycholate resistance. All four virulence factors were present among strains of human and poultry origin, with strong differences observed among strains. For invasion and IL-8 induction, no difference was observed between the two populations. However, on average, human isolates arrested more HEp-2 cells in their cell cycle than did the poultry isolates ($P=0.041$), suggesting higher CDT production by the former. The ability to survive 16 000 µg sodium deoxycholate ml⁻¹ was significantly more pronounced ($P=0.006$) among human isolates than poultry isolates, although all strains possessed the *cmeABC* operon. These data suggest that all four virulence properties are widespread among *C. jejuni* isolates, but that a higher degree of bile-salt resistance and more pronounced CDT production are associated with strains causing enteritis in humans.

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INTRODUCTION

Campylobacter jejuni is recognized as one of the most common causes of human gastrointestinal disease in industrialized countries, and is frequently associated with the handling and consumption of contaminated poultry meat and cross-contaminated food products (Hänninen *et al.*, 2000; Pearson *et al.*, 2000; Wingstrand *et al.*, 2006). A study in the UK showed that 80 % of retail poultry is contaminated with *C. jejuni* (Corry & Atabay, 2001), and approximately 39 % of the broiler flocks tested in a Belgian study conducted during 1998 and 2000 were positive for *C. jejuni* (Herman *et al.*, 2003). This emphasizes the

importance of poultry as a reservoir and source of *C. jejuni* infection.

Several virulence factors are considered to be important for the induction of gastroenteritis, such as resistance to bile salts (Lin *et al.*, 2003), invasion of epithelial cells (Russell *et al.*, 1993) and cytolethal distending toxin (CDT) production (Konkel *et al.*, 2001). Cell invasion could result in cellular injury, leading to reduced absorptive capacity of the intestine, whereas CDT production is important for interleukin-8 (IL-8) release by intestinal cells *in vitro* (Hickey *et al.*, 1999, 2000) and thus plays an important role in the host mucosal inflammatory response.

Although poultry meat is considered an important source of *C. jejuni* infections for humans, it is not known whether all poultry-associated *C. jejuni* strains are capable of

Abbreviations: CDT, cytolethal distending toxin; IL-8, interleukin-8; TNF, tumour necrosis factor.

causing disease in humans. We therefore compared the capability for epithelial cell invasion, CDT production, bile-salt resistance and IL-8 induction of poultry and human isolates.

METHODS

Experimental animals. Day-of-hatch White Leghorn Chickens (Charles River Laboratories) were kept in brooder batteries and provided with food and water *ad libitum*. Husbandry, euthanasia methods, experimental procedures and biosafety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University. Prior to use, chicks were screened for the presence of *Campylobacter* in the faeces. All of the chicks tested proved to be negative for *Campylobacter*.

Bacterial strains. In this study, 20 *C. jejuni* strains of poultry origin and 24 clinical isolates (kindly provided by Professor Peter Vandamme, Ghent University) were used. The choice of strains was based on their genotypic heterogeneity, which was determined by PFGE with *KpnI* and *SmaI* (Amersham Biosciences) according to the PulseNet USA protocol (http://www.cdc.gov/pulsenet/protocols/campy_protocol.pdf). PFGE profiles were clustered with BioNumerics software (version 4.0, Applied Maths) using the Dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm. PFGE clusters were defined on a similarity level of 80 %. All strains were routinely cultured in Nutrient Broth No. 2 (Oxoid), supplemented with *Campylobacter*-specific growth supplements (SR117 and SR0232, Oxoid) and 5 % lysed horse blood (E&O Laboratories) at 42 °C under microaerobic conditions (5 % O₂, 5 % CO₂, 5 % H₂, 85 % N₂). For certain experiments, horse blood was omitted. Care was taken to pass the strains no more than five times *in vitro*.

Before use in virulence assays, all *C. jejuni* strains were passed through day-old specific pathogen-free (SPF) White Leghorn Chickens. Chicks were orally inoculated with 2×10^7 organisms in 0.2 ml PBS. Controls were sham-inoculated with PBS alone. At 24 h post-inoculation, cloacal swabs were streaked on modified charcoal cefoperazone deoxycholate agar plates (mCCDA; CM0739, Oxoid) containing *Campylobacter*-specific growth supplements (SR0155 and SR0232, Oxoid) for 48 h at 42 °C under microaerobic conditions. Bacteria were collected by washing plates with PBS, and the aliquoted suspension was stored in horse blood at -80 °C.

For the CDT assay, bacterial cell lysates were prepared by sonication. Bacterial cultures were grown in 40 ml selective broth with 5 % lysed horse blood for 19 h at 42 °C in a microaerobic atmosphere to a density of 5×10^7 c.f.u. ml⁻¹ and harvested by centrifugation at 900 g for 20 min at 4 °C. The bacteria were washed once with 3 ml PBS and resuspended in 3 ml PBS. The pellet was sonicated on ice by eight 30 s pulses with an XL 2015 Sonicator (Misonix) followed by centrifugation at 6000 g for 10 min at 4 °C. The supernatant was filter-sterilized by subsequent passage through 0.45 and 0.20 µm pore-size filters (IWAKI, International Medical) and stored at -80 °C.

Cell lines. The HEp-2 cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in Eagle's Minimum Essential Medium (EMEM; Gibco, Invitrogen) supplemented with 10 % fetal calf serum, 1 % glutamine, 1 % non-essential amino acids and, unless stated otherwise, 100 U penicillin ml⁻¹ and 0.1 mg streptomycin ml⁻¹ (Gibco) at 37 °C in a 5 % CO₂ atmosphere.

The human colon carcinoma cell line T84 (ECACC) was grown in 44 % Dulbecco's Minimal Essential Medium (DMEM), 44 % F-12

(Gibco), 10 % fetal calf serum (Integro), 1 % L-glutamine, 100 U penicillin ml⁻¹ and 0.1 mg streptomycin ml⁻¹ at 37 °C in a 5 % CO₂ atmosphere.

Invasion of T84 cells by *C. jejuni* strains of poultry and human origin. T84 cells were seeded in 96-well plates at 1×10^5 cells per well. After 78 h, the cells were inoculated with bacterial cells grown overnight in broth without horse blood at an m.o.i. of 200. The plates were centrifuged at 500 g for 10 min at 37 °C to enhance bacterial contact with the cell monolayer. The plates were incubated for 3 h at 37 °C in a 5 % CO₂ atmosphere. Cells were washed with Hanks' Buffered Salt Solution (HBSS; Gibco) with Ca²⁺ and Mg²⁺ to remove non-invaded bacteria. To kill extracellular bacteria, 100 µg gentamicin ml⁻¹ (Gibco) was added to the wells. This concentration proved to be lethal for all strains tested. Plates were further incubated for 2 h at 37 °C in a 5 % CO₂ atmosphere. Gentamicin was washed away with HBSS without Ca²⁺ and Mg²⁺. Intracellular bacteria were released from T84 cells with 0.25 % sodium deoxycholate and titrated on mCCDA plates.

CDT production by poultry and human isolates. The CDT production of *C. jejuni* isolates was determined by the assessment of the number of HEp-2 cells blocked in the G₂ phase after exposure to *C. jejuni* lysate (Young *et al.*, 2000). The DNA content of HEp-2 cells incubated with the *Campylobacter* cell sonicates was determined quantitatively by flow cytometry. HEp-2 cells were seeded at a density of 1×10^5 cells ml⁻¹ in 25 ml culture flasks. The cells were treated with 100 µl bacterial sonicate and incubated for 72 h at 37 °C with 5 % CO₂. Sonicates from *C. jejuni* NCTC 11168 were used as a positive control (Purdy *et al.*, 2000). Negative controls were treated likewise, but incubated with 100 µl PBS. After incubation, cells were washed once with medium without antibiotics and trypsinized. The cells were collected in a Falcon tube and pelleted by centrifugation at 1000 g for 5 min at 37 °C. DNA was stained according to Chien *et al.* (2000). Briefly, the pellet was resuspended in 0.5 ml staining solution (3 % PEG, 4 µg propidium iodide ml⁻¹, 9 U RNase A ml⁻¹, 0.1 % Triton X-100, 0.0001 % BSA, in 4 mM sodium citrate) for 20 min at 37 °C, followed by the addition of 0.5 ml salt solution (3 % PEG; 4 µg propidium iodide ml⁻¹, 9 U RNase A ml⁻¹, 0.1 % Triton X-100, 0.0001 % BSA, in 0.4 M NaCl) and the mixture was stored for 1.5 h at 4 °C in the dark. The DNA content was analysed using a FACSCalibur flow cytometer (Becton-Dickinson) and data acquisition was performed using CellQuest software. For each experiment, 10⁴ cells were counted.

Presence of the *virB11*, *cdtA*, *cdtB* and *cdtC* genes and the CmeABC multidrug efflux pump in human and poultry isolates of *C. jejuni*. The presence of the *virB11* gene as a marker for the plasmid pVir, the genes involved in CDT production *cdtA*, *cdtB* and *cdtC*, and the *cmeABC* genes for the multidrug efflux pump, was assessed using PCR. Bacterial chromosomal DNA was isolated from an overnight liquid culture without horse blood, using the DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions.

Primers (Operon Biotechnologies) used in this study and their sources are listed in Table 1. PCR was performed in a volume of 10 µl containing 0.03 U µl⁻¹ Polymerase Taq Platinum, 1 × PCR buffer, 3 mM MgCl₂ (Invitrogen Life Technologies), 40 µM dNTP (Qiagen), 0.5 pmol µl⁻¹ primers and 20 ng genomic DNA. Annealing temperature conditions varied according to the set of primers used. PCR products (3 µl) were separated by electrophoresis in an agarose gel containing 1.5 % agarose (Boehringer Mannheim) in 1 × Tris-acetate/EDTA (TAE) buffer, pH 8, and stained with ethidium bromide. Gels were visualized using Image Master VDS (Pharmacia Biotech).

Resistance to deoxycholate of *C. jejuni* strains of poultry and human origin. MICs were determined using a modification of the

Table 1. Primers used in this study

Target and annealing temperature	Primer	Sequence (5'–3')	Size (bp)	Reference
<i>cmeB</i> gene, 55 °C	cmeBF	GGTACAGATCCTGATCAAGCC	819	Lin <i>et al.</i> (2002)
	cmeBR	AGGAATAAGTGTTCACGGAAATT		
<i>cmeA</i> gene, 55 °C	cmeAF	TTTGGATCCTTGATGGCTAAGGCAACTTTC	781	Lin <i>et al.</i> (2002)
	cmeAR	CTCCAATTTCTTAAGCTTCGCTACC		
<i>cmeC</i> gene, 53 °C	cmeCF	GCTTGGATCCTTATCTTGGGAAAAA	641	Lin <i>et al.</i> (2002)
	cmeCR	TTTTTAAAGCTTTAAGGTAATTTTCTT		
<i>virB11</i> gene, 52 °C	virFW	GAACAGGAAGTGGAAAACTAGC	708	Bacon <i>et al.</i> (2000)
	virRV	TTCCGCATTGGGCTATATG		
<i>cdtA</i> gene, 52 °C	cdtAFW	GGAAATTGGATTGGGGCTATACT	165	Pickett <i>et al.</i> (1996)
	cdtARV	ATCACAAGGATAATGGACAAT		
<i>cdtB</i> gene, 52 °C	cdtBFW	GTTAAATCCCTGCTATCAACCA	495	Pickett <i>et al.</i> (1996)
	cdtBRV	GTTGGCACTTGGAAATTTGCAAGGC-		
<i>cdtC</i> gene, 52 °C	cdtCFW	TGGATGATAGCAGGGGATTTTAAC	555	Pickett <i>et al.</i> (1996)
	cdtCRV	TTGCACATAACCAAAAGGAAG		

method described by Lin *et al.* (2002). Briefly, *Campylobacter* strains were plated on plates containing twofold dilutions of sodium deoxycholate (Sigma) between 1000 and 16 000 µg ml⁻¹ in mCCDA. Overnight cultures supplemented with horse blood were spotted at a density of 1 × 10⁴ bacteria, and the presence of growth was determined after 48 h at 42 °C under microaerobic conditions.

Induction of IL-8 production by T84 cells exposed to *C. jejuni* strains of poultry and human origin. T84 cells were seeded in 96-well plates at a concentration of 1 × 10⁵ cells per well. After 78 h the cells were inoculated with a bacterial culture grown overnight in broth without horse blood at an m.o.i. of 200. Wells were incubated with PBS as the negative control and with tumour necrosis factor (TNF)-α (200 ng ml⁻¹; Sigma) as the positive control. The plate was centrifuged at 500 g for 10 min to facilitate contact of bacteria with cells, and incubated for 24 h at 37 °C and 5 % CO₂. Supernatant was collected and centrifuged (2300 g, 10 min, 4 °C). The concentration of IL-8 in cell culture supernatant was measured by an ELISA (Amersham Biosciences) according to the manufacturer's instructions.

Statistical analysis. Differences in occurrence of the *virB11* gene in both populations were evaluated by means of Fisher's exact test. Differences in T84 invasion, HEP-2 cell cycle arrest and IL-8 production were tested by means of one-way ANOVA. For the T84 results, log₁₀ transformation was performed to obtain normally distributed data. The capacity of the different strains to survive on different concentrations of sodium deoxycholate was analysed by means of Cox proportional hazard analyses. All statistical tests were performed in SPSS 14.0.

RESULTS AND DISCUSSION

Genotyping of poultry and human strains

The aim of this study was to compare *C. jejuni* isolates of human origin with those of poultry origin with respect to their virulence properties. To assure genetic heterogeneity in this study, isolates were chosen on the basis of their PFGE pattern after passage through day-old chicks. The combined PFGE *Sma*I and *Kpn*I profile showed that the 20 poultry isolates clustered in 17 different clusters, while the

24 human isolates clustered into 19 clusters. Both human strains and poultry isolates were spread homogeneously over the dendrogram (not shown) and none of the PFGE patterns was identical. Only two poultry and human clinical clusters showed a similarity of at least 80 % with each other.

To test the genetic stability of the isolates during passage, nine poultry isolates and nine clinical isolates recovered from cloacal swabs after passage were compared by PFGE to the original isolates that were used for oral inoculation of the chick. It was confirmed that six poultry isolates recovered after passage showed 100 % similarity to the original isolates by PFGE, while two isolates were slightly different (>90 % similarity) and one isolate was somewhat more different (80 % similarity). Four of the nine clinical isolates recovered after passage were identical to the original isolates by PFGE (>97 % similarity), while four isolates were slightly different (>92 % similarity) and one strain was more different (67 % similarity).

The 44 *C. jejuni* isolates tested in this study belonged to a large variety of genetic types, as none of the strains was identical as shown by PFGE. A certain degree of genetic instability was observed with PFGE after passage through day-old chicks for some of these strains, confirming earlier observations with passage through chick intestines (Hänninen *et al.*, 1999).

Prevalence of the *virB11* gene is equal among poultry and human isolates

Since the *virB11* gene is associated with invasiveness (Bacon *et al.*, 2000), we screened the *C. jejuni* collection for its presence using PCR. The *virB11* gene was present equally often in the poultry strains as in the human strains ($P=0.20$), suggesting that some poultry isolates are capable of invading the human intestine. It should be noted, however, that the prevalence of the *virB11* gene was low:

the *virB11* gene was present in only six of the 24 human isolates and two of the 20 poultry isolates.

C. jejuni strains from poultry and humans invade T84 cells equally

Invasion is generally considered to be important in pathogenesis, and a higher invasive capacity is generally reported for clinical isolates than non-clinical isolates (Fauchere *et al.*, 1986; Konkel & Joens, 1989; Prasad *et al.*, 1996; Tay *et al.*, 1996). In our study, a total of 20 strains of poultry origin and 24 strains of human origin were compared with respect to their ability to invade T84 monolayers. Strains were considered invasive from $\log_{10}(\text{c.f.u. ml}^{-1})=1.0$ and above. Data from four independent experiments for the poultry isolates and three independent experiments for human clinical isolates are summarized in Table 2. All but one isolate from poultry and one isolate of human origin were invasive. There was no significant difference ($P=0.48$) in mean invasiveness between the poultry isolates [mean $\pm$ SEM $\log_{10}(\text{c.f.u. ml}^{-1})=2.5 \pm 0.1$] and the human clinical isolates [mean $\pm$ SEM $\log_{10}(\text{c.f.u. ml}^{-1})=2.7 \pm 0.1$], supporting the findings of Lindblom & Kaijser (1995). However, strong differences in invasiveness between individual strains were noticed, and a greater proportion of human isolates than poultry isolates was highly invasive with $\log_{10}(\text{c.f.u. ml}^{-1}) > 3.5$, but this tendency was not statistically significant.

Lysates from human C. jejuni strains arrest more HEp-2 cells in the G₂ phase than poultry isolates

To investigate CDT production, lysates from 42 strains were tested for their ability to block the HEp-2 cell cycle. After 78 h incubation with bacterial sonicates or PBS, the DNA content of the cells was analysed by flow cytometry. Isolates which produced a cell cycle arrest of more than 16.9% (mean of negative controls plus $2 \times \text{SEM}$) were considered positive. The positive control strain NCTC 11168 caused a cell cycle arrest in $56.1 \pm 3.8\%$ of the HEp-2 cells, while the negative control treated with PBS arrested $7.5 \pm 4.7\%$ of the cells. On average, human isolates ($n=23$; mean $\pm$ SEM $55.3 \pm 3.6\%$) were able to block more cells in

their cycle progression ($P=0.041$) than the poultry isolates ($n=19$; mean $\pm$ SEM $43.5 \pm 4.4\%$) (Table 3), confirming the findings on differences in toxigenicity between clinical and poultry isolates of Gilbert & Slavik (2004) and Prasad *et al.* (1996). For the extreme values, no differences in proportions between the poultry isolates and the human isolates could be found: one isolate from poultry (5.3%) and two isolates from humans (8.7%) were considered to be negative in their ability to produce functional CDT, while four poultry isolates (21.1%) and seven human isolates (30.4%) arrested more than 70% of the HEp-2 cells in their cell cycle progression. The proportion of human isolates (34.8%) which could induce between 50.0 and 70.0% of the HEp-2 cells to remain locked in the cell cycle was greater than the equivalent proportion of poultry isolates (5.3%).

All strains possessed the *cdtA*, *cdtB* and *cdtC* genes. It is indeed generally accepted that the *cdt* genes are widespread amongst poultry and human isolates (Bang *et al.*, 2003; Eyigor *et al.*, 1999; Rozynek *et al.*, 2005). While our data suggest an association between CDT production and clinical outcome, two clinical isolates were CDT negative, while possessing the three *cdt* genes. The occurrence of CDT-negative strains in clinical isolates is in accordance with other observations (Abuoun *et al.*, 2005; Bang *et al.*, 2001; Eyigor *et al.*, 1999), and shows that CDT might not be the sole determinant in the final clinical outcome of *C. jejuni* infection in humans.

C. jejuni isolates from humans are more resistant to deoxycholate than poultry isolates

The ability of *C. jejuni* to reach the intestinal tract is associated with gastric-acid and bile-salt tolerance, and is a prerequisite for successful colonization and thus the induction of disease. Lin *et al.* (2003) showed a relationship between bile-salt resistance, colonization ability and the expression of the *cmeABC* operon. In our study, the prevalence of the *cmeABC* complex among the *C. jejuni* isolates was assessed using PCR. Although all 44 strains examined in this study possessed the three genes, strains were markedly different in their capacity to survive different concentrations of sodium deoxycholate. Human

Table 2. Invasion of *C. jejuni* strains of poultry and human origin in T84 monolayers, exposed to an m.o.i. of 200 for 3 h

Type of strain	Number* (percentage of the total sample) of strains with an intracellular bacterial count of:			
	<1.0	1.0–2.0	2.0–3.5	>3.5
Poultry isolates ($n=20$)	1 (5.0)	3 (15.0)	14 (70.0)	2 (10.0)
Human isolates ($n=24$)	1 (4.2)	2 (8.3)	16 (66.7)	5 (20.8)

*Mean $\log_{10}(\text{c.f.u. ml}^{-1})$.

Table 3. CDT-induced cell cycle arrest in the HEp-2 cell line 78 h after exposure to bacterial lysates of *C. jejuni* strains of poultry and human origin

Type of strain	Number (percentage of the total sample) of strains that induced G ₂ arrest of:			
	<16.9 %	16.9–50.0 %	50.0–70.0 %	>70.0 %
Poultry isolates ($n=19$)	1 (5.3)	13 (68.4)	1 (5.3)	4 (21.1)
Human isolates ($n=23$)	2 (8.7)	6 (26.1)	8 (34.8)	7 (30.4)

isolates appeared to be significantly more resistant to bile salts than the poultry isolates ($P=0.006$) (Fig. 1), with 85 % of the human strains surviving 16 000 μg sodium deoxycholate ml^{-1} compared to 54 % of the poultry isolates. Increased bile-salt tolerance might thus predispose to clinical campylobacteriosis in humans.

***C. jejuni* strains from poultry and humans induce IL-8 secretion by T84 monolayers to an equal extent**

Because invasion and CDT production are two mechanisms which are held to be responsible for the induction of IL-8 release (Hickey *et al.*, 1999, 2000), we examined the capacity of the two *C. jejuni* populations to elicit an IL-8 response in T84 cells. Data from three independent experiments are shown in Table 4. Strains inducing more than the mean of the negative control plus $2 \times \text{SEM}$ (346.0 pg ml^{-1}) were considered to be positive in their ability to induce IL-8 production. The positive TNF- α (200 ng ml^{-1}) control induced a mean of 899.3 ± 57.0 pg IL-8 ml^{-1} . All strains mediated IL-8 production from the T84 monolayers. Although isolates of poultry origin ($n=18$; mean $\pm \text{SEM}$ 643.9 ± 31.3 pg ml^{-1}) were not statistically different ($P=0.29$) in their ability to induce IL-8 production from the human clinical isolates ($n=24$; mean $\pm \text{SEM}$ 672.6 ± 23.5 pg ml^{-1}), it was notable that more human isolates (66.7 %) induced an IL-8 response between 600 and 700 pg ml^{-1} than did poultry isolates (33.3 %). In both *C. jejuni* populations, highly specialized strains exist: the lysate of the poultry strain KC 44 arrests only a small percentage of HEP-2 cells and is a poor invader, but causes the highest IL-8 response. Likewise, the human strain R-27478 induces an IL-8 response, but is a low CDT producer and invades T84 cells poorly. In contrast, two highly invasive and CDT-producing strains elicit the IL-8 response only moderately.

Fig. 1. Effect of sodium deoxycholate on the survival of different strains of *C. jejuni* of poultry (white bars; $n=19$) and human (black bars; $n=24$) origin. The data represent the mean $\pm \text{SEM}$ percentage of three independent experiments.

Table 4. IL-8 induction by T84 monolayer after 24 h exposure to *C. jejuni* isolates of poultry and human origin at an m.o.i. of 200

Type of strain	Number (percentage of the total sample) of strains that induce:		
	<600*	600–700*	>700*
Poultry isolates ($n=18$)	8 (44.4)	6 (33.3)	4 (22.2)
Human isolates ($n=24$)	3 (12.5)	16 (66.7)	5 (20.8)

* pg IL-8 ml^{-1} .

Conclusion

In this study, no correlation between PFGE type and phenotype could be observed, nor between PFGE type and the virulence properties themselves. The appearance of low CDT-producing and poorly invading strains, which are nevertheless able to elicit an IL-8 response, raises questions about the strategies employed by *C. jejuni* to cause pathology. We conclude that based on invasiveness and the ability to induce IL-8 production, all poultry isolates studied should be considered capable of causing human enteritis, and that enhanced CDT production and bile-salt resistance might predispose to *C. jejuni* clinical infections in humans.

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