Campylobacter jejuni invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines

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Campylobacter jejuni is a major food-borne bacterial pathogen, which is capable of causing diarrhoea containing blood and leukocytes. *C. jejuni* invasion of the intestinal epithelial cells and the release of proinflammatory molecules contribute to the pathophysiology of campylobacteriosis. Given the commensal relationship of *C. jejuni* with chickens, we hypothesized that *C. jejuni* invasion of chicken cells and the release of host cell cytokines would be significantly less than with human cells. To test our hypothesis, we examined the interactions of *C. jejuni* with chicken LMH cells, and performed *in vivo* experiments with chickens. The binding and internalization assays revealed that *C. jejuni* was significantly less invasive of LMH cells relative to human INT 407 cells, even though the bacteria bound to each host cell species equally. We also assessed interleukin-8 (IL-8) transcript, IL-8 secretion, and the release of chemoattractant molecules from the inoculated cells. Inoculation of LMH cells with *C. jejuni* stimulated expression of both chicken IL-8 orthologues, *chCXCLi2* and *chCXCLi1*, but at levels significantly less than human IL-8 (*huCXCL8*) expressed from human INT 407 cells inoculated with *C. jejuni*. Moreover, the supernatant fluids of the *C. jejuni*-inoculated LMH cells resulted in little heterophil migration. *In vivo*, *C. jejuni* were observed bound to the cells lining the glandular crypts, but overt signs of cell invasion or pathology were not observed. These results indicate that cytokine expression in chicken LMH cells in response to *C. jejuni* is distinct from that of *Salmonella typhimurium*.

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INTRODUCTION

Campylobacter jejuni, a Gram-negative, spiral-shaped bacterium, is a common cause of gastrointestinal illness throughout the world. In developing countries, individuals are continuously exposed to *C. jejuni* from an early age. As a result of repeated exposure, *C. jejuni*-infected individuals can be asymptomatic or suffer from mild diarrhoea characterized by loose, watery stools (Blaser *et al.*, 1985;

Wassenaar & Blaser, 1999). In contrast, *C. jejuni*-infected individuals in developed countries experience fever, abdominal cramps and diarrhoea that may be accompanied with gross blood and leukocytes (Black *et al.*, 1988; Blaser *et al.*, 1983). This observed variation in disease severity is likely to be due to differences in both bacterial virulence (i.e. host cell invasion) and the host innate immune response.

A complex set of events is necessary for a robust host inflammatory response (Kelly *et al.*, 2005; Nathan, 2006). In one instance, pathogen-associated molecular patterns (PAMPs) are recognized by host pattern recognition

Abbreviations: C_t, cycle threshold; IL-8, interleukin-8; p.i., post-inoculation; PMA/I, phorbol 12-myristate 13-acetate and ionomycin; TEM, transmission electron microscopy.

receptors (PRRs) on the surface of intestinal epithelial cells (O'Hara & Shanahan, 2006). This interaction triggers host cell signal transduction pathways and the transcription of genes encoding proinflammatory molecules. For example, Toll-like receptor 5 (TLR5) is found on the apical surface of intestinal epithelial cells. TLR5 recognizes conserved epitopes within some bacterial flagellins and initiates signalling that leads to the expression of the gene encoding interleukin-8 (IL-8, also known as huCXCL8) (Ramos *et al.*, 2004). The release of IL-8 and other proinflammatory molecules from cells activates innate immune defences (i.e. inflammation) and promotes acquired immune responses. Other evidence suggests that PRR-dependent expression of proinflammatory molecules alone is not always sufficient to initiate the inflammatory response, but that the release of a second class of molecules from injured host cells may be required for robust inflammation (see reviews by Blikslager *et al.*, 2007; Nathan, 2006). The host microflora can also influence the innate immune response. Specifically, bacteria can dampen the immune response and block the release of proinflammatory molecules in order to colonize a host. A commensal strain of *Salmonella*, which harbours PAMPs, blocks NF- κ B-dependent expression of proinflammatory cytokines to prevent host inflammation (Neish *et al.*, 2000).

A hallmark of gastrointestinal infection with *C. jejuni* is an acute host inflammatory response (Allos, 2001). Central to this acute inflammatory response is the secretion of IL-8 from the cells lining the intestinal tract (Wassenaar & Blaser, 1999). IL-8 is a potent chemoattractant and activator of macrophages and polymorphonuclear leukocytes (Fleckenstein & Kopecko, 2001). In humans, serum IL-8 levels peak between 3 and 10 days after the onset of the disease (Baqar *et al.*, 2001), and it is common to observe gross blood and leukocytes in stool samples from these individuals (Konkel *et al.*, 2001). The characteristics of clinical disease support the notion that proinflammatory mediators and tissue damage both participate in the robust inflammatory response (Baqar *et al.*, 2001; Black *et al.*, 1988). *In vitro* studies have revealed that *C. jejuni* invades and stimulates the release of IL-8 from numerous human colonic cell lines, and that the level of IL-8 released is related to the invasiveness of the *C. jejuni* strain and the host cell lineage (Bakhiet *et al.*, 2004; Everest *et al.*, 1992; Hickey *et al.*, 1999; MacCallum *et al.*, 2006).

C. jejuni infections frequently result from eating undercooked poultry or foods cross-contaminated with raw poultry products. *C. jejuni* is a commensal organism in chickens. Indeed, the caeca of chickens can be colonized by in excess of 10^7 *C. jejuni* per gram of caecal contents without signs of pathology (e.g. host cell invasion, cell necrosis, inflammation, influx of neutrophils) (Beery *et al.*, 1988; Cawthraw *et al.*, 1994). The characteristics that distinguish the commensal relationship of *C. jejuni* with chickens from its pathogenic relationship with humans are unclear.

In vitro cell culture models have been employed to explore the interaction between *C. jejuni* and chicken epithelial cells. Specifically, studies have been performed to determine whether *C. jejuni* invades primary chicken epithelial cells and induces the expression of proinflammatory cytokines.

It has been reported that *C. jejuni* invades human epithelial cells and primary chicken cells at similar levels (Byrne *et al.*, 2007; Smith *et al.*, 2005). The array of proinflammatory cytokines produced by humans and chickens is distinct. Human IL-8 is expressed from the *CXCL8* gene, for which chickens have two orthologues, termed *CXCLi1* (*K60*) and *CXCLi2* (*CAF/IL-8*, also described as *9E3/CEF4*) (Kaiser *et al.*, 1999; Sick *et al.*, 2000). As with *CXCL8* gene expression in human epithelial cells, researchers have observed an increase in *CXCLi1* and *CXCLi2* expression in primary chicken cells inoculated with *C. jejuni* (Borrmann *et al.*, 2007; Smith *et al.*, 2005). Based on the results from these studies, investigators have concluded that the interaction of *C. jejuni* with human cells is similar to that observed with chicken cells. We undertook this study with a continuous chicken epithelial cell line (LMH) as an *in vitro* model to further dissect the interactions of *C. jejuni* with chicken cells. To our knowledge, this is the first study to evaluate the use of LMH cells as an *in vitro* model to investigate the interaction of *C. jejuni* with chicken epithelial cells.

In this study, we report differences in invasiveness, expression and secretion of IL-8, as well as the functional activity of chemoattractants between chicken and human cells inoculated with *C. jejuni*. Throughout this manuscript, we refer to IL-8 from humans as huCXCL8 and IL-8 from chickens as chIL-8. In instances where they can be distinguished, we will refer to the specific *CXCL8* orthologues as chicken *CXCLi1* (*chCXCLi1*) and chicken *CXCLi2* (*chCXCLi2*). We tested whether the inoculation of chicken LMH cells with *C. jejuni* induces these cells to secrete IL-8 and other chemoattractant molecules. In addition, chickens were inoculated with *C. jejuni* to assess colonization, the immune response, and the pathology of the caecum. These data provide insights regarding the differences that distinguish the interactions of *C. jejuni* with chicken and human cells, and clearly show that chicken LMH cells respond differently to *C. jejuni* versus *Salmonella typhimurium*.

METHODS

Culture of bacterial strains. *C. jejuni* F38011 and 81-176 human clinical strains and *C. jejuni* D34a and A2a poultry strains were cultured under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) on Mueller–Hinton agar plates supplemented with 5% (v/v) citrated bovine blood (MH–blood agar). *C. jejuni* F38011 was recovered from an individual with diarrhoea containing blood and leukocytes in the stool sample. *C. jejuni* strain A2a was recovered from a Kansas poultry plant that processed organic broiler chickens. Organic poultry do not receive any coccidiostats, antibiotics, pesticides or insecticides (e.g. on their feed or in their environment), and must have access to the outside. *C. jejuni* strain D34a was

recovered from an Iowa poultry processor plant from chickens reared using conventional methods. Cultures were passaged to a fresh MH-blood agar plate every 24–48 h. *S. typhimurium* 85-102840 and *Escherichia coli* XL1-Blue MRF' (Stratagene) were cultured on Luria-Bertani (LB) agar plates.

Motility assay. Motility assays were performed using MH medium supplemented with 0.4 % Select agar (Invitrogen). Briefly, 10 µl of a bacterial suspension was added to the centre of a plate, and the plate was incubated at 37 °C under microaerobic conditions for 48 h. The relative motility of each bacterial strain was determined by measuring the diameter of the migration zone.

Culture of LMH and INT 407 cells. LMH chicken hepatocellular carcinoma epithelial cells (ATCC CRL-2117), INT 407 human embryonic epithelial cells (ATCC CCL-6) and HepG2 cells (ATCC HB-8065) were obtained from the American Type Culture Collection. Stock cultures of LMH cells were grown in flasks coated with 0.1 % gelatin in Waymouth's MB 752/1 medium supplemented with 10 % fetal bovine serum (FBS). Stock cultures of INT 407 and HepG2 cells were grown in minimal essential medium (MEM) supplemented with 10 % FBS (HyClone Laboratories). Cultures were maintained at 37 °C in a humidified, 5 % CO₂ incubator.

***C. jejuni*–host cell binding and internalization assays.** For the adherence assays, each well of a 24-well tissue-culture tray was seeded with LMH, INT 407 or HepG2 cells ($1.5\text{--}3.0 \times 10^5$ cells per well) and incubated for 18 h at 37 °C in a humidified, 5 % CO₂ incubator. The cells were rinsed with the appropriate medium and inoculated with approximately 5×10^7 c.f.u. of the various *C. jejuni* strains. The cells were also inoculated with the same number of *S. typhimurium* and *E. coli* MRF', which were used as positive and negative controls, respectively. Bacteria–host cell contact was promoted by centrifugation at 600 g for 5 min. Following a 30 min incubation at 37 °C in a humidified, 5 % CO₂ incubator, the epithelial cells were rinsed three times with PBS (20 mM sodium phosphate, 150 mM NaCl, pH 6.9) to remove non-adherent bacteria. The epithelial cells were then lysed with a solution of 0.1 % (v/v) Triton X-100 in PBS. The suspensions were serially diluted and the number of viable, adherent bacteria determined by counting the resultant colonies on MH–blood agar plates. For internalization, each 24-well plate containing the *C. jejuni*-inoculated cells was incubated for 3 h. The media were aspirated and replaced with culture media containing 1 % FBS and 250 µg gentamicin µl⁻¹. The plates were incubated for an additional 3 h, and cell lysis and plating of serial dilutions were performed as outlined above. The values reported represent the mean counts $\pm$ SD derived from triplicate wells.

RNA isolation from eukaryotic cell lines. For isolation of RNA from LMH or INT 407 cells, six-well plates were seeded with 1.2×10^6 and 6×10^5 cells, respectively. Bacterial suspensions were added at an m.o.i. of 1:100 in 6 ml MEM 1 % FBS. Samples were centrifuged at 600 g for 5 min and placed in a humidified 37 °C incubator with 5 % CO₂ for 4 h. Cells were washed once with ice-cold PBS and lysed with 1 ml TRIzol reagent (Invitrogen). A cell scraper was used to remove adherent cells and the resulting lysate was vortexed thoroughly. For phase separation, 200 µl chloroform:iso-amyl alcohol (24:1) was added, vortexed for 15 s, incubated at room temperature for 3 min, and spun at 14 000 g for 15 min at 4 °C. The upper aqueous layer was transferred to a fresh tube and precipitated with 0.5 ml isopropyl alcohol. To collect the RNA precipitate, samples were centrifuged at 14 000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed twice with 75 % ethanol and resuspended in 100 µl nuclease-free water. Samples were loaded on a 2 % agarose gel to verify extraction efficacy prior to RNA cleanup using the RNeasy kit (Invitrogen) according to the manufacturer's instructions. RNA samples were generated on three separate days and used for reverse-transcription reactions.

Real-time RT-PCR of IL-8. Reverse transcription of RNA extracts was performed using the ThermoScript RT-PCR System (Invitrogen) according to the manufacturer's protocol. Briefly, 1 µg total mRNA was combined with 50 µM Oligo(dT)₂₀ random primer in a 20 µl total volume with ThermoScript reverse transcriptase. Thermal reaction conditions were as follows: preheating of the RNA sample for 5 min at 65 °C, addition of the reaction mixture on ice, heating for 60 min at 50 °C, 85 °C for 5 min for enzyme denaturation, and rapid cooling to 4 °C. Real-time RT-PCR analysis was performed with three cDNA sample sets.

Transcript quantification was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with primers and PCR conditions described elsewhere (Borrmann *et al.*, 2007). Real-time quantitative PCR was carried out with 7.5 ng cDNA template using the following primers: *chCXCLi2* (CAF) Fwd 5'-ATG AAC GGC AAG CTT GGA GCT-3', Rev 5'-GCC ATA AGT GCC TTT ACG ATC AG-3'; *chCXCLi1* (K60) Fwd 5'-TGG CTC TTC TCC TGA TCT CAA TG-3', Rev 5'-GCA CTG GCA TCG GAG TCT A-3'; and chicken beta actin Fwd 5'-CAT CAC CAT TGG CAA TGA GAG G-3', Rev 5'-GAT TCA TCG TAC TCC TGC TTG C-3'; *huCXCL8* Fwd 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3', Rev 5'-TCT CAG CCC TCT TCA AAA ACT TCT C-3'; human GAPDH Fwd 5'-TGG GTG TGA ACC ATG AGA AG-3', Rev 5'-GCT AAG CAG TTG GTG GTG C-3'. For the *chIL-8* and beta actin samples, 450 nM of each primer was used; and for *huCXCL8* and GAPDH samples, 300 nM of each primer was used. Duplicate 50 µl reactions were performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) for each sample with each primer set. RNA samples without a reverse transcriptase step were included for every sample and primer combination to ensure the absence of genomic DNA. Thermal cycling conditions for RT-PCR were as follows: 2 min at 50 °C, 15 min at 95 °C followed by 40 repeats of 15 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C. PCR efficiencies were determined from standard curves of cDNA dilutions and their respective cycle thresholds (*C_s*). A reference gene was used to correct for variance in mRNA template concentration and efficiency of the reverse-transcription reaction. The chicken beta actin gene was used as the reference gene for *chIL-8* and the human GAPDH gene was used as the reference gene for *huCXCL8*. These data were analysed using the comparative *C_t* method, and the results are presented as the fold increase of gene expression of the bacteria-inoculated samples relative to the uninoculated sample, as described by Applied Biosystems.

Generation of *C. jejuni*-inoculated LMH and INT 407 cell culture supernatant fluids. Overnight cultures of bacteria were washed twice by centrifugation for 10 min at 8000 g and resuspended in the appropriate tissue-culture medium supplemented with 1 % FBS. The epithelial cells were co-cultured with 0.5 ml (per well) of a bacterial suspension containing a 1:10 dilution of 5×10^7 c.f.u. of the appropriate *C. jejuni* strain. The plate was centrifuged at 600 g for 5 min before being transferred to a 37 °C incubator. Supernatant fluids were collected 24 h after inoculation, and stored at -20 °C. Supernatant fluids were also collected from uninoculated cells as a negative control and cells inoculated with a 1:1000 dilution of 5×10^7 c.f.u. *S. typhimurium* as a positive control. In certain instances, a solution of phorbol 12-myristate 13-acetate and ionomycin (PMA/I, 10 pg ml⁻¹ of each) was included as a separate, stimulation (positive) control.

IL-8 ELISAs. ELISA was performed to detect the presence of IL-8 secreted by LMH or INT 407 cells. Polystyrene plates (96-well, Costar) were coated with 50 µl per well of 0.4 µg ml⁻¹ mouse anti-*huCXCL8* (Pierce Endogen) and stored at 4 °C overnight under humid conditions. Plates were blocked with 150 µl 1 % (w/v) BSA (fraction V, Sigma) in PBS for 30 min at room temperature. After blocking, plates were washed three times with PBS containing Tween

20 (0.005 % v/v) (PBS-Tween). Supernatant fluid samples and chicken sera (diluted 1:10 in PBS) were added at 100 µl per well and incubated for 2 h at room temperature. Wells were washed three times with PBS-Tween, after which 100 µl of 0.1 µg ml⁻¹ biotin-labelled mouse anti-huCXCL8 (Pierce Endogen) in PBS with 1 % BSA was added. Samples were incubated for 1 h at room temperature. The plate was washed three times with PBS-Tween and 100 µl of 0.25 µg ml⁻¹ horseradish peroxidase conjugated streptavidin (SA-HP) in PBS with 1 % BSA was added. After a 30 min incubation period, the plate was washed three times with PBS-Tween. Fifty microlitres of 3,3',5'-tetramethylbenzidine (TMB; Pierce) per well was then added and allowed to incubate for 15 min. Colorimetric development was stopped by adding 50 µl 0.18 M H₂SO₄. A₄₅₀ was read with a Titertek Multiscan MCC/340. All experimental samples were assayed in triplicate. Control samples of recombinant huCXCL8 (Pierce Endogen) ranging from 20 to 5000 pg ml⁻¹ were included as an internal control for reproducibility and dose-dependent response. The mean and SDs of the absorbance readings were calculated from triplicate measurements. Statistical analysis to determine significance was performed using Student's *t* test.

To demonstrate specificity of the chIL-8 ELISA, one microtitre plate (plate 1) was coated with 50 µl mouse anti-huCXCL8 antibody (Pierce Endogen) in serial twofold dilutions ranging from 7 to 1000 pg ml⁻¹ and incubated overnight at 4 °C. A second ELISA plate (plate 2) was coated with 50 µl per well 0.4 µg ml⁻¹ mouse anti-huCXCL8 and stored at 4 °C overnight, as above. A 100 µl volume of *S. typhimurium*-inoculated LMH supernatant fluids was added to plate 1 for 45 min at room temperature. Supernatant fluids were then transferred to plate 2. Both plate 1 and plate 2 were blocked and analysed for bound chIL-8 as described above. Specificity was demonstrated by the dose-dependent capture of chIL-8 on plate 1, and the inverse trend of chIL-8 capture on plate 2.

Heterophil cell migration assays. One-day-old broiler chickens were obtained from a commercial source (Phinney Hatchery), raised in wired cages, and given free access to feed and water. At 3–4 weeks of age, 3 ml blood was drawn from the brachial wing vein using a sterile plastic syringe containing 0.3 ml 10 % disodium EDTA. Heterophils were isolated from blood using a published technique (Andreasen & Latimer, 1989) with some modifications. Briefly, for each 1.5 ml of EDTA-anticoagulated blood, an equal volume of 1 % (w/v) methylcellulose was added, mixed, and centrifuged at 25 *g* for 10 min. The plasma and extended buffy-coat layers were removed, diluted in 0.5–1 ml serum-free Dulbecco's Modified Eagle's Medium (MEM; Invitrogen) and layered on to a discontinuous Ficoll-Hypaque gradient (3 ml at specific gravity 1.077; 3 ml at 1.119) (Sigma). Following centrifugation at 250 *g* for 35 min, the plasma–1.077 interface was discarded. The remaining gradient (partial 1.077 and 1.119) containing heterophils was collected and washed three times in serum-free MEM. Viable cells were counted using a haemocytometer and resuspended in serum-free MEM to achieve concentration of 1–2 × 10⁴ cells per 100 µl.

The presence of chemoattractants in the supernatant fluids collected from *C. jejuni*-inoculated LMH cells was assessed by chicken heterophil cell migration with a QCM Chemotaxis 96-well Cell Migration Assay kit ECM515 (Chemicon). Briefly, freshly isolated chicken heterophils (2 × 10⁴) in 100 µl serum-free medium were loaded into the upper migration chamber of the transwell plate and 150 µl of supernatant from *C. jejuni*-inoculated LMH cells was added to the lower chamber (feeder tray). Supernatant fluids were collected from uninoculated and *S. typhimurium*-inoculated cells as negative and positive controls, respectively. Cells were incubated in a humidified, 5 % CO₂ incubator for 45 min at 37 °C. After incubation, the upper chamber was incubated with 150 µl prewarmed cell detachment solution for 30 min at 37 °C to dislodge migrated cells

from the underside of the insert. From the feeder tray, 75 µl of the medium was transferred to a 96-well plate and 75 µl of the cell detachment solution was transferred to the same plate. To this 150 µl mixture, 50 µl lysis buffer/dye solution containing CyQuant GR dye was added. The fluorescence was measured using a Wallac 1420 fluorescence plate reader set at excitation/emission wavelengths of 485/535 nm, and the total number of migrated heterophils in each sample was calculated using a standard curve obtained for known concentrations of heterophils. All samples were tested in triplicate, and the statistical significance between samples was determined using Student's *t* test (*P* < 0.05).

Inoculation of chickens. Thirty-six nine-day-old broiler chickens were divided into six groups and placed into isolation chambers (Horsfall–Bauer isolators) on wire mesh. Water and a commercial chick starter feed were provided *ad libitum*. The chickens were inoculated by oral gavage with 0.5 ml of a suspension of *C. jejuni* F38011 strain, which was cultured in Bolton's broth at 42 °C for 16 h under microaerobic conditions. One group of six chickens was kept as the uninoculated control group. The remaining five groups of chickens were inoculated with: (1) 10² c.f.u. *C. jejuni* F38011; (2) 10³ c.f.u. *C. jejuni* F38011; (3) 10⁴ c.f.u. *C. jejuni* F38011; (4) 10⁵ c.f.u. *C. jejuni* F38011; and (5) 10⁶ c.f.u. *C. jejuni* F38011. The bacterial suspensions were serially diluted and plated onto cefoperazone–vancomycin–amphotericin B (CVA) agar to confirm the c.f.u. of each dose. Six days later the chickens were euthanized, and the number of *C. jejuni* per caecal sample was determined by direct plating. More specifically, the caecum with its contents was collected, placed into a whirl-pack, sliced open, weighed, diluted 1:10 (w/v) in Bolton's broth medium, and thoroughly stomached. The serial 10-fold dilutions were made in 3 ml quantities of Bolton's broth medium. The diluted samples were spread onto the surface of CVA agar plates and incubated in a microaerobic environment at 37 °C. The c.f.u. were determined for each sample after 48 h of incubation. Blood was also collected from each chicken.

A second *in vivo* colonization experiment was performed with 12 broiler chickens. Six chickens were inoculated with 10⁷ c.f.u. *C. jejuni* F38011 strain and six uninoculated chickens were used as a control. Three of the *C. jejuni*-inoculated chickens and three uninoculated chickens were euthanized at 8 days post-inoculation (p.i.), and the remaining six chickens at 15 days p.i. Caecal and blood samples were collected as described above. All of the chicken studies and procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC protocol #3248) at Washington State University.

Immunoblot analysis and ELISA of chicken serum antibodies. Blood samples were collected from chickens sacrificed 6, 8 and 15 days p.i., as described above in the inoculation protocol. Sera were separated by centrifugation and stored at –20 °C. For immunoblot analysis, *C. jejuni* F38011 whole-cell lysates were normalized to 0.1 OD₅₄₀ units in electrophoresis sample buffer and incubated in a boiling water bath for 5 min. The proteins were separated in a 12 % acrylamide gel matrix using Laemmli's discontinuous buffer system and transferred to PVDF membranes (Immobilon P, Millipore). The membranes were rinsed in PBS and incubated for 16 h at room temperature with a 1:50 dilution of chicken sera in PBS (pH 7.4) containing 0.01 % Tween-20 and 9 % (w/v) dried milk. Bound immunoglobulins were detected with either anti-chicken IgM (ICL) or anti-chicken IgG horseradish peroxidase conjugates (Sigma). 4-Chloro-1-naphthol (Sigma) was used as the chromogenic substrate. The sera were also tested for anti-*C. jejuni* antibodies by ELISA. For these assays, 96-well microtitre plates (Costar 3797, Corning) were coated with 1 µg *C. jejuni* F38011 whole-cell lysate overnight at 4 °C. After blocking with PBS/0.5 % BSA (w/v) for 2 h at room temperature, the chicken sera were added in twofold serial dilutions

(ranging from 1:50 to 1:6400) and incubated for 2 h at room temperature. The wells were rinsed three times with PBS-Tween and incubated for 30 min with a 1:1000 dilution of anti-chicken IgG-horseradish peroxidase antibody (Sigma). The plates were developed as described above.

Histopathology and transmission electron microscopy (TEM).

Intestinal and caecal tissues were collected at the time of necropsy and placed in 10 % buffered formalin. Each sample was coded to conceal whether it was collected from a *C. jejuni*-inoculated or uninoculated chicken, and given to the core facility for processing. The samples were prepared with an automatic processor (Tissue-Tek VP, Sakura) and embedded in paraffin, and 4 µm thick sections were mounted onto glass slides. The sections were de-paraffinized with xylene and stained with haematoxylin and eosin. All the samples were examined by a board-certified pathologist. The sample identification code was shared with the pathologist after the final summary report was provided to the investigators. In total, intestinal and caecal tissues were examined from two control chickens (i.e. uninoculated) and two *C. jejuni*-inoculated chickens euthanized at 8 days p.i., and from one control chicken and one *C. jejuni*-inoculated chicken euthanized at 15 days p.i.

For TEM, chicken caecum samples were removed from 10 % formalin, cut into 1 × 2 mm pieces and placed in 2 % paraformaldehyde/2 % glutaraldehyde in 0.1M cacodylate buffer overnight at 4 °C. Samples were rinsed in buffer and post-fixed in 2 % OsO₄ 0.1M cacodylate buffer overnight at 4 °C and dehydrated in an ethanol series, followed by two changes of propylene oxide. Samples were infiltrated with 1:1 Spurr's epoxy resin for 2 h, followed by two changes with 100 % Spurr's epoxy resin, and polymerized overnight in 100 % Spurr's 2 × at 70 °C. Thin sections were collected on nickel grids using a Reichert Ultra Cut R ultramicrotome (Leica Microsystems), stained with 4 % uranyl acetate/1 % potassium and Reynold's Lead, and viewed with a JEOL JEM 1200 EX transmission electron microscope. Images were obtained with a MegaView III camera using analySIS 3.2 software [Olympus Soft Imaging Systems Corp. (SIS)].

RESULTS

C. jejuni is less invasive of LMH cells compared with human INT 407 cells

Binding and internalization assays were performed with four *C. jejuni* strains and chicken LMH hepatocellular

carcinoma epithelial cells. Two *C. jejuni* strains (F38011 and 81-176) were recovered from individuals exhibiting clinical signs of campylobacteriosis, and two *C. jejuni* strains (A2a and D34a) were recovered from poultry processing plants. All four *C. jejuni* strains were motile (not shown). Earlier work has indicated that *S. typhimurium* efficiently invades primary chick cells, while a laboratory strain of *E. coli* poorly invades these cells (Kaiser *et al.*, 2000). In this study, *S. typhimurium* 85-102840 was used as a positive control for host cell invasion and *E. coli* XL1-Blue MRF' as a negative control. Assays were performed in parallel with human INT 407 epithelial cells.

The results of the bacterial binding assays are shown in Table 1. Similar numbers of *S. typhimurium* and *E. coli* bound to the LMH and INT 407 cells. In addition, all four *C. jejuni* strains bound LMH cells and INT 407 cells in comparable numbers ($P > 0.05$). The variations in adherence among the four *C. jejuni* strains to the LMH cells did not correlate with the isolation source of the strain (i.e. human versus poultry). These data indicated that *C. jejuni* binding to the host epithelial cells is independent of host cell species (i.e. chicken versus human cells).

The results of the bacterial invasion assays are shown in Table 1. *S. typhimurium* invaded LMH cells at $4.5 \pm 1.9 \times 10^5$ c.f.u. per well and invaded INT 407 cells at $6.2 \pm 0.9 \times 10^5$ c.f.u. per well. *E. coli* invaded LMH cells at $2.9 \pm 0.5 \times 10^3$ c.f.u. per well and invaded INT 407 cells at $2.8 \pm 0.6 \times 10^3$ c.f.u. per well. There was no significant difference in the numbers of either *S. typhimurium* or *E. coli* that invaded the LMH cells when compared with INT 407 cells. In contrast to the controls, the number of *C. jejuni* that invaded LMH cells was significantly lower ($P < 0.05$) than the number of *C. jejuni* that invaded the INT 407 cells. Because the number of *C. jejuni* that invade host cells is dependent on the number bound, we calculated I/A values [(number of intracellular bacteria/number of cell-associated bacteria) × 100] to normalize the number of internalized bacteria relative to the number of

Table 1. Binding and internalization of *C. jejuni* human clinical and poultry strains to LMH chicken hepatocellular carcinoma epithelial cells and INT 407 human embryonic epithelial cells

Isolate	LMH cells			INT 407 cells		
	Number of cell-associated bacteria*	Number of internalized bacteria†	(I/A) × 100 (%)‡	Number of cell-associated bacteria*	Number of internalized bacteria†	(I/A) × 100 (%)‡
<i>S. typhimurium</i>	$(1.2 \pm 0.1) \times 10^7$	$(4.5 \pm 1.9) \times 10^5$	3.8	$(1.3 \pm 0.2) \times 10^7$	$(6.2 \pm 0.9) \times 10^5$	4.8
<i>C. jejuni</i> F38011	$(9.6 \pm 1.0) \times 10^6$	$(1.3 \pm 0.4) \times 10^4$	0.1	$(9.8 \pm 1.5) \times 10^6$	$(3.3 \pm 0.6) \times 10^5$	3.4
<i>C. jejuni</i> 81-176	$(6.6 \pm 2.1) \times 10^6$	$(1.2 \pm 0.7) \times 10^4$	0.2	$(7.8 \pm 1.0) \times 10^6$	$(6.2 \pm 0.4) \times 10^5$	8.0
<i>C. jejuni</i> A2a	$(8.8 \pm 1.4) \times 10^6$	$(1.6 \pm 0.6) \times 10^4$	0.2	$(1.1 \pm 0.2) \times 10^7$	$(4.6 \pm 1.4) \times 10^5$	4.2
<i>C. jejuni</i> D34a	$(4.7 \pm 0.8) \times 10^6$	$(7.0 \pm 3.0) \times 10^3$	0.2	$(5.1 \pm 0.3) \times 10^6$	$(2.8 \pm 1.5) \times 10^5$	5.5
<i>E. coli</i> MRF	$(1.4 \pm 0.2) \times 10^7$	$(2.9 \pm 0.5) \times 10^3$	0.02	$(1.3 \pm 0.1) \times 10^7$	$(2.8 \pm 0.6) \times 10^3$	0.02

*Number of cell-associated bacteria per well of a 24-well plate.

†Number of internalized bacteria per well of a 24-well plate, as determined by the gentamicin-protection assay.

‡(I/A) × 100 indicates the percentage of cell-associated bacteria that were internalized.

adherent bacteria for the different strains used in this study. Comparisons of *S. typhimurium*, *E. coli* and *C. jejuni* I/A values for the LMH and INT 407 cells revealed that while *C. jejuni* does invade LMH chicken cells, it is significantly less invasive for LMH cells than INT 407 cells. Collectively, we concluded that each *C. jejuni* strain used in this study: (1) exhibited similar binding potential to LMH and INT 407 cells; (2) demonstrated a low invasive potential for LMH cells and a high invasive potential for INT 407 cells; and (3) variations in binding and invasion of the *C. jejuni* strains did not correlate with the source (i.e. human versus poultry) of the isolate. We concluded that each of the four *C. jejuni* strains is virulent based on the fact that the *C. jejuni* strains invaded the human INT 407 cells in numbers that were similar with one another and that of the *S. typhimurium* strain, and two of the four *C. jejuni* strains were recovered from individuals with campylobacteriosis.

***C. jejuni* stimulates greater *chCXCLi1* expression than *chCXCLi2* in LMH cells**

Real-time RT-PCR was used to measure *chCXCLi1*, *chCXCLi2* and *huCXCL8* transcripts in epithelial cells inoculated with *C. jejuni* or *S. typhimurium*, PMA/I treated cells (i.e. positive stimulation control), and media only-treated cells (i.e. mock-inoculated, negative stimulation control) (Table 2). Relative to the uninoculated LMH cells, *S. typhimurium* induced a 13.04 ± 1.44 -fold increase and a 21.18 ± 5.52 -fold increase in *chCXCLi1* and *chCXCLi2* expression, respectively. Each of the four *C.*

jejuni strains induced the expression of both *chCXCLi1* and *chCXCLi2*. The greatest level of *chCXCLi1* and *chCXCLi2* expression from the *C. jejuni*-inoculated cells was noted for strain A2a. The expression of *chCXCLi2* in the *C. jejuni* A2a-inoculated LMH cells was significantly less than that observed with *S. typhimurium*. Also, in contrast with *S. typhimurium*, the expression of *chCXCLi1* relative to *chCXCLi2* was greater in the *C. jejuni*-inoculated LMH cells. Noteworthy is the fact that the level of *huCXCL8* expression in INT 407 cells inoculated with *C. jejuni* 81-176 was greater than *huCXCL8* expression induced by *S. typhimurium*. Based on these assays, we concluded that the expression of *huCXCL8* orthologues in chicken LMH cells in response to *C. jejuni* is different from that of *S. typhimurium*.

***C. jejuni* does not induce chicken IL-8 secretion from LMH cells, as judged by ELISA**

Due to the unavailability of antibodies against *chCXCLi1* and *chCXCLi2*, we used capture and detection antibodies generated against human IL8 (*huCXCL8*) in a sandwich ELISA, with the caveat that it is not known whether these antibodies would detect either *chCXCLi1* or *chCXCLi2*. Thus, we refer to the protein detected by the ELISA as 'chIL-8' rather than *chCXCLi1* or *chCXCLi2*. ELISAs were performed with supernatant fluids collected from epithelial cells inoculated with *C. jejuni* 81-176 or *S. typhimurium*, and media only-treated cells (i.e. uninoculated cells) (Fig. 1). Epithelial cells were inoculated and 24 h after inoculation supernatant fluids were collected. LMH cells incubated with *S. typhimurium* released significant amounts of chIL-8. In contrast, a significant difference was not observed in chIL-8 in the supernatants from LMH cells inoculated with *C. jejuni* strain 81-176 when compared with cells treated with media only. Recombinant *huCXCL8* protein standards were run in parallel to assess dose-dependency and specificity of the sandwich ELISA. Additional assays revealed that none of the four *C. jejuni* strains induced the release of detectable amounts of chIL-8 from LMH cells, but each stimulated *huCXCL8* protein secretion from the INT 407 cells (results not shown). To further demonstrate that the *huCXCL8* antibody used in the sandwich ELISA reacts with chIL-8, the supernatant fluids collected from *S. typhimurium*-inoculated LMH cells were incubated in wells of an ELISA plate coated with serial dilutions of the capture antibody. Specificity of the reaction was demonstrated by the observation that as the concentration of capture antibody increased, a decrease was observed in the amount of chIL-8 detected in the supernatant fluid (results not shown). It is not known whether the capture antibody reacts with either *chCXCLi1* and/or *chCXCLi2*. Inoculation of the INT 407 cells with both *C. jejuni* and *S. typhimurium* induced *huCXCL8* secretion (Fig. 1). The IL-8 secretion responses obtained with HepG2 cells were similar to those of the INT 407 cells (results not shown).

Table 2. Analysis of *chCXCLi2* (*CAF/IL-8*) and *chCXCLi1* (*K60*) expression in LMH cells, and *huCXCL8* expression in INT 407 cells

The relative fold changes in transcript levels were determined from *C. jejuni*- and *S. typhimurium*-inoculated cells using the comparative C_t method. All samples were normalized as described in Methods.

Bacterial isolate	Relative fold change of transcript	
	<i>chCXCLi1</i> (<i>K60</i>)	<i>chCXCLi2</i> (<i>CAF</i>)
Chicken LMH cells		
81-176	16.84 ± 3.25	2.87 ± 0.77
F38011	3.84 ± 0.53	2.52 ± 1.13
D34a	6.05 ± 2.06	1.25 ± 0.32
A2a	28.45 ± 5.87	3.56 ± 2.30
85-102840	13.04 ± 1.44	21.18 ± 5.52
PMA/I*	596.16 ± 194.77	92.41 ± 10.86
<i>huCXCL8</i>		
Human INT 407 cells		
81-176	1283.94 ± 201.29	
85-102840	39.67 ± 0.41	
PMA/I*	11.80 ± 0.12	

*PMA/I was included as a separate, stimulation-positive control.

* Indicates that the absorbance value was significantly different from the media-only control ($P < 0.05$).

Fig. 1. *C. jejuni* 81-176 does not elicit chIL-8 secretion from LMH cells, as judged by ELISA. *C. jejuni* was incubated with LMH and INT 407 cell monolayers for 24 h, and supernatant fluids were assayed for IL-8 as outlined in Methods. *S. typhimurium* 85-102840-inoculated cells were used as a positive control and uninoculated cells (media only) were used as a negative control for IL-8 induction.

Supernatants from *C. jejuni*-inoculated LMH cells stimulate minimal chemotactic activity

Supernatant fluids were collected from LMH cells inoculated with *C. jejuni* at 24 h p.i. and used for the heterophil migration assays (Fig. 2). Supernatants of LMH cells inoculated with *S. typhimurium* and uninoculated cells were used as positive and negative controls, respectively. The supernatant fluids from the LMH cells inoculated with *C. jejuni* and *S. typhimurium* induced heterophil migration. However, the *S. typhimurium* supernatants induced the migration of significantly more heterophils than the *C. jejuni* supernatants ($P < 0.05$).

C. jejuni-colonized chickens produce anti-*Campylobacter* antibodies but not detectable chicken IL-8

A preliminary experiment revealed that a dose of 10^6 *C. jejuni* F38011 is sufficient to obtain 100 % colonization of chickens (not shown). A second experiment was

performed to assess antibody and chIL-8 production in *C. jejuni*-inoculated chickens. All chickens inoculated with *C. jejuni* were colonized at 8 and 15 days p.i., whereas all of the uninoculated chickens were *Campylobacter*-free (Fig. 3a). IgG and IgM antibodies reactive against *C. jejuni* were detected in the serum of the *C. jejuni*-colonized chickens at 15 days p.i., but not at 8 days p.i., as judged by immunoblot analysis (not shown). ELISAs were performed to further assess the antibody responses of the chickens. The amount of anti-*Campylobacter* antibodies in the sera from the *C. jejuni*-colonized chickens at 15 days p.i. was significantly greater than that in the sera from uninoculated chickens at 15 days (Fig. 3b). In contrast, the amount of anti-*Campylobacter* antibodies in the sera collected from the *C. jejuni*-colonized chickens at 8 days p.i. was not significantly different from the amount for the sera of the uninoculated chickens at 15 days. chIL-8 was not detected in any of the serum samples, as judged by ELISA.

Fig. 2. Chicken heterophil cell migration assays were performed with supernatant fluids of *C. jejuni* 81-176-inoculated LMH cells to assess the presence of cytokines. Supernatant fluids from *S. typhimurium* 85-102840-inoculated LMH cells and uninoculated cells (media only) were used as positive and negative controls for heterophil cell migration, respectively.

C. jejuni F38011 does not result in overt pathology in the caeca of chickens

Intestinal and caecal tissues were examined from the *C. jejuni*-inoculated and uninoculated chickens euthanized at 8 and 15 days p.i. None of the tissues collected from the *C. jejuni*-inoculated chickens revealed signs of histopathology. Shown is a representative caecal tissue sample from a chicken colonized with *C. jejuni* F38011 at 15 days p.i. (Fig. 4). Rod-shaped bacteria were observed in the deep colonic crypts of both the *C. jejuni*-inoculated and uninoculated chickens, but *Campylobacter* organisms were only recovered from the *C. jejuni*-inoculated chickens. None of the chickens exhibited epithelial necrosis, epithelial hyperplasia, or infiltration of heterophils, lymphocytes or plasma cells into the stroma of the lamina propria. Examination of the caecal tissues by TEM revealed numerous *C. jejuni* present in the glandular crypts of the inoculated chickens, but no bacteria were observed in the surrounding tissue (Fig. 5). *C. jejuni* were also observed in direct contact with the host tissue (Fig. 5c, d).

DISCUSSION

This study was conducted to further characterize the commensal-type interactions of *C. jejuni* in chickens and to compare these results with the pathogen-type interactions of *C. jejuni* with human cells. We hypothesized that the *C. jejuni* strains used in this study would behave similarly in a particular cell line, but their behaviour might differ depending on the host cell species (i.e. chicken or human). We selected four *C. jejuni* strains and assessed their interactions with chicken LMH hepatocellular carcinoma epithelial cells. The *C. jejuni* F38011 and 81-176 strains were recovered from humans with campylobacteriosis and the *C. jejuni* A2a and D34a strains were recovered from

poultry-processing plants. Many of the experiments were conducted with human INT 407 embryonic epithelial cells to quantitatively compare the virulence behaviour of *C. jejuni* strains with chicken cells versus human cells. This study differs in two fundamental aspects from earlier studies designed to characterize the interaction of *C. jejuni* with chicken cells. First, we used a continuous chicken epithelial cell line (LMH) as an *in vitro* model to examine the interactions of *C. jejuni* with chicken cells. Second, we concomitantly assessed *chCXCLi2* and *chCXCLi1* expression, *chIL-8* secretion, and production of functional chemoattractants from the chicken LMH epithelial cells inoculated with *C. jejuni*. Noteworthy is that the LMH cell line is the only chicken epithelial cell line currently available to researchers.

The *C. jejuni* strains used in this study were first tested for their ability to bind to LMH chicken hepatocellular carcinoma and INT 407 human embryonic epithelial cells. While the number of bacteria bound to the epithelial cells varied between the four *C. jejuni* strains, the differences in bacteria–host cell binding did not correlate with the source of a bacterial strain. The binding assays also revealed that the host cell species had little quantitative effect on the binding of *C. jejuni* to host cells. The results from the binding assays did not identify distinguishing characteristics to explain why *C. jejuni* is a commensal microbe in chickens and a pathogen in humans.

Striking differences were observed in the invasiveness of *C. jejuni* with LMH cells when compared with the INT 407 cells. The number of *C. jejuni* internalized by the LMH cells was significantly lower than the number of *C. jejuni* internalized by the INT 407 cells (7×10^3 – 1.6×10^4 versus 2.8×10^5 – 6.2×10^5 , respectively). For all four *C. jejuni* strains, the percentage of adherent bacteria internalized by the chicken cells was reduced more than 20 times

Fig. 3. Anti-*C. jejuni* antibodies can be detected in the sera of chickens 15 days p.i. with *C. jejuni*. (a) Caecal samples were collected at 8 and 15 days p.i. from *C. jejuni*-inoculated chickens (◇) and from uninoculated chickens (○). The c.f.u. per gram of caecal content was determined as described in Methods. (b) Sera were collected at 8 and 15 days p.i. from *C. jejuni*-inoculated and -uninoculated chickens. ◆, Sera from the *C. jejuni*-inoculated chickens at 15 days p.i.; ◇, sera from the *C. jejuni*-inoculated chickens at 8 days p.i.; ○, sera from the uninoculated chickens at 15 days p.i. The ELISAs were performed as described in Methods. chIL-8 was not detected in the sera of any of the chickens.

compared with human cells. The reduction in *C. jejuni* invasion of LMH cells was judged to be host-cell-specific because *S. typhimurium* invaded the LMH and INT 407 cells at comparable levels. To ensure that the low efficiency observed for *C. jejuni* invasion of LMH cells was not due to the hepatocellular origin of the cells, binding and invasion assays were performed with the human HepG2 hepatocellular cells. All of the *C. jejuni* strains used in this study bound and invaded the HepG2 cells at levels comparable with the INT 407 cells (results not shown). In contrast to these results, Smith *et al.* (2005) and Byrne *et al.* (2007) reported that *C. jejuni* isolates invade chicken primary cells and human cells at comparable levels. Byrne *et al.* (2007) did find that one *C. jejuni* isolate (i.e. poultry strain 1646DF) invades human cells in greater numbers than it

invades chicken cells. Interestingly, Van Deun *et al.* (2007) reported that *C. jejuni* does not invade chicken embryonic fibroblast cells. Van Deun *et al.* (2007) also reported that *C. jejuni* invades primary caecal epithelial cells, but at a level that is significantly less than observed with T84 human colonic epithelial cells. The differences reported in *C. jejuni* invasion of chicken cells suggest that the specific cell type may dictate the invasive phenotype observed rather than the virulence of the specific *C. jejuni* strain, and raises the possibility that the behaviour of LMH cells may not mimic that of cells *in vivo*. These differences also highlight the need for additional chicken continuous cell lines for comparative studies. In summary, we found that *C. jejuni* invades chicken LMH epithelial cells in significantly lower numbers than it invades human INT 407 epithelial cells.

Fig. 4. Deep caecal glands from an uninoculated (a) and *C. jejuni* F38011-inoculated (b) chicken at 15 days p.i. Note the colonization of the deep mucosal glands by slender rod-shaped bacteria (arrow heads) in both chickens and the lack of any epithelial hyperplastic response or stromal leukocyte infiltration (arrows). The caecal muscularis mucosae is indicated with an asterisk. *C. jejuni* bacteria were not recovered from the caecal sample of the uninoculated chicken (a), but were recovered from the caecal sample of the *C. jejuni* F38011-inoculated chicken (b). Both images were acquired at $\times 400$ magnification. Bars, 50 μm .

Fig. 5. Transmission electron micrographs of caecal tissues from a *C. jejuni* F38011-inoculated chicken at 15 days p.i. (a, b) *C. jejuni* in the glandular crypt. Infiltration of the tissue surrounding the crypt by the bacteria was not apparent. (c, d) Enlarged images of the boxed areas in (b). Contact between *C. jejuni* and host tissue is indicated by the arrows.

The transcript levels of *chCXCLi2* and *chCXCLi1* in LMH cells and of *huCXCL8* in INT 407 cells inoculated with *C. jejuni* were assessed by real-time RT-PCR. Each of the four *C. jejuni* strains induced the expression of both *chCXCLi1* and *chCXCLi2* from LMH cells. The expression of *chCXCLi1* in *C. jejuni*-inoculated LMH cells was greater than that of *chCXCLi2*. While this finding is in agreement with the results of Li *et al.* (2008) with primary chicken embryo intestinal cells, it is in contrast to the findings of Borrmann *et al.* (2007) and Smith *et al.* (2005) with primary chicken cells. We also assessed *chCXCLi1* and *chCXCLi2* transcript levels in response to *S. typhimurium* as a biological control (Withanage *et al.*, 2004). In contrast to *C. jejuni*, the expression of *chCXCLi2* was greater than that of *chCXCLi1* in *S. typhimurium*-inoculated LMH cells. The relevance of the differences in *chCXCLi1* and *chCXCLi2* expression from *C. jejuni*-inoculated LMH cells is not known. While *chCXCLi2* is chemotactic for macrophages and lymphocytes (Martins-Green & Feugate, 1998), the function of *chCXCLi1* has been less intensively studied and is not yet defined.

Earlier studies have revealed that *C. jejuni* induces secretion of *huCXCL8* from a variety of human epithelial cells, dendritic cells and macrophages (Hickey *et al.*, 1999; Hu *et al.*, 2006; Johanesen & Dwinell, 2006; MacCallum *et al.*, 2006). Therefore, we performed experiments to determine whether *chIL-8* (*chCXCLi1* and/or *chCXCLi2*) is secreted from LMH epithelial cells. *chIL-8* was detected in LMH cells inoculated with *S. typhimurium*, but not in supernatants from LMH cells inoculated with *C. jejuni*, as judged by ELISA. We also found that all four *C. jejuni* strains induced *huCXCL8* secretion from human HepG2 cells (not shown), indicating that the absence of detectable *chIL-8* was not an effect resulting from the hepatocellular origin of LMH cells.

Transcriptional, post-transcriptional and post-translational regulatory mechanisms determine whether cytokines are secreted to initiate the inflammatory response (Hamilton *et al.*, 2007). Earlier studies report that cytokine transcript levels are increased in primary chicken cells inoculated with *C. jejuni*, but these studies did not assess secretion and activity of cytokines (Borrmann *et al.*, 2007; Smith *et al.*, 2005). We evaluated whether chicken cells inoculated with *C. jejuni* secrete chemoattractants capable of recruiting chicken heterophils. We found that supernatant fluids collected from *C. jejuni*-inoculated LMH cells induced an increase in chicken heterophil migration relative to the untreated control supernatant fluids. However, the number of heterophils that migrated in response to supernatants from *C. jejuni*-infected cells was significantly lower than the number of heterophils that migrated in response to *S. typhimurium*-inoculated chicken cells. Based on these assays, we concluded that the response of chicken LMH cells to *C. jejuni* is distinct from that to *S. typhimurium*, in that *S. typhimurium* induces the production of cytokines that trigger a robust heterophil migration response.

Two *in vivo* experiments were conducted with broiler chickens inoculated with the *C. jejuni* F38011 strain to assess colonization, pathology of the caecal mucosa, production of serum antibodies, and serum *chIL-8* levels. In the first experiment, we found that 10^6 *C. jejuni* F38011 were sufficient for colonization of all chickens at 6 days p.i. In the second experiment, chickens were inoculated with 10^7 *C. jejuni* F38011, and caecal tissue and serum samples were collected at 8 and 15 days p.i. Anti-*Campylobacter* antibodies were not detected in the sera of *C. jejuni*-inoculated chickens at 8 days p.i., but both IgG and IgM antibodies were present in the serum of the *C. jejuni*-inoculated chickens at 15 days p.i., as judged by immunoblot analysis and ELISAs. More specifically, the level of anti-*C. jejuni* IgG antibodies detected in colonized chickens at 15 days p.i. was significantly greater than the antibody levels in the *C. jejuni*-uninoculated chickens at both 8 and 15 days p.i., as well as in the chickens colonized by *C. jejuni* at 8 days p.i. *chIL-8* was not detected in the sera from any of the chickens over the course of the experiment. Collectively, these data demonstrate that anti-*Campylobacter* antibodies are produced in *C. jejuni*-inoculated chickens between 1 and 2 weeks p.i., and that the production of these antibodies occurs in the apparent absence of stimulation of the *chIL-8* pro-inflammatory cytokine. While it is possible that *chIL-8* secretion occurred at times other than at 8 and 15 days p.i., overt pathology was not observed in the *C. jejuni*-inoculated chickens (see below). These observations distinguish the immune response of chickens to *C. jejuni* from that of humans. First, humans infected with *C. jejuni* have detectable serum levels of *huCXCL8* that peak between 3 and 10 days p.i. (Baqar *et al.*, 2001). Second, the kinetics of antibody production against *C. jejuni* is distinct in chickens compared with humans. In humans, an increase in anti-*C. jejuni* antibodies is detected at approximately 7 days post-infection (Baqar *et al.*, 2001). However, we were unable to detect anti-*C. jejuni* antibodies in the chickens inoculated with a large dose of *C. jejuni* F38011 ($\sim 10^7$ organisms) until 15 days p.i.

Caecal tissues were collected to assess pathology of tissues in chickens harbouring *C. jejuni*. It was not possible to distinguish the tissue samples of the *C. jejuni*-inoculated chickens from those of the uninoculated chickens by either haematoxylin and eosin staining or TEM. None of the *C. jejuni*-inoculated chickens exhibited epithelial necrosis or hyperplasia. In addition, we did not observe infiltration of heterophils, lymphocytes or plasma cells into the stroma of the lamina propria. *C. jejuni* were observed in loose association with the cells lining the caecal gland; areas of bacteria-host-cell binding were observed in which small fibres appeared to serve as a bridge between the cells. *C. jejuni* invasion of the tissue was not observed.

Beery *et al.* (1988) and Lee *et al.* (1986) concluded that *C. jejuni* does not adhere to the gut mucosa of chickens or mice, respectively, and that bacterial adherence is likely not important for the colonization of a host. Although not the

primary focus of this study, *C. jejuni* were observed in association with the cells of the caecal gland by TEM. The tissue-associated bacteria were confirmed to be *C. jejuni* by TEM coupled with immunogold labelling using anti-*Campylobacter*-specific antibodies (results not shown). Moreover, studies have been published since the mid-1980s demonstrating that specific proteins play a role in, and in some cases are required for, *C. jejuni* colonization of chickens. Two *C. jejuni* proteins involved in colonization, presumably due to their adhesive properties, are CadF and CapA (Ashgar *et al.*, 2007; Konkel *et al.*, 1997). Ziprin *et al.* (1999) reported that a *C. jejuni* cadF mutant is incapable of colonizing chickens. Furthermore, by comparative proteomics, Seal *et al.* (2007) found that a robust colonizing isolate of *C. jejuni* synthesizes the CadF protein in a greater amount than a poorly colonizing isolate. Additional studies are warranted to dissect the specific interactions of *C. jejuni* with cells lining the glandular crypts.

The data obtained from the ELISA, RT-PCR and heterophil migration experiments raise the concern that the ELISA may not detect both chCXCLi1 and chCXCLi2. As stated above, we found that the inoculation of LMH cells with *C. jejuni* stimulated expression of both *huCXCL8* orthologues, *chCXCLi1* and *chCXCLi2*, but at levels significantly lower than *huCXCL8* expressed from human INT 407 cells inoculated with *C. jejuni*. The supernatant fluids of LMH cells co-cultured with *C. jejuni* did not contain detectable amounts of chIL-8, as judged by the sandwich ELISA, but these same supernatant fluids did induce some chicken heterophil migration. Interestingly, chIL-8 was detected from LMH cells inoculated with *S. typhimurium* using the sandwich ELISA. Taken together, it is not clear whether the capture and detection antibodies reacted against both chCXCLi1 and chCXCLi2. The RT-PCR and heterophil migration data support the finding by ELISA that *S. typhimurium* stimulates chIL-8 secretion, but both assays also indicate that the ELISA has low sensitivity, since chIL-8 was not detected in supernatant fluids from *C. jejuni*-inoculated LMH cells. The detection of chIL-8 in the sera collected from the *C. jejuni*-inoculated broiler chickens was dependent on the sandwich ELISA, and due to the possibility that the assay may not be sensitive enough to detect both chCXCLi1 and chCXCLi2, it is feasible that at least one of these chemokines is produced and participates in inducing the humoral immune response against *C. jejuni*.

In summary, binding and internalization assays revealed that *C. jejuni* was significantly less invasive for LMH cells relative to human INT 407 cells, whereas *S. typhimurium* was able to invade both cell types at similar levels. We also found that the expression of *chCXCLi2* was greater than that of *chCXCLi1* for *S. typhimurium*-inoculated LMH cells, but that *chCXCLi1* expression was greater than *chCXCLi2* expression in LMH cells inoculated with *C. jejuni*. chCXCLi1 and chCXCLi2 have approximately 48 and 50 % amino acid identity to huCXCL8, respectively, and share 67 % identity with one another (Sick *et al.*, 2000). Based on our data, it is possible that either

chCXCLi1 or chCXCLi2 is not recognized by the huCXCL8 capture and detection antibodies. Because the production of chCXCLi1 from *C. jejuni*-inoculated LMH cells was similar with *S. typhimurium*-inoculated LMH cells, we hypothesize that the huCXCL8 antibodies used in the ELISA have greater affinity for chCXCLi2 than chCXCLi1. This hypothesis is also supported by the heterophil migration assay, in which *S. typhimurium* stimulated greater heterophil migration than *C. jejuni*. Noteworthy is that chCXCLi2 has been shown to be chemotactic for monocytes and lymphocytes (Martins-Green & Feugate, 1998), whereas the chemotactic potential of chCXCLi1 has not yet been demonstrated. Based on these data, we conclude that chicken LMH cells and human INT 407 cells respond differently to *C. jejuni* versus *S. typhimurium*. Additional studies are needed to elucidate the role of chCXCLi1 and chCXCLi2 in response to *C. jejuni*.

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