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# Induction of chicken cytokine responses *in vivo* and *in vitro* by lipooligosaccharide of *Campylobacter jejuni* HS:10

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# ABSTRACT

Campylobacter jejuni is a pathogen of the gastrointestinal tract of humans, but colonizes chickens for prolonged periods without causing disease. It is unclear what host and bacterial mechanisms maintain a non-inflammatory state in chickens. The present work was undertaken to characterize cytokine responses of chickens to purified lipooligosaccharide (LOS) of C. jejuni HS:10. Chickens were injected with purified LOS, and expression of interleukin (IL)-1 $\beta$  (pro-inflammatory cytokine), IL-8 (pro-inflammatory chemokine), interferon (IFN)γ (Th1-like cytokine), IL-10 (immune regulatory/antiinflammatory cytokine) and IL-13 (Th2-like cytokine) was evaluated in spleen using quantitative RT-PCR, up to 24 h post-injection. In an in vitro study, splenocytes were incubated with LOS, and cytokine expression followed up to 18 h. Chickens injected with LOS had increased expression of IL-1 $\beta$  up to 24 h later. Expression of IL-8 was significantly increased at 2 h but then declined below baseline. Expression of IFN $\gamma$  and IL-10 was increased significantly at 2 h, but declined thereafter. Splenocytes incubated with LOS had increased expression of IL-1 $\beta$  and IL-8 up to 18 h of incubation. Expression of IFN $\gamma$  was increased at 6 and 18 h, IL-10 was increased at 2 h, but expression of IL-13 did not differ significantly up to 18 h. It is concluded that LOS of C. jejuni can induce expression of proinflammatory IL-1 $\beta$  and IL-8, as well as IFN $\gamma$  and IL-10 in chickens. More extensive studies with more prolonged exposure to LOS are needed to further clarify the interaction between C. jejuni and the chicken host.

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# 1. Introduction

*Campylobacter jejuni* is a major bacterial cause of foodborne illness in humans (Scallan et al., 2011) but colonizes chickens for prolonged periods without causing inflammation or clinical disease (Dhillon et al., 2006). It is unclear what host and bacterial mechanisms maintain a non-

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inflammatory state in chickens. Lipopolysaccharides (LPS) and lipooligosaccharides (LOS) are integral structural components of the outer membranes of Gram-negative bacteria, that interact with pattern recognition receptors (PRR) of innate immune defenses and induce inflammatory responses. The diverse forms of LOS expressed by different strains of *C. jejuni* play an important role in colonization of chickens (Javed et al., 2012), and may assist in immune evasion. Chickens express PRR homologous to mammalian TLR1/6/10, TLR2, TLR3, TLR4, TLR5, TLR7 (Boyd et al., 2007). A homolog to mammalian TLR9 has not been identified, but unmethylated CpG can activate NF-κB *via* chicken TLR21 (Brownlie and Allan, 2011). In mammals TLR4, in







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association with myeloid differentiation protein-2 (MD-2), can mediate activation of NF-κB by LPS (Akira, 2003). In chickens, a dimer of chicken TLR2 type 2 (chTLR2t2) with MD-2 has been reported to bind LPS of *Escherichia coli* and activate NF-κB (Fukui et al., 2001). More recently, Keestra and van Putten (2008) have demonstrated activation of NFκB by LPS, mediated by a dimer of chicken TLR4 (chTLR4, an ortholog of mammalian TLR4) and chicken MD-2. Lysates of *C. jejuni* (but not live organisms) have been reported to activate NF-κB via chTLR2t2/16 and chTLR4 (de Zoete et al., 2010). In the present study, the effects of purified *C. jejuni* serotype HS:10 LOS on cytokine expression by splenocytes were examined, *in vivo* and *in vitro*, in order to better characterize the interactions between *C. jejuni* and the chicken host.

# 2. Materials and methods

# 2.1. Chickens and housing

One-day-old female commercial broiler chickens were obtained from Stratford Chick Hatchery (Stratford, ON, Canada). Chickens were housed in the animal isolation facility of the Ontario Veterinary College, University of Guelph during the experimental period. The research was approved by the University of Guelph Animal Care Committee, and adhered to the guidelines of the Canadian Council for Animal Care.

#### 2.2. Lipooligosaccharide of C. jejuni serotype HS:10

There were two experiments in this study to assess the ability of LOS to stimulate immune responses. For the *in vivo* experiment, purified LOS of serotype HS:10 containing an outer core decorated with sialic acid, inner core and lipid A was used (Fig. 1). For the *in vitro* experiment two different types of oligosaccharide were used: the complete serotype HS:10 LOS, and purified core OS of serotype HS:10 (LOS lacking lipid A and sialic acid). *C. jejuni* HS:10 cells were grown in brain heart infusion broth at 37 °C under microaerophilic conditions. The LOS was extracted as previously described by us (Shin et al., 1998). Within the limits of detection, no oligonucleotide-phosphates or protein was detected in the NMR spectra. The lipid A

#### А

#### Neu5Ac $\rightarrow$ Neu5Ac $\rightarrow$ Gal $\rightarrow$ GalNAc $\rightarrow$ Gal $\rightarrow$ LDHep $\rightarrow$ LDHep[P] $\rightarrow$ Kdo $\rightarrow$ lipid A $\uparrow$ $\uparrow$ Glc Glc

# В

**Fig. 1.** Structure of lipooligosaccharide (A) and core oligosaccharide (B) of *C. jejuni* HS:10 used in the present study. For structural details see Shin et al., 1998. Note the presence of sialic acid and lipid A in the lipooligosaccharide, but not in the core oligosaccharide. *Abbreviations*: Neu5Ac, N-acetylneuraminic acid (sialic acid); Gal, galactose; GalNAc, N-acetylgalactosamine; Hep, heptose; Kdo, 2-keto-3-deoxymannooctulosonic acid; Glc, glucose.

and sialic acid moieties were removed by 1% acetic acid at 100 °C with removal of lipid A as an insoluble material by bench-top centrifugation (5000 rpm). The oligosaccharide free of lipid A (core OS) was collected from the supernatant and purified by size exclusion chromatography (Bio-Gel P2). The LOS and core OS structures were analyzed and confirmed by Nuclear Magnetic Resonance Spectroscopy in a 400 MHz instrument with deuterium oxide as solvent as described previously in Shin et al., 1998.

# 2.3. Experimental design of in vivo experiment

In the first trial, which was conducted as an in vivo experiment, 96 fourteen-day-old chickens were divided into four groups (n = 24/group). Chickens were injected intramuscularly (IM) with either a low  $(100 \mu g [250 \mu g/$ kg]) or a high (500 µg [1250 µg/kg]) dose of purified LOS. Positive and negative control groups received lipopolysaccharide (LPS, 100 µg [250 µg/kg]) from E. coli O111:B4 (Sigma-Aldrich Canada) or PBS (100 µl), respectively. LOS and LPS doses were selected based on previous experiments in chickens involving LPS as a TLR agonist (St. Paul et al., 2011). Chickens were euthanized at 2, 6, 12 and 24 h post-treatment (n = 6 at each time point). Spleen tissue was collected and preserved in RNAlater (Qiagen, Valencia, CA) at -80 °C for RNA extraction. Blood samples were taken from the femoral vein into a tube containing disodium ethylenediaminetetraacetic acid (EDTA) and plasma was obtained after centrifugation  $(400 \times g \text{ for})$ 10 min at  $4 \,^{\circ}$ C) and stored at  $-80 \,^{\circ}$ C until analysis.

#### 2.4. Preparation of spleen single-cell suspensions

For the in vitro experiment, 6 six-week-old chickens were euthanized and spleens were harvested. Splenocytes were isolated and treated with different doses and preparations of LOS. Single-cell suspensions were prepared as described previously by Brisbin et al. (2010). Briefly, spleens were rinsed in  $1 \times$  Hanks' balanced salt solution (HBSS) and then minced with sterile scalpels. The tissue was further disrupted with the flat end of a 10-ml syringe plunger and strained through a 40 µm nylon cell strainer to obtain a single-cell suspension. The cell suspension was layered over Histopaque-1077 (Sigma, Oakville, ON, Canada) and centrifuged at  $400 \times g$  for 30 min; mononuclear cells at the interface were collected and washed twice in RPMI (Invitrogen, Burlington, ON, Canada). Cells were counted on a haemocytometer using trypan blue dye exclusion before being suspended in complete RPMI 1640 (Invitrogen) containing 10% fetal bovine serum, 200 U/ml penicillin, 80 µg/ml streptomycin, and 50 µg/ml gentamicin. The cell density was adjusted to  $1 \times 10^7$  cells/ml and stored on ice until used.

# 2.5. In vitro stimulation of spleen cells

Spleen mononuclear cells were seeded into 48-well flat-bottom plates ( $5 \times 10^6$  cells/well). Cells were stimulated with different doses ( $0.2 \mu g/ml$ ,  $1 \mu g/ml$ ,  $5 \mu g/ml$ , and  $25 \mu g/ml$ ) of *C. jejuni* LOS (which contained outer core, inner core and lipid A). Core oligosaccharide (core OS)

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