Title: Expression profiles of genes in Toll-like receptor-mediated signaling of broilers infected with *Clostridium perfringens*

Running Title: Toll-like receptor expression in *C. perfringens*–challenged chickens

Yang Lu$^{1,2†}$, Aimie J. Sarson$^2$, Joshua Gong$^{2**}$, Huaijun Zhou$^3$*, Weiyun Zhu$^1$, Zhumei Kang$^2$, Hai Yu$^2$, Shayan Sharif$^4$, and Yanming Han$^5$

$^1$Laboratory of Gastrointestinal Microbiology, College of Animal Science & Technology, Nanjing Agricultural University, Nanjing, China 210095

$^2$Guelph Food Research Centre, Agriculture & Agri-Food Canada, Guelph, Ontario, Canada N1G 5C9

$^3$Department of Poultry Science, Texas A &M University, College Station, Texas, USA 77843

$^4$Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

$^5$Nutreco Canada Agresearch, Guelph, Ontario, Canada N1G 4T2

*Corresponding author: Dr. Joshua Gong. Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ontario, Canada N1G 5C9. Telephone: +1 (519) 780-8027. Facsimile: +1 (519) 829-2600. E-mail: gongj@agr.gc.ca.

**Co-corresponding author: Huaijun Zhou; Department of Poultry Science, Texas A &M University, College Station, Texas, USA 77843. Telephone: +1 (979) 845-2994; Facsimile: +1 (979) 845-1921; E-mail: hjzhou@poultry.tamu.edu.

† Visiting graduate student to J. Gong’s laboratory.
Abstract

Toll-like receptors (TLRs) participate in detecting microbial pattern molecules for activation of host immune response. We investigated possible roles of TLRs in the chicken response to *Clostridium perfringens* infection by examining the expression of TLRs and other genes involved in TLR-mediated signaling within the spleen and ileum of *C. perfringens*-challenged broilers. When comparing challenged to naïve chickens, up-regulation of TNF-α-inducing factor-homolog was observed regardless of the incidence of necrotic enteritis (NE). In addition, the members of TLR2 subfamily were found to be most strongly involved in the host response to *C. perfringens*-challenge, although the expression of TLR4 and 7 were also up-regulated in spleen. While a combination of TLR1.2, TLR2.1 and TLR15 appeared to play a major role in the splenic response, TLR2.2 and TLR1.1 were positively correlated to the expression of adaptor genes MyD88, TRAF6, TRIF and RIP-1 in the ileum, demonstrating a dynamic spatial and temporal innate host response to *C. perfringens*.

Key words: Toll-like receptors, chickens, *Clostridium perfringens*, necrotic enteritis, Q-PCR
Necrotic enteritis (NE) is an enteric disease in chickens caused by *Clostridium perfringens* (23, 36, 40). Despite that *C. perfringens* is part of the normal intestinal microbiota, flock management changes, such as diet or exposure to additional pathogens such as coccidia, can change the population of *C. perfringens* in the small intestine. Overgrowth of *C. perfringens* often leads to NE disease through the accumulation of multiple extracellular toxins that damage the intestinal wall (40). α-toxin has historically been considered to be the major disease determinant (41). However, this notion has been challenged by the newly-discovered NetB (Necrotic Enteritis Toxin B-like) toxin that was shown to be critical for the production of NE (15). The acute form of the disease leads to high mortality in broiler flocks, while the damaged intestinal mucosa observed in the sub-clinical form of *C. perfringens* infection can result in decreased production output due to decreased digestion and nutrient absorption, reduced weight gain and increased feed conversion ratio (22).

*C. perfringens* has been well-controlled in the past through the prophylactic use of antibiotics in commercial chicken production. However, this practice of feeding growth-promoting antibiotics has been banned in the European Union countries (1), and is under review by other countries due to public concerns over widespread antibiotic resistance in bacterial pathogens. Since the reduction of dietary antibiotic use, the incidence of NE disease in chicken production has significantly increased (23). Therefore, effective alternatives to dietary antibiotics including those that can improve overall host immune response are urgently required.

A full understanding of *C. perfringens* infection and its related pathogenesis and immunology is essential for developing effective control strategies other than antibiotic therapies. Recently, there has been significant progress in understanding the molecular mechanisms of pathogenesis of *C. perfringens* infection in chickens. Specifically, necrotic enteritis toxin B-like (NetB) toxin...
and bacteria-secreted collagenases were described as major determinants for NE lesions, in
addition to α-toxin that had been considered to cause the disease (15, 16, 28). In contrast to this
progress, the immunology relating to *C. perfringens* infection is still poorly understood,
including immune recognition of the pathogen.

The Toll-like receptor (TLR) family is a highly conserved group of proteins that
participate in pathogen detection and initiation and regulation of innate and adaptive immune
response (18, 39). In avian species, ten TLRs have been discovered to date, including orthologs
of mammalian TLR3, 4, 5 and 7. In humans, TLR1 and 6 have been shown to form a
heterodimer with TLR2 to recognize bacterial lipoproteins and peptidoglycan. In chickens,
TLR1.1 and TLR1.2, two members of TLR1/6/10 family, demonstrated the same function,
respectively (4, 10). The chicken genome also contains duplicated TLR2 genes (TLR2.1 and
TLR2.2) and unique TLRs (TLR 15 and 21) that might function in association with TLR2
subfamily (31). In mammals, many of the TLR signaling pathways have been defined. Typically,
TLRs activate innate and adaptive responses through a series of intracellular signaling cascades
that are MyD88-dependent or –independent/TRIF-dependent (18). Following MyD88 or TRIF
activation, several adaptor molecules such as IRAK molecules are recruited to initiate
transcription factor activation and subsequently induce cytokine (e.g. IL-6) and chemokine (e.g.
IL-8) production (18). Although the genes that encode some of these adaptor molecules,
transcription factors and immune mediators have been identified in chickens, little is known
about chicken TLR-regulated pathogen recognition and subsequently initiated systemic host
responses to *C. perfringens* infection. As a first step to identify the relationship between key
molecules in TLR signaling during *C. perfringens* infection, the present study sought to examine
gene expression profiles of TLR-mediated signaling molecules in the spleen and ileum tissues of
broiler chickens challenged with *C. perfringens*.

**MATERIAL AND METHODS**

**Bacterium.** A type A strain of *C. perfringens* was grown in Mueller-Hinton broth or on Mueller-Hinton agar containing 5% (vol/vol) sheep blood at 37°C under an anaerobic atmosphere (85% N2, 10% CO2, and 5% H2). The bacterium has routinely been used to induce NE in broiler chickens at Nutreco Canada Agresearch as described previously (5).

**Chicken trial.** The chickens used for the present study were the same birds for our previous challenge study, from which the gene expression of α-toxin in the chicken intestine (35) and host response to *C. perfringens* infection with both low-density chicken immune cDNA microarray and 44K whole chicken genome Agilent microarray (34, 42) had been reported. The chickens were cared for under the guidelines of the Canadian Council on Animal Care (1993). Briefly, six hundred one-day-old chicks (Ross × Ross) were originally used and randomly allocated to twelve pens fed an all-vegetable starter diet (Shur-Gain; Nutreco Canada). Three hundred of these birds (in six pens) were fed an antibiotic-medicated diet and showed no NE disease and low *C. perfringens* colonization in the ileum (35), and thus were not subject to further analysis in the present study. On day 18 post-hatch, birds were challenged with the Type A strain of *C. perfringens* (5) at the stationary phase at 10⁷ colony-forming units (CFU) per gram feed and 40 grams feed per chicken for 16 hr after 8 hr starvation. The day of *C. perfringens* challenge was designated D0 post-infection (PI). On D0 PI, two birds from each pen were randomly selected for downstream molecular analyses to examine the host responses, and euthanized with CO₂ before the *C. perfringens* challenge. The remaining birds in each pen were originally used for the statistical analysis of animal performance that had been reported earlier.
The same sampling procedure was repeated for 4 days (D1, D2, D3, and D4 PI) after the challenge. Spleen and ileum tissues were collected from each bird for total RNA isolation. The 5-cm long section of ileal tissue was collected 1-cm away from the end of the cecal junction, digesta was removed and the tissue was rinsed with 3 - 5 ml saline. Tissues were snap-frozen until RNA extraction. RNA extracted from frozen samples collected on D0, D1, D2, and D4 PI was used for quantitative reverse transcription PCR (QRT-PCR) assays.

**RNA isolation.** Spleen or ileum tissue was homogenized using a PRO200 Homogenizer (DiaMed, Mississauga, ON, Canada). Total RNA was isolated from each homogenized tissue using Trizol extraction method as described by the manufacturer (Invitrogen, Carlsbad, CA). The RNA samples were treated to remove DNA using TURBO DNase-free™ Kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The RNA quantity was determined by NanoDrop ND-1000 spectrophotometer at 260/280nm (Nano Drop Technologies, Wilmington, Delaware). cDNA for QRT-PCR assays was synthesized from 1 µg of purified RNA using 50 ng of random hexamers and the Superscript II First Strand cDNA Synthesis kit (Invitrogen, Mississauga, ON) following the manufacturer’s instructions.

**Quantitative reverse transcription PCR.** QRT-PCR primers were either synthesized based on published sequences or designed for this experiment. PCR primers for the genes of TNF-α-inducing-factor-homolog, IL-6 and β-actin were those described previously by Mohammed *et al.*, (25) Hong *et al.*, (12) and Abdul-Careem *et al.*, (2), respectively. PCR primers for IL-8 (CXCLi2) gene were obtained from Dr. Sharif’s laboratory (unpublished). PCR primers for remaining target genes were developed through the present study. The primers were designed using the Vector NTI 8 software program (Invitrogen Corporation) according to
sequences acquired from GenBank employing keyword searches. The specificity of designed
primers was firstly examined by comparison with database sequences (NCBI,
http://blast.ncbi.nlm.nih.gov/Blast.cgi) through BLAST analysis and then experimentally verified
through PCR assays. The PCR conditions for each target gene were optimized and the optimal
conditions are listed in Table 1.

QRT-PCR assays were performed on a Stratagene MX3005 thermal cycler with brilliant
SYBR green QRT-PCR Master Mix (Stratagene, La Jolla, CA). cDNA was diluted 10-fold, and
1 µl of each diluted sample was added to a 25 µl reaction solution containing 12.5 µL of 2X
master mix, 150 nM of each primer, and 30 nM ROX reference dye. Cycling parameters were as
follows: 10 min at 95°C, then 35 - 40 cycles of 95°C for 30s, annealing temperature for 30s, and
72°C for 30s, followed by an extension for 2 min at 72°C.

For a relative quantitative measurement, a standard curve of each target gene was
acquired. The standard curves were established through amplification and cloning of individual
target genes (including β-actin that served as a reference) into a plasmid (Topo 10 vector from
Invitrogen; Burlington, Ontario, Canada) followed by a log₁₀ series dilution of the plasmid
harbouring cloned genes. Each experiment was performed in duplicate with the same
plasmid dilution used as a calibrator. The amplification efficiency (E) was calculated as 1x10⁻¹/slope and
the relative expression was calculated with efficiency correction as $E_T \times (C_{pT(c)} - C_{pT(s)}) \times E_R \times (C_{pR(s)} - C_{pR(c)})$, where $E_T$ stands for the efficiency of target gene determined by the standard curve; $E_R$ for
the efficiency of reference gene determined by the standard curve; $C_p$ for the crossing point
(threshold); S for the sample; C for the calibrator (the plasmid) (30). Fold changes were then
calculated by taking ratios of the relative expression between CP-challenged birds compared to
naïve birds at D0 PI.

**Statistical analysis.** The relative expression of each gene was log2-transformed before proceeding with statistical analysis. Statistical computations were performed using the Statistical Analysis System (SAS version 9.0, SAS Institute Inc., Cary, NC). The gene expression data were subjected to the analysis of variance using GLM procedure. Least square means of the relative gene expression were calculated using the option of LSMEANS and statistical differences between the treatments were identified at $p < 0.05$ using the option of PDIFF.

Spearman correlation was used to examine relationships among genes.

**RESULTS**

**Challenge results.** The response to *C. perfringens* challenge was previously described (35) and therefore will not be elaborated on within this study. However, it is important to note that birds challenged with *C. perfringens* became infected beginning at D1 PI and showed both ileal *C. perfringens* counts and lesion scores beginning on D1 and D2 PI, respectively.

Furthermore, spleen tissue from this study was examined and tested positive for bacterial presence by Q-PCR of alpha-toxin gene expression across all time points (data not shown).

**Generation of QRT-PCR Standard Curves.** Standard curves for relative quantification of target and housekeeping genes were generated on the Stratagene MX3005 thermal cycler, using 3 technical replicates and 10 serial dilutions. The values identified for PCR efficiency (E) of standard curves ranged from 1.89-2.03 with the majority of efficiencies ranging from 1.97-2.02. R-squared values of the standard curve reactions ranged from 0.991-0.999, with the majority of values above 0.995.

**TLR gene expression profile during CP challenge.** QRT-PCR analysis was performed
to further define and compare TLR signaling gene expression profiles in both ileum and spleen tissues. TLR expression data acquired from QRT-PCR are presented in Figure 1, which demonstrates that the expression of TLR7 was increased on D2 PI in spleen and TLR1.2, TLR2.1 and TLR15 expression was increased on D1 and D2 PI followed by a decrease on D4 PI, which was only significant for TLR1.2 (Fig. 1). While TLR1.1 and TLR4 only showed up-regulation on D1 PI in spleen tissue, TLR2.2 expression was not significantly affected by *C. perfringens* infection at any time point. In the ileum, significant differences in expression were only observed for some members of the TLR2 subfamily. TLR1.1 was up-regulated at D2 PI and TLR1.2 and TLR15 were down-regulated at D4 PI.

**Expression of adaptor and signaling molecule genes involved in the TLR signaling pathway.** Several adaptor and signaling molecules involved in the TLR signaling pathway have been shown to play important roles in bacterial infections of mammals. In this study, the expression of TLR pathway-associated adaptors in chickens before and after *C. perfringens* challenge was investigated (Fig. 2). Compared with D0 PI, no significant differences were observed for most adaptor or signaling molecule genes in the spleen, except for a decrease in IRF3 (interferon regulatory factor 3) expression on D2 and D4 PI. In the ileum, the expression of MyD88, TRAF6 (TNF receptor associated factor-6), and TRIF (TIR-domain-containing adapter-inducing interferon-β) genes all increased after *C. perfringens* challenge from D2 to D4 PI. However, IRF3 and RIP-1 (receptor interacting protein-1) genes were also up-regulated on D2 PI. MAL adaptor expression was not affected by *C. perfringens* challenge in spleen or ileum at any time point.

**Expression of cytokines.** Gene expression of four cytokines, including TNF-α-inducing
factor-homolog, interferon (IFN)-β, interleukin (IL)-6, and CXCL2 (IL-8) that can be produced as a result of TLR activation, was assessed by QRT-PCR assays. Splenic and ileal tissues demonstrated different expression patterns of the cytokines in response to clostridial infection (Fig. 3). IL-6 expression was not detected ileal samples. The cytokine was expressed in the spleen, but showing no significant changes in response to *C. perfringens* challenge. IFN-β also exhibited no significant differences in gene expression before and after *C. perfringens* challenge.

In contrast to these observations, chicken TNF-α-inducing factor-homolog expression was decreased significantly on D4 PI in the spleen, but increased after *C. perfringens* challenge (D1 and D2 PI) in the ileum. CXCL2 (IL-8) expression increased significantly at all time points in the ileum after *C. perfringens* challenge, but this pattern was not observed in the spleen.

**Correlation of the members of TLR2 subfamily and other genes.** To investigate the relationship between expressed TLR2 subfamily members, the correlation coefficient (CC) has been estimated between pairs of TLR signaling pathway after *C. perfringens* challenge (D1, D2 and D4 PI). A significant correlation is defined by the correlation coefficient with a *p* value less than 0.05. A positive correlation between TLR2 subfamily gene expression was observed after *C. perfringens* challenge (Table 2) in the spleen, with TLR1.2 and TLR15 being the most highly correlated. In the ileum, correlations within the TLR2 subfamily were also observed. The highest correlation was found between TLR21 and TLR2.2. Among the four members of chicken TLR1 and TLR2 subfamily (TLR1.1/1.2 and TLR2.1/2.2), the highest correlations were TLR1.2 and TLR2.1 in spleen and TLR1.1 and TLR2.2 in ileum (Table 2). In the present study, TLR15 in spleen and TLR21 in ileum were the most frequently observed to have significant correlations within the chTLR2 subfamily. When comparing the expression profiles of TLR genes to adaptor and cytokine genes, positive correlations were also observed. Table 3 outlines the correlations.
between significantly changed genes after \textit{C. perfringens} challenge and TLRs, whereby TNF-\(\alpha\)-inducing factor-homolog was the only gene significantly correlated with the TLR family members in spleen tissue. In contrast, gene expression of more than one adaptor molecules and CXCLi2 (IL-8) were correlated to the expression of multiple TLR genes. TLR15 in the ileum demonstrated no correlation with the genes encoding adaptor molecules and CXCLi2 (IL-8).

\section*{Discussion}

The Toll-like receptor (TLR) family is a highly conserved group of proteins that participate in pathogen recognition and initiation and regulation of immune response \cite{6}. The avian TLR repertoire comprises both orthologous and distinct TLR genes, since avian and mammalian lineages diverged from a common ancestor about 300 million years ago. For example, the chicken genome contains genes with homology to mammalian TLR3, 4, 5 and 7, fish/amphibian TLR21 and the novel chicken gene TLR15 \cite{4, 37}. In the present study, several TLR genes appear to be involved in the host response to \textit{C. perfringens} infection, with emphasis on the up-regulation of the TLR2 subfamily, although both TLR4 and TLR7 expression also increased in the spleen. These results are consistent with observations in mammals, where gram-positive bacteria present a tendency for signaling through the TLR2 pathway. The signaling via TLR4 was also observed, however, to a lesser extent \cite{38}.

\textit{C. perfringens} infection occurs in the small intestine of chickens. Previous studies relating to the host response to \textit{C. perfringens} has mainly involved the examination of pathological effects on the intestinal tissue and the identification of NE-disease-causing bacterial components. Nonetheless, the pathogen has also been detected systemically, in the spleen of NE-positive chickens by Collier \textit{et al.} \cite{8} and our group (unpublished data). Similarly, systemic
response was indicated by elevated serum antibodies to *C. perfringens* from approximately 2-4 weeks post-infection (20, 21). Given these reports of systemic host response to *C. perfringens* and our previous findings of differential TLR gene expression in the spleen of *C. perfringens*-infected chickens (34), our aim was to determine the host recognition and response to *C. perfringens* that surrounds TLR pathway signaling during the local and systemic host responses.

*C. perfringens* challenge had a robust effect on gene expression profiles. In many cases, expression of several TLR genes was up-regulated after challenge, however, for certain time points, only significant down-regulation was observed (Fig. 1-3). These results show the variability of the TLR response to *C. perfringens* antigens in both the spleen and ileum, where one TLR signaling pathway does not appear to be dominant over the other in terms of host response to *C. perfringens* infection. This pattern is also true of the adaptor and cytokine genes measured throughout the study, suggesting that the innate host response to *C. perfringens* can only be characterized by a complex combination of responses varying based on temporal as well as spatial influences. Of these TLRs, some have no previously-identified functions in chickens, and the others have only been described for their response to other bacterial pathogens rather than *C. perfringens*. Specifically, TLR15 gene expression has only been previously noted to be up-regulated in the cecum of *Salmonella enterica* serovar Typhimurium (SE)-infected chickens, and in heterophils stimulated with SE and thus until this study, has been considered specific to chicken host responses to *Salmonella* pathogenesis alone (9, 27). Similar to TLR15, TLR21 expression has only briefly been described in chickens in response to *Salmonella* and *Campylobacter* infection (24). Further to these previous observations, our results indicate that TLR15 and TLR21 expression changes are differentially regulated following *C. perfringens* exposure, which could considered central to host responses to intestinal bacterial infection in
Upon examining the gene expression profiles in ileal and splenic tissues, large differences were detected between tissue responses, in regards to the specific gene or to the direction of the change (i.e. up- or down-regulated in comparison with naïve chickens) (Fig. 1-3). Primarily, we observed that *C. perfringens* infection caused a change in expression of most TLRs within the spleen. Since TLR 1.2, 2.1 and 15 expression was up-regulated on D1 and D2 PI, TLR4 and TLR7 expression was up-regulated on D1 and D2 PI respectively, we would have predicted up-regulation of MyD88-dependent downstream signaling events leading to pro-inflammatory cytokine production, which are typically in mammalian counterparts. However, no significant changes were observed for MyD88 or TRAF6 pathway expression, except for the decrease in TNF-α-inducing factor-homolog expression. Similarly, TRIF and its downstream signaling molecules, which are involved in mammalian MyD88-independent TLR3 and TLR4 activation, also showed no significant changes in the spleen except for the decrease in IRF3 expression. The relationship between TLR4 activation and IRF3 up-regulation has been fairly well investigated. In concert with our results, Sakaguchi (33) shows that stimulation with bacterial LPS activates TLR4 in an IRF3-dependent response in mice, which extends to IFN-B-dependent when IRF7 is also activated. To interpret the failure of up-regulation of MyD88 pathway-dependent adaptors, one could hypothesize that these TLRs may not signal through the MyD88 pathway as TLR2 molecules do in mammals. Alternatively, our sampling may have missed the time window to capture transcription; as well, we did not assess protein mechanisms that may be more indicative of MyD88 activation, such as docking or phosphorylation. Lastly, in the case of TLR4, over-stimulation of TLR4 by both endogenous ligands and chronic exposure to exogenous ligands following bacterial lysis has been described to cause systemic
inflammation leading to death (32). Whether the typical TLR4 signaling pathway involving MyD88 is activated during this exacerbated response, is yet to be determined, and may explain the up-regulation of the TLR4 that is not necessarily linked to MyD88 gene expression.

Seemingly important to the host response to *C. perfringens*, chicken TNF-α-inducing factor-homolog has been previously suggested to be involved with initiation of TNF-α production. TNF-α in mammals is a member of a group of NF-κB-activated signaling cytokines that stimulates systemic inflammation. In chickens, a TNF-α modulating factor production has been shown to increase along with inflammatory cytokines after exposure to *Eimeria* and *Salmonella* species (7, 11, 17, 29). Given the inflammatory nature of NE disease, and the predominance of innate and inflammatory based responses observed in the present study, TNF-α-inducing factor-homolog expression could be indicative of the initiation of a TNF-α-type response in chickens infected with *C. perfringens* (3, 13).

In the ileum, the gene expression of TLR4 and adaptor MAL was unchanged. In addition, there was no IL-6 expression. Previously, *C. perfringens*-derived β-toxin failed to induce the production of IL-6 in mice, despite its typical role as a pro-inflammatory cytokine produced following TLR pathway activation (26), suggesting that this cytokine is not involved in TLR regulation of *C. perfringens*-induced NE. In contrast to IL-6, the expression of MyD88, TRAF6, TRIF and RIP-1 was all increased in the ileum after *C. perfringens* challenge in the present study. Considering that activation of adaptor genes including MyD88, which is typically followed by production of inflammatory immune mediators such as IL-6, IL-1β and TNF-α in mammals (14, 18), one could expect that such genes would be up-regulated in the ileum of *C. perfringens*-infected chickens. The present data shows that TNF-α-inducing factor-homolog and
CXCLi2 (IL-8) expression were both up-regulated in the ileum, in contrast to the spleen where the only significant change was the decreased TNF-α-inducing factor-homolog expression.

In addition to the statistical significance of gene expression changes, correlations between gene expression patterns were investigated to further infer functional relationships of TLR-mediated genes following *C. perfringens* challenge (Table 2 and 3). In particular, genes were highlighted when their expression profile was correlated with significant changes after *C. perfringens* challenge. In the spleen, the cooperative effect of TLR1.2, TLR2.1 and TLR15 in the initiation of immune response on D1 or D2 PI was confirmed by positive Pearson’s correlation between TLR1.2, TLR2.1, TLR15 and TNF-α-inducing factor-homolog expression. However, IRF3, which was the only gene that significantly decreased after *C. perfringens* challenge, yet was not significantly correlated with TLR expression. In contrast, ileal gene expression profiles showed no change of these genes on D1 or D2 PI. Yet similar to the spleen, TLR1.2, TLR2.1 and TLR15 expression was decreased on D4 PI, and all expression profiles were positively correlated, suggesting an overlap in functionality of these genes amongst immune-related tissues at the later time points.

In summary, the present study has revealed the possible role of the TLR2 subfamily and less-studied, novel chicken TLRs, TLR15 and TLR21, in innate chicken responses to *C. perfringens* infection. Chickens seem to differ in part from mammals in TLR-mediated signaling, particularly based on the divergence of the TLR2 subfamily and potential functional differences. These observations warrant further functional studies to determine the TLR signaling pathway in chickens that may lead to a discovery of potential targets in innate immunity for control of NE disease.
ACKNOWLEDGMENTS

This research was supported by Agriculture & Agri-Food Canada through the A-base program. The chicken trial with sample collection was made possible through the support from Nutreco Canada Agresearch and Poultry Industry Council. Y.L. was a visiting graduate student to the laboratory of J.G. supported by the China Scholar Council through the MOE-AAFC Ph.D. Research Program. A.J.S. was a NSERC Visiting Fellow to Canadian Federal Government Laboratories.
REFERENCES


bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily.


immunization of broiler chickens against necrotic enteritis with an attenuated Salmonella vaccine vector expressing Clostridium perfringens antigens. Vaccine 26:4194-203.


Figure 1. Fold changes in the gene expression of TLRs in the spleen and ileum of *C. perfrigens*-challenged chickens determined by QRT-PCR assays. Relative expression of Toll-like receptors (TLRs) was measured by QRT-PCR in the spleen and ileum of chickens. Relative expression data were logarithm (to base 2) transformed to acquire normal distribution after normalizing against β-actin expression and a constant concentration of plasmid calibrator using GLM statistical analysis within the same tissue (spleen or ileum) (n=8). Data are presented as fold changes (FC) by comparing D1, D2 and D4 PI with D0 PI. FC > 1 represents up-regulation; FC < -1, down-regulation. The bars denoted with stars (*) are significantly different before and after challenge (p < 0.05).

Figure 2. Fold changes in the gene expression of adaptor molecules involved in the TLR signalling pathway in the spleen and ileum of *C. perfrigens*-challenged chickens determined by QRT-PCR assays. Relative expression of adaptors of TLR signalling pathway was measured by QRT-PCR in the spleen and ileum of chickens. Relative expression data were logarithm (to base 2) transformed to acquire normal distribution after normalizing against β-actin expression and a constant concentration of plasmid calibrator using GLM statistical analysis within the same tissue (spleen or ileum) (n=8). Data are presented as fold changes (FC) by comparing D1, D2 and D4 PI with D0 PI. FC > 1 represents up-regulation; FC < -1, down-regulation. The bars denoted with stars (*) are significantly different before and after challenge (p < 0.05).

Figure 3. Fold-changes in the gene expression of cytokine genes in the spleen and ileum of *C. perfrigens*-challenged chickens determined by QRT-PCR assays. Relative expression of cytokine genes was measured by QRT-PCR in the spleen and ileum of chickens. Relative expression data were logarithm (to base 2) transformed to...
acquire normal distribution after normalizing against β-actin expression and a constant concentration of plasmid calibrator using GLM statistical analysis within the same tissue (spleen or ileum) (n=8). Data are presented as fold changes (FC) by comparing D1, D2 and D4 PI with D0 PI. FC > 1 represents up-regulation; FC < -1, down-regulation. The bars denoted with stars (*) are significantly different before and after challenge ($p < 0.05$).
<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Product Size (bp)</th>
<th>Genbank Accession Number</th>
<th>Annealing Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1-1 F</td>
<td>CTGTCTTGGCAATCTGTC</td>
<td>194</td>
<td>AY633574</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>GTGAAGGCTCCGTGATT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1-2 F</td>
<td>AGCTGCAGACTTCTTCGCG</td>
<td>264</td>
<td>NM_001098854</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>TTGCTTGGTCACCTGCCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2-1 F</td>
<td>TTAAAAAGGTTGGGCCAGGAG</td>
<td>271</td>
<td>AB050005</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>GTCCAAAACCATGAAAGAGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2-2 F</td>
<td>AGGCACCTGAGATGGAGAC</td>
<td>314</td>
<td>AB046533</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>CCTGTATGGGCCAGGTCCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4 F</td>
<td>GTCTCTTCTCTACATGCAGTCGTC</td>
<td>187</td>
<td>AY064697</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>AGGGAGAAGAACAGGAGTATTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR7 F</td>
<td>GAAAACGCTACTAACCCTG</td>
<td>282</td>
<td>DQ780342</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>GTTGGACTCCAGACTCAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR15 F</td>
<td>GTTCTCTCCAGGTGGTTAATAAGC</td>
<td>262</td>
<td>NM_001037835</td>
<td>56</td>
<td>c</td>
</tr>
<tr>
<td>R</td>
<td>GTGGTCATTGGTTGTTTTTAGGAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Forward/Reverse</td>
<td>Sequences</td>
<td>Accession</td>
<td>Cnt</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>TLR21</td>
<td>F</td>
<td>ATGATGGAGACAGCGGAGAAGG</td>
<td>NM_001030558</td>
<td>62  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGATGCAGCGGAAGTACAAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyD88</td>
<td>F</td>
<td>AGAAGGTGTCGGAGGATGGTG</td>
<td>NM_001030962</td>
<td>57  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGGCTCCAAATGCTGACTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP1</td>
<td>F</td>
<td>AGTGCTCCAAAAGTCCCATACC</td>
<td>AB108485</td>
<td>56  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGTCTCTTCTTTGGTCAGCCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAF6</td>
<td>F</td>
<td>GAGTGTCCAAGGCGTCAAGTCTG</td>
<td>XM_421089</td>
<td>57  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTGTGCTGCCCAGTTCACTCCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF3</td>
<td>F</td>
<td>CGTACTTCTCAGATCCCTTGG</td>
<td>U20338</td>
<td>56  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGTCGGTGCACCTTTGGAGCCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRIF</td>
<td>F</td>
<td>TCAGCCATTCTCCGTCTCTCTC</td>
<td>EF025853</td>
<td>57  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTCACGCAGAAGGATAAGGAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAL</td>
<td>F</td>
<td>CTCATAGCACCACCCAGCCACTC</td>
<td>DQ019929</td>
<td>56  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGTAATCCTCAGGTCATTGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-β</td>
<td>F</td>
<td>ACCACGGCTCTTCCCATCAAC</td>
<td>X14455</td>
<td>56  c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTCATTTCTTCAGGTCATCCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-like F</td>
<td>TGGTTTTATGAGGCGCC</td>
<td>AY765397</td>
<td>57  18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTTTCAGAGCATCAACGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>Reverse</td>
<td>Accession</td>
<td>Length</td>
<td>bp</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>---------------</td>
<td>-----------</td>
<td>--------</td>
<td>----</td>
</tr>
<tr>
<td>IL-6 F</td>
<td>CAAGGTGACGGAGGAGGAC</td>
<td>TGGCGAGGAGGGATTTCT</td>
<td>AJ309540</td>
<td>254</td>
<td>56</td>
</tr>
<tr>
<td>IL-8 F</td>
<td>ATGAACGGCAAGCTTGGAGCTG</td>
<td>TCCAAGCACACCTCTCTTCCATCC</td>
<td>AJ009800</td>
<td>233</td>
<td>57</td>
</tr>
<tr>
<td>β-actin F</td>
<td>CAACACAGTGCTGTCTGCTGG</td>
<td>ATCGTACTCTGCTGTGCTGAT</td>
<td>X00182</td>
<td>205</td>
<td>55</td>
</tr>
</tbody>
</table>

* Forward.  
* Reverse.  
* Primers developed for the present study.  
* Chicken TNF-α-inducing factor-homolog.  
* Primers obtained from Dr. Sharif’s laboratory (unpublished).

26
Table 2. Spearman’s rank correlation coefficients for TLR gene expression within spleen and ileum of *C. perfrigens*-challenged chickens.

<table>
<thead>
<tr>
<th>Spleen</th>
<th>TLR1.1</th>
<th>TLR1.2</th>
<th>TLR2.1</th>
<th>TLR2.2</th>
<th>TLR15</th>
<th>Ileum</th>
<th>TLR1.1</th>
<th>TLR1.2</th>
<th>TLR2.1</th>
<th>TLR2.2</th>
<th>TLR15</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1.2</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2.1</td>
<td>0.32</td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
<td>0.29</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2.2</td>
<td>0.42</td>
<td>0.21</td>
<td>0.05</td>
<td></td>
<td>0.55</td>
<td>0.31</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR15</td>
<td>0.54</td>
<td>0.71</td>
<td>0.68</td>
<td>0.21</td>
<td>-0.01</td>
<td>0.56</td>
<td>0.21</td>
<td>-0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR21</td>
<td>0.13</td>
<td>-0.11</td>
<td>-0.06</td>
<td>0.38</td>
<td>-0.04</td>
<td>0.56</td>
<td>0.24</td>
<td>0.43</td>
<td>0.75</td>
<td>-0.05</td>
<td></td>
</tr>
</tbody>
</table>

Correlation coefficients (CC) were calculated in a pair-wise manner, where high CC values (e.g. approaching 1.00 or -1.00) indicate a high degree of correlation. Positive CC values indicate positive correlation between gene expression and negative (-) CC values indicate negative correlation between gene expression. Significant correlations (*p* < 0.05) are in bold font.
Table 3. Spearman’s rank correlation coefficient for TLR gene expression with adaptor molecule and cytokine genes within spleen and ileum of *C. perfrigens*-challenged chickens.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Spleen</th>
<th>TLR1.1</th>
<th>TLR1.2</th>
<th>TLR2.1</th>
<th>TLR2.2</th>
<th>TLR15</th>
<th>TLR21</th>
<th>TLR7</th>
<th>TLR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyD88</td>
<td></td>
<td>0.53</td>
<td>0.41</td>
<td>0.29</td>
<td>0.79</td>
<td>0.01</td>
<td>0.85</td>
<td>0.71</td>
<td>0.58</td>
</tr>
<tr>
<td>TRAF6</td>
<td></td>
<td>0.54</td>
<td>0.09</td>
<td>0.25</td>
<td>0.49</td>
<td>-0.25</td>
<td>0.61</td>
<td>0.47</td>
<td>0.39</td>
</tr>
<tr>
<td>TRIF</td>
<td></td>
<td>0.36</td>
<td>0.17</td>
<td>0.19</td>
<td>0.55</td>
<td>-0.05</td>
<td>0.67</td>
<td>0.43</td>
<td>0.58</td>
</tr>
<tr>
<td>IRF3</td>
<td></td>
<td>0.49</td>
<td>0.08</td>
<td>0.46</td>
<td>0.63</td>
<td>-0.02</td>
<td>0.70</td>
<td>0.59</td>
<td>0.28</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>0.14</td>
<td>0.19</td>
<td>0.45</td>
<td>0.43</td>
<td>0.12</td>
<td>0.48</td>
<td>0.37</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Correlation coefficients (CC) were calculated in a pair-wise manner, where high CC values (e.g. approaching 1.00 or -1.00) indicate a high degree of correlation. Positive CC values indicate positive correlation between gene expression and negative (-) CC values indicate negative correlation between gene expression. Significant correlations (*p* < 0.05) are in bold font.
Figure 1.
Figure 1. continued
Figure 2.

**MyD88**

**MAL**

**TRAF6**

**TRIF**

**IRF3**

**RIP-1**

Fold change vs. D1, D2, D4 in Spleen and Ileum.

Legend:
- Spleen
- Ileum
- Fold change
- D1
- D2
- D4
- * Significant difference

Downloaded from http://cvl.asm.org/ on January 10, 2021 by guest
Figure 3.