



# Systemic cytokine response in pigs infected orally with a *Lawsonia intracellularis* isolate of South Korean origin

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**ABSTRACT.** In the swine industry, *Lawsonia intracellularis* is one of the main enteric pathogens; it causes acute intestinal hemorrhage (proliferative hemorrhagic enteropathy) in naïve adult pigs and a wasting disease (proliferative enteropathy) in growing pigs. Among many kinds of cytokines, interferon- $\gamma$  (IFN- $\gamma$ ) has previously been reported to play a significant role in limiting intracellular infection and increasing cellular proliferation associated with *L. intracellularis*. However, the levels of various circulating inflammatory cytokines, including IFN- $\gamma$ , in animals infected with *L. intracellularis* is still an area of considerable interest for understanding immunity against this bacterium. In addition, there has been no information on cytokine response in animals infected with any *L. intracellularis* isolate of South Korean origin or Asian origin. To determine the relationship between the changes in the systemic inflammatory cytokine response in the peripheral blood of the host after *L. intracellularis* infection, we measured the levels of some pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and IFN- $\gamma$ ), anti-inflammatory cytokines (IL-4, IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ )), and a chemokine (IL-8) in pigs infected with *L. intracellularis* isolated from South Korea. This study demonstrated that a *L. intracellularis* isolate of South Korean origin induced cytokine (TNF- $\alpha$ , IL-6, and IFN- $\gamma$ ) responses in infected animals within 15 days post-infection although the circulating levels of IL-4, IL-10, IL-8 and TGF- $\beta$  were induced relatively late.

**KEY WORDS:** antibody, cytokine response, diagnosis, infection, *Lawsonia intracellularis*

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Proliferative enteropathy (PE) is an infectious intestinal disease characterized by thickening of the distal small and proximal large intestinal mucosa as a result of enterocyte proliferation associated with the presence of an intracellular bacterium [25]. *Lawsonia intracellularis* is the etiological agent that causes PE in various species, especially pigs, horses, and hamsters. *L. intracellularis* is a highly fastidious obligate intracellular Gram-negative bacterium. In the swine industry, *L. intracellularis* is one of the main enteric pathogens; it causes acute intestinal hemorrhage (proliferative hemorrhagic enteropathy, PHE) in naïve adult pigs and a wasting disease (PE) in growing pigs [8, 13]. Variations in microbial virulence, the spectrum of symptoms, and antibody responses have been described for *L. intracellularis* infection or vaccination [1, 16, 17, 19].

Generally, cytokines are involved in many pathophysiological processes of the body. They act as intercellular messengers and exert potent biological effects at extremely low concentrations. Among various cytokines, interferon- $\gamma$  (IFN- $\gamma$ ) has been reported to play a significant role in limiting intracellular infection and increasing cellular proliferation associated with *L. intracellularis* and so far most immunological researches on *L. intracellularis* infection have focused mainly on IFN- $\gamma$  [2, 3, 20, 22]. The relevance of IFN- $\gamma$  in *L. intracellularis* infection has been assessed mainly using IFN- $\gamma$  receptor knockout mice [2, 22], normal pigs [3, 16, 17, 20, 21], and horses [18].

Certainly, IFN- $\gamma$  is one of the most important immunological molecules because IFN- $\gamma$  is produced in large amounts and is crucial for the control of infectious diseases. Although several papers on mucosal, systemic, or primed cell-mediated immune responses to *L. intracellularis* have recently been published [1, 16, 17, 21], the levels of various circulating inflammatory cytokines, including IFN- $\gamma$ , in animals infected with *L. intracellularis* is still an area of considerable interest for understanding immunity against PE. In addition, there has been no information on cytokine response in animals infected with any *L. intracellularis* isolate of South Korean origin or Asian origin.

To determine the relationship between the changes in systemic inflammatory cytokine response in the peripheral blood and the immunity of the host after *L. intracellularis* infection, we measured the levels of pro-inflammatory cytokines (tumor necrosis

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factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and IFN- $\gamma$ ), anti-inflammatory cytokines (IL-4, IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ )), and a chemokine (IL-8, also known as NAP-1, AMCF-1, and CXCL8) in pigs infected with *L. intracellularis* isolated from South Korea. In addition, the results of this study may elucidate the effects of pro-inflammatory and anti-inflammatory cytokine response to *L. intracellularis* infection.

## MATERIALS AND METHODS

### *Preparation of the pure L. intracellularis culture inoculum*

Murine fibroblast-like McCoy cells (American Type Culture Collection [ATCC] CRL 1696) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Invitrogen Corp., Carlsbad, CA, U.S.A.) with 1% glutamine (Gibco BRL, Invitrogen Corp.) and 5% fetal bovine serum (FBS) (Gibco BRL, Invitrogen Corp.) without antibiotics at 37°C in 5% CO<sub>2</sub>. The infection of McCoy cells with the *L. intracellularis* isolate PHE/KK421 (Korean Collection for Type Cultures [KCTC] 10686BP) has been described in detail elsewhere [26]. Briefly, the contents of a 1-ml cryogenic vial containing approximately 10<sup>5</sup> *L. intracellularis*, at passage five from origin, were added to a 175 cm<sup>2</sup> flask containing a 1-day-old, 30% confluent McCoy cell monolayer. The flask was placed in a container, which was then evacuated to 500 mmHg and refilled with medical-grade hydrogen. Half of the medium was replaced 3 days after infection. At 7 days after infection, the monolayer of highly infected cells was harvested, and the infection was passaged by scraping the monolayer with a cell scraper, centrifuging the scraped cells with the supernatant medium for 20 min at 3,400 ×g, resuspending the pellet in fresh medium, and infecting three 175 cm<sup>2</sup> flasks containing 1-day-old, 30% confluent McCoy cell monolayers. The monolayer of McCoy cells highly infected with *L. intracellularis* was harvested, and the infection was passaged weekly, using the same technique described above. The number of flasks containing *L. intracellularis*-infected McCoy monolayers was tripled weekly from passage six to passage nine. At the last passage, bacteria were released from infected McCoy cells by 0.1% potassium chloride treatment and mechanical rupture by passage through a 20-gauge needle [12]. These bacteria were then combined with the bacteria present in the supernatant, homogenized briefly for 15 sec, and centrifuged for 20 min at 3,400 ×g. The bacteria pellet was resuspended in sucrose-potassium glutamate (pH 7.0) (SPG) solution containing 0.218 M sucrose, 0.0038 M KH<sub>2</sub>PO<sub>4</sub>, 0.0072 M K<sub>2</sub>HPO<sub>4</sub> and 0.0049 M potassium glutamate with 5% FBS and kept at 4°C until pigs were inoculated later on the same day.

### *Quantification and microbiological screening of the inoculum*

Quantification of the inoculum was accomplished by making serial 1:10 dilutions of each inoculum in phosphate-buffered saline (PBS; pH 7.2), coating 12-well glass slides (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) with 10  $\mu$ l of each dilution, drying the slides at 37°C for 30 min, fixing with cold acetone, and staining by an indirect immunoperoxidase method using hyperimmune antiserum against *L. intracellularis*. The number of organisms per well from the highest countable dilution (50 to 500 organisms) was evaluated in duplicate using light microscopy.

### *Animals and experimental design*

All animal procedures carried out in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee (IACUC) of National Veterinary Research and Quarantine Service (NVRQS, Anyang-si, Gyeonggi-do, Republic of Korea) (permit number: 2009-10-01). All procedures were conducted in accordance with the guidelines of the "Animal Care and Use Manual" of NVRQS. Ten 5-week-old *L. intracellularis*-seronegative pigs that had not been vaccinated for *L. intracellularis*, weighing between 10 and 15 kg, were obtained from a herd with no history or recorded case of PE; they were divided in 2 groups with 5 pigs per group. All of the pigs were bled prior to inoculation. Two days before challenge, serum and heparinized whole-blood samples were collected from all pigs and tested by an immunoperoxidase monolayer assay (IPMA), as reported in a previous paper [5, 7]. In addition, fecal samples were collected and tested for *L. intracellularis* DNA by PCR [9, 11, 24] to assure PE negativity. One day before the challenge, the animals were divided in two groups, randomized by weight: 5 pigs in the control group and 5 pigs in the infection group. Each group was housed in a different room in isolation barns at the NVRQS. The pigs were orally inoculated with the PHE/KK421 strain of *L. intracellularis* at 5.25 × 10<sup>8</sup> organisms of the *L. intracellularis* isolate PHE/KK421 in SPG with 5% FBS solution per pig on day 0. Pigs in the control group received the same volume of SPG solution. Animals in the control and infection groups were intra-gastrically dosed with 25 ml of the respective inoculum using a stomach tube. Pigs challenged with *L. intracellularis* and mock-infected pigs were euthanized on 40 days post-infection (DPI).

### *Clinical sign, fecal and serum sample analysis*

Clinical signs based on the consistency of the feces were evaluated every five days. The clinical conditions of the pigs were observed and recorded for at least 10 min, including appetite, feces, body condition, and changes in behavior. The clinical scores ranged from 0 to 2, i.e., 0=normal; 1=slightly to moderately gaunt, depressed and appetite loss, or listless, but still standing; 2=severely gaunt, depressed, and recumbent. The fecal score was determined based on the following characteristics: 0=no diarrhea; 1=semi-solid feces with no blood; 2=watery without dark or bloody feces; 3=blood-tinged feces, loose or formed; and 4=profuse diarrhea with frank blood or dark tarry feces. PCR analysis was conducted on fecal samples every five days for a period of 40 DPI, as described in a previous paper [9, 11, 24]. In serum, anti-*L. intracellularis*-specific IgG concentration was measured by IPMA.

### Cytokine bioassays

Peripheral blood samples for cytokine and chemokine analyses were obtained from the experimental animals, and sera were frozen at  $-70^{\circ}\text{C}$  for batch analyses. The levels of TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IL-4, IL-10, TGF- $\beta$  and IL-8 in the sera were determined using commercial ELISA kits (R & D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. A standard curve was constructed using a known standard. All detections were performed in parallel.

### Statistical analysis

Statistical significance was set at the 5% level, and two-sided  $p$ -values were calculated. Statistical analysis was performed by the paired  $t$ -test, and a  $P$ -value of  $<0.05$  was accepted as statistically significant. The data are presented as the mean  $\pm$  standard deviation. All data were analyzed using GraphPad PRISM software (version 6.07 for Windows; GraphPad Software Inc., La Jolla, CA, U.S.A.).

## RESULTS

The concentration of the pathogenic *L. intracellularis* isolate PHE/KK421 in the inoculum was  $2.1 \times 10^7$  organisms/ml, and the total dose per pig was  $5.25 \times 10^8$  organisms. Before this exposure, all animals were found to be negative for *L. intracellularis* by fecal PCR and serology.

The results of the PCR testing of fecal samples are shown in Table 1. All of the pigs inoculated with *L. intracellularis* had persistent or intermittent diarrhea from 10 to 40 DPI. Fecal shedding was initially detected at 10 DPI and lasted, continuously (4 pigs in the infection group) or intermittently (1 pig in the infection group), up to 40 DPI with *L. intracellularis*, whereas fecal shedding was not detected in uninfected control pigs. The observation of persistent or intermittent diarrhea in animals in the infection group during the study agrees with the results reported for other experimental studies [7, 10]. In one previous study [23], fecal shedding was detected 10 weeks after experimental challenge of pigs with a pure culture of a European pathogenic *L. intracellularis* isolate. In a follow-up study at a commercial farm after an outbreak of the acute form of PE in the breeding herd [6], intermittent fecal shedding in growing-finishing pigs was detected for up to 12 weeks after the first positive PCR result.

As shown in Fig. 1a, the pigs in the test group inoculated with *L. intracellularis* showed appetite loss, depression, and significant diarrhea from 10 to 40 DPI. The fecal scores began to rise at 15 DPI, as seen in Fig. 1b. Otherwise, no clinical signs or fecal changes were observed in uninfected control pigs.

Detection of serum IgG has been shown to be a useful tool to assess previous exposure to *L. intracellularis*. The IPMA serology test has been shown to be a highly specific (100%) and fairly sensitive (89%) test [5]. Serum IgG antibodies against *L. intracellularis* began to be detected between 15 and 40 DPI in the challenged pigs. Serum IgG titer elevated progressively during the period of this study, as seen in Fig. 2. Uninfected control pigs had no detectable serum IgG antibody against *L. intracellularis* throughout the study.

Pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6 and IFN- $\gamma$  showed the pattern similar to clinical signs, fecal changes, and IgG antibody demonstration. Overall, the cytokine level changes of TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IL-4, IL-10, TGF- $\beta$  and IL-8 in blood collected from pigs from 0 to 40 DPI are shown in Fig. 3 and Fig. 4. The increase was statistically significant only for TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IL-4 and IL-8 in the serum of infected animals ( $P < 0.03$ ,  $P < 0.001$ ,  $P < 0.03$  and  $P < 0.02$ , respectively).

The TNF- $\alpha$ , IL-6 and IFN- $\gamma$  levels were closely related to *L. intracellularis* infection, as serum levels of these proteins were higher in *L. intracellularis*-infected pigs than in uninfected control animals. However, circulating levels of IL-4, IL-8, IL-10 and TGF- $\beta$  were relatively late affected by the presence of *L. intracellularis* and were similar between infected and uninfected pigs for the whole experimental period.

The increase was statistically significant for IL-10 and TGF- $\beta$  and in the serum of infected animals only after 30 DPI and 25 DPI ( $P < 0.0001$  and  $P < 0.006$ , respectively) although IL-10 and TGF- $\beta$  levels were also similar in *L. intracellularis*-infected and uninfected pigs ( $P < 0.05$ ) for the whole experimental period.

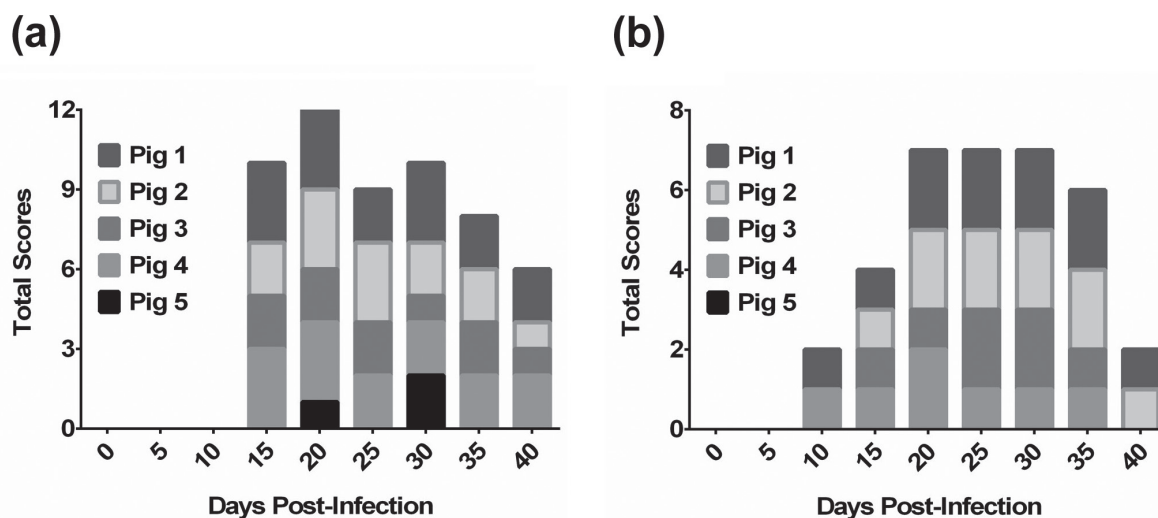
Infected pigs showed significantly higher TNF- $\alpha$  levels than uninfected pigs. The levels of TNF- $\alpha$  in the infected group quickly reached the first peak at 10 DPI ( $544.0 \pm 75.4$  pg/ml). The levels of TNF- $\alpha$  started to rise again at 20 DPI and reached the second peak at 40 DPI ( $712.0 \pm 132.1$  pg/ml). The levels of IL-6 and IL-4 for the infected group reached  $427.4 \pm 151.9$  pg/ml and  $324.4 \pm 112.3$  pg/ml, respectively, at 25 DPI and returned nearly to baseline levels at 40 DPI. IL-8 rose progressively from 20 DPI ( $258.6 \pm 52.1$  pg/ml) and peaked at 35 DPI ( $430.4 \pm 150.4$  pg/ml), then declined to  $275.8 \pm 73.8$  pg/ml. The IL-6, IL-4 and IL-8 levels were increased 2- to 3-fold in sera collected from infected animals as compared with uninfected control animals from 20 DPI to 35 DPI. The TNF- $\alpha$  level began to rise earlier than those of IL-6, IL-4, IL-8, and IFN- $\gamma$  cytokines investigated in this study.

IFN- $\gamma$  began to rise at 10 DPI ( $192.0 \pm 81.5$  pg/ml) and at 15 DPI ( $400.4 \pm 163.5$  pg/ml). The level of IFN- $\gamma$  continued to rise again at 40 DPI ( $870.0 \pm 174.0$  pg/ml). These results support the suggestion of Smith and others that IFN- $\gamma$  plays a significant role in limiting infection and cell proliferation caused by *L. intracellularis* [2, 22]. They demonstrated that infection rate of IFN- $\gamma$  receptor knockout mice was substantially higher than that of wild-type mice in a *L. intracellularis* challenge model [2, 22].

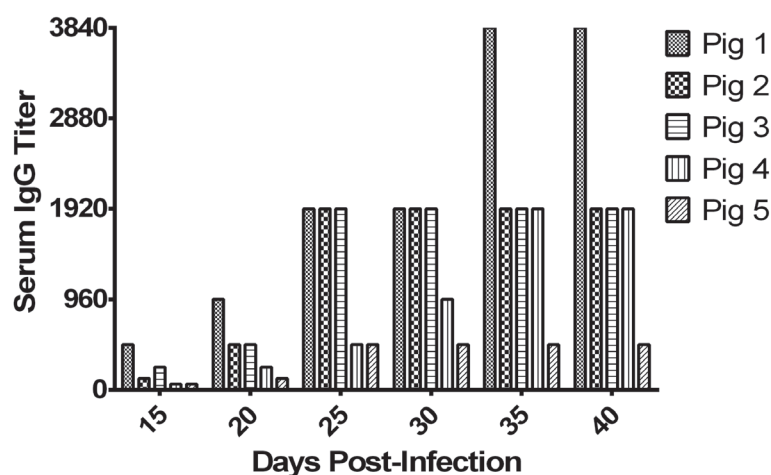
**Table 1.** Results of PCR testing of fecal samples from pigs infected with *Lawsonia intracellularis*

DPI	Pig				
	1	2	3	4	5
0	–	–	–	–	–
5	–	–	–	–	–
10	+	+	–	+	–
15	+	+	+	+	–
20	+	+	+	+	+
25	+	+	+	+	–
30	+	+	+	+	+
35	+	+	+	+	–
40	+	+	+	+	–

DPI, days post-infection; +, PCR positive for *L. intracellularis* DNA; –, PCR negative for *L. intracellularis* DNA.



**Fig. 1.** Total scores of body condition, behavior, and feces in pigs challenged with *Lawsonia intracellularis*. Clinical signs based on the consistency of the feces were evaluated every five days. The clinical conditions of the pigs were observed and recorded for at least 10 min, including appetite, feces, body condition, and changes in behavior. (a) The clinical scores ranged from 0 to 2, i.e., 0=normal; 1=slightly to moderately gaunt, depressed and appetite loss, or listless, but still standing; 2=severely gaunt, depressed, and recumbent. (b) The fecal score was determined based on the following characteristics: 0=no diarrhea; 1=semi-solid feces with no blood; 2=watery without dark or bloody feces; 3=blood-tinged feces, loose or formed; and 4=profuse diarrhea with frank blood or dark tarry feces.

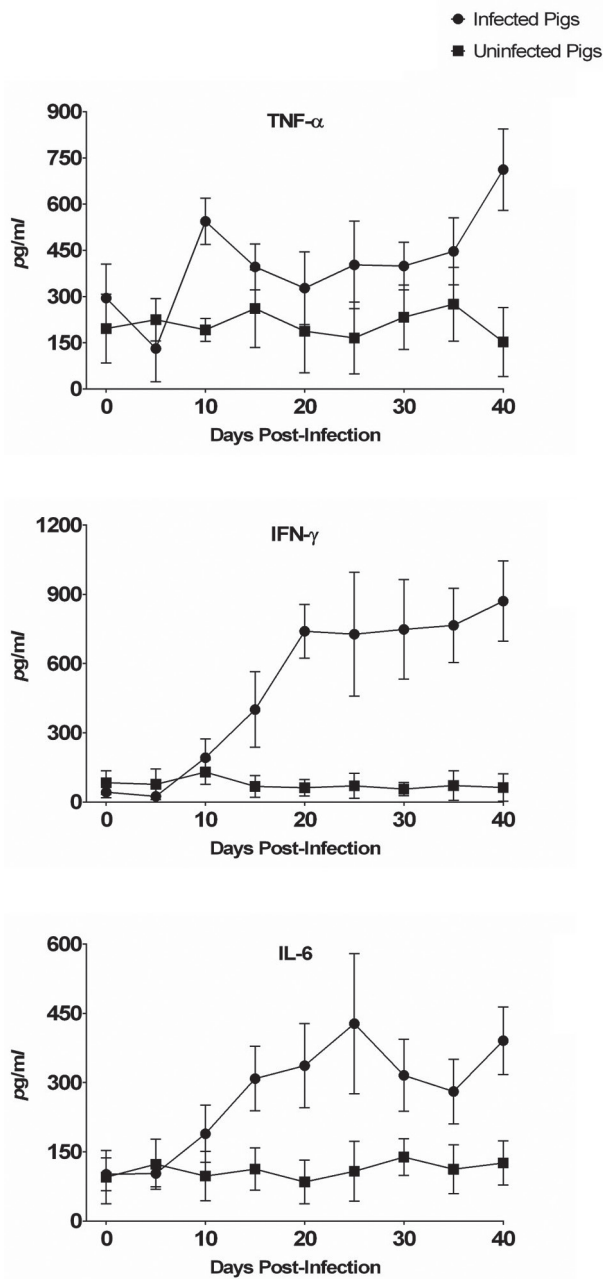


**Fig. 2.** *Lawsonia intracellularis*-specific antibody titer in serum of infected pigs. Sera were collected at the time points indicated from *L. intracellularis*-inoculated pigs and IgG was measured by an immunoperoxidase monolayer assay (IPMA).

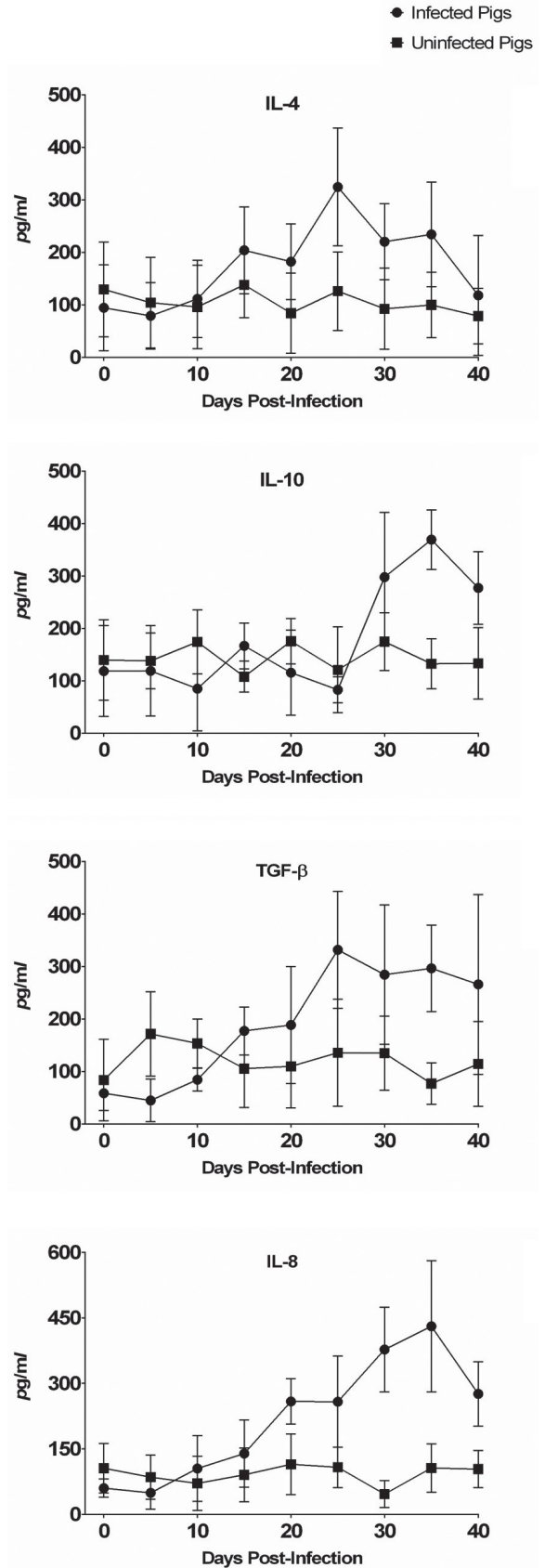
## DISCUSSION

There are numerous *in vitro* studies of the actions and interactions of cytokines but relatively fewer *in vivo* studies of cytokines in animal models of infectious disease. The response of cells to cytokines can be markedly affected by the “context”, and the systemic cytokine response *in vivo* is more valid for immunology than for any other type of research. Although several papers on mucosal, systemic, or primed cell-mediated immune responses to *L. intracellularis* have recently been published [1, 16, 17, 21], most immunological researches on *L. intracellularis* have focused mainly on IFN- $\gamma$ . Here we focused on *in vivo* research on cytokine involvement in porcine PE caused by *L. intracellularis* infection using a Korean isolate.

In a previous study [3], the detection of IFN- $\gamma$ -producing cells purified from the peripheral blood of pigs challenged with *L. intracellularis* after *in vitro* specific stimulation with *L. intracellularis* antigen demonstrated the induction of systemic cell-mediated immune responses against this organism despite the localization and proliferation of *L. intracellularis* in the cytoplasm of enterocytes. In another study [14], a lymphocyte stimulation assay performed in PBMCs of pigs naturally affected with PE showed an apparently specific relationship between the concentration of *L. intracellularis* test antigen ( $3 \times 10^{-5}$ ,  $3 \times 10^{-4}$ ,  $3 \times 10^{-3}$ ,  $3 \times$



**Fig. 3.** Pro-inflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$  and IL-6) level quantitated by ELISA in the sera of control and *Lawsonia intracellularis*-infected pigs after oral challenge at various time points.



**Fig. 4.** Anti-inflammatory cytokines (IL-4, IL-10 and TGF- $\beta$ ) and a chemokine (IL-8, also known as NAP-1, AMCF-1 and CXCL8) level quantitated by ELISA in the sera of control and *L. intracellularis*-infected pigs at various time points after oral challenge.

$10^{-2}$ , 0.3 pg/ml) and the lymphocytic response. In the control group, there were no significant changes in the levels of any tested cytokine or chemokine before or during the experiment.

Although this study focused mainly circulating and systemic inflammatory cytokines, actually researches on cytokine response encounter some particular problems. For example, in case of immunological research on mucosal immune response, samples of the gastrointestinal tract, such as intestinal contents in the local area, are needed to study cytokine production, and these are difficult to obtain from living animals. There is a high risk of contamination induced by fecal inhibitors when determining the local cytokine levels, although *L. intracellularis*-induced cytokine expression within the intestinal mucosa may be more clinically relevant than circulating cytokine levels. The lack of real-time endoscopy or sequential necropsy findings that would allow correlation among local inflammation, gastrointestinal symptoms, and circulating cytokines is also an important consideration and for this reason many previous study did not showed a consistent correlation between the severity of enteritis and local cytokine expression yet [2, 4, 20, 22].

The immune system evolved to resist rapidly proliferating pathogens that, in the absence of an immediate response, have the potential to overwhelm and kill a host. Slowly replicating pathogens, by comparison, pose a less immediate threat and thus require less of an immediate response [15]. When hosts are infected by *L. intracellularis*, PE may be seen as various proliferating manifestations including acute intestinal hemorrhage or acute to chronic diarrhea with mucosal proliferation or necrosis. The specific mechanisms by which the immune system reacts on the cellular and subcellular levels are still unknown. This is one of the important studies to assess systemic cytokine response in animals infected with *L. intracellularis*.

Here, we were able to determine the cytokine profiling in pigs challenged with a pathogenic Korean *L. intracellularis* isolate. *L. intracellularis* induced cytokine and chemokine responses in infected animals. Detection of pro-inflammatory and anti-inflammatory cytokines may elucidate whether *L. intracellularis* infection is affected by other aspects of the host immune system. Further studies need to be conducted to better understand the host–pathogen interaction and the importance of cytokines and chemokines in controlling *L. intracellularis* infection.

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