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# *Eimeria pragensis* Induce Immune Mediated Intestinal Hypomotility in C57BL/6 Mice

Riki Siswandi[1], Ayako Yoshida[1], Hiroyuki Satoh[2], Nariaki Nonaka[1]*

[1]Laboratory of Veterinary Parasitic Diseases, [2]Laboratory of Veterinary Clinical Radiology, Graduate School of Medicine and Veterinary Medicine, University of Miyazaki, Japan
*corresponding author: nnonaka@cc.miyazaki-u.ac.jp

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## INTRODUCTION

The manifestation of intestinal dysmotility of coccidiosis has never been briefly reported before. From murine *Eimeria* study we concluded that *Eimeria pragensis* (Ep) is capable to induce intestinal hypomotility described to be similar to pan-enteric ileus.

In comparison with the intestinal dysmotility in postoperative ileus which is orchestrated by $T_H1$ immune response [1], a local injury to some part of intestine could trigger pan-enteric ileus. If the $T_H1$ immune response could also be induced in Ep infection, the delay in intestinal motility could be explained as similar mechanism as the post-operative ileus. Since the Ep infestation occurred in the ileocecal part of the intestines, with similar mechanism of post-operative ileus, the pan-enteric ileus could be triggered. In this study, we tried to find a relationship between intestinal dysmotility and $T_H1$ immune response in murine coccidiosis.

## MATERIAL AND METHODS

Animals used in this study were C57BL/6 male mice. All mice were purchased at six weeks of age from SLC Inc. Japan. All mice were kept as outlined in the "Guide for the Care and Use of Laboratory Animals" by University of Miyazaki.

After one week of adaptation, mice were assigned into different groups of treatments. The infected group was inoculated with 300 sporulated Ep oocysts per mouse and then divided into three groups in which observation was done at 4 (EP 4D), 7 (EP 7D), and 10 (EP 10D) days post-infection (dpi). The control group was inoculated with distilled water instead of Ep oocysts. Each group consisted of 5 mice.

The intestinal motility was observed by contrast gastrography of barium sulfate ($Ba_2SO_4$). The observation was performed at 2, 6, 12, 24, and 48 hours post $Ba_2SO_4$ administration. $Ba_2SO_4$ (4 gr/ml) were administered 0,3 ml on each mouse by oral gavage. The intestinal content transition was analyzed by the methods described by Giron, et al. [2]. Alterations in GI motility were semi quantitatively determined from the images by assigning a compounded value to each region of the GI tract considering the following parameters: percentage of the GI region filled with contrast (0–4); intensity of contrast (0–4); homogeneity of contrast (0–2); and sharpness of the GI region profile (0–2). Each of these parameters was scored and a sum (0–12points) was made.

To understand the effects of Ep infection on host immune response, the expression of splenic IL-1β, IL-6, TNF-α, IFN-γ, IL-12p35, IL-12/23p40, STAT3, IL-17, IL-10, iNOS, TGFβ and T-bet genes was analyzed by real-time PCR using sense and antisense primers. Five mice were distributed into control group and Ep infected group. Tissue were collected on 9 dpi after Ep infection (300 sporulated oocyst) in infected group or sham inoculation of DW in control group.

Total cellular RNA was extracted from spleen tissue using an TRI Reagent kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. First strand cDNA was synthesized from 3 µg RNA by PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, JPN) according to the manufacturer's protocol. Real-time PCR was performed using GoTaq qPCR Master MIX kit (Promega, USA). PCR was performed in a 10-µl reaction volume containing 5 µl of qPCR master mix (2×), 0.2 µM of each primer (sense and antisense) 2 µl of each cDNA template, and 2.6 µl of nuclease-free water. All primers were purchased from Invitrogen (Japan). Dissociation of the PCR products by a melting curve analysis protocol consistently showed specific single melting peaks for all used primer pairs.

The real-time PCR cycling program consisted of initial PCR activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and a dissociation curve was added to the protocol whenever necessary. The assay was performed using a Lightcycler 96 real-time PCR system (Roche, Swiss). Changes in gene expression were calculated from the obtained cycle threshold (Ct) values provided by real-time PCR instrumentation using the $2-\Delta\Delta Ct$ calculation, where ΔCt indicates the Ct changes in target genes in comparison to a reference (housekeeping) gene (β-actin).

Statistical associations between variables

were analyzed using the Spearman's rank correlation or Wilcoxon rank-sum test. All analyses were performed using the statistical program R (www.r-project.org).

## RESULTS AND DISCUSSION

The intestinal dysmotility described as delay in intestinal transition were observed in stomach and caecum of infected mice on EP 7D and EP 10D groups (Figure 1). There was no difference in intestinal transition between control and EP 4D groups.

There was a delay in caecum filling in EP 7D and EP 10D groups compare to control group and EP 4D (Figure 1). These delay indicated the hypomotility occurred in the upper intestinal tract. As the retention of barium occurred in the caecum, some barium was also observed in the colon up to 48 hours. With 24 and 48 hours of x-ray observation, the dysmotility continues to occur from 7 dpi up to 12 dpi. These delay was occurred only during the patency of Ep, which could be resulted for more extent damage due to oocyst shedding. After 7 dpi, oocyst production became increasing and the damage in intestinal tract could be more profound, thus also producing intestinal hypomotility during oocyst shedding.

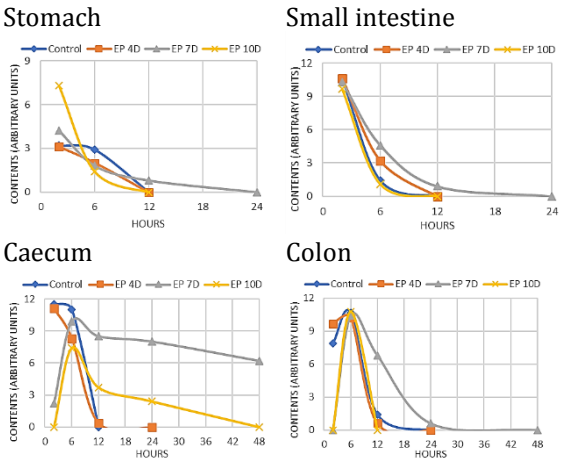


*Figure 1. Transition of intestinal content observed by barium contrast gastrography.*

There was no notable clinical sign observed in the infected group besides reduction in body weight gain. Therefore, we proposed 300 sporulated oocysts as a dose for subclinical coccidiosis, thus avoiding pain related bias in the clinical coccidiosis. The infected groups ceased to gain body weight during 14 days of observation, meanwhile on the same time the control group showed increase in body weight. This growth impediment in infected group could resulted in impairment in gastrointestinal function. Further study needs to be conducted to evaluate how long this growth impediment affects.

Intestinal dysmotility in post-operative ileus is typically accompanied by increased expression of proinflammatory cytokines in intestinal tissue such as IL-1, IL-6 and tumor necrosis factor-α (TNF-α), followed by infiltration of polymorphonuclear neutrophilic granulocytes (PMNs) and macrophages [3]. Activated macrophages in the intestinal muscularis expressed inducible nitric oxide synthase (iNOS), whose product, nitric oxide, directly inhibited smooth muscle cell function [4]. In our study, IL-1, IL-6, and TNF-α were found to be expressed in the spleen (Figure 2). The absence of iNOS expression in spleen might indicated that macrophage is not essential in Ep infection-induced immune response. However, further study needs to be conducted to evaluate the intestinal cytokines expression.

We found that mRNA for IL-12 p35 and T-bet were upregulated in spleen, suggesting that a $T_H1$ response was generated. IFN-γ mRNA was also increased consistent with the presence of $T_H1$ cells (Figure 2).

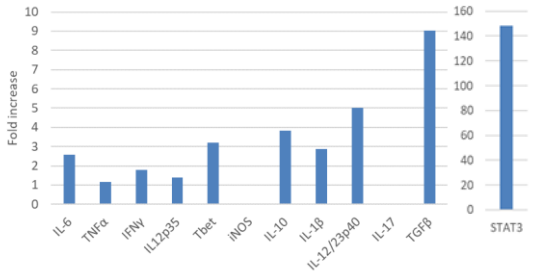


*Figure 2. Fold increase of immune mediators' expression in the spleen of infected group on 9 dpi compare with uninfected healthy mice.*

Daughcies [5] reported that coccidiosis in cattle resulted in increase in feed apparent digestibility as compensatory mechanism of impaired intestinal function. It is not clear yet whether the delay of intestinal motility is compensatory mechanism in mice or merely a meaningless defect due to lesion in motor function.

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