The effect of excretory/secretory product released by L_3 of *A. galli* on villous compact in intestine of laying hens

[1]Ummu Balqis, [2]Darmawi, [3]Risa Tiuria, [4]Bambang P. Priosoeryanto, and [5]Muhammad Hambal

[1]Laboratory of Pathology, Faculty of Veterinary Medicine of Syiah Kuala University, Banda Aceh 23111, Indonesia; [2]Laboratory of Microbiology, Faculty of Veterinary Medicine of Syiah Kuala University, Banda Aceh 23111, Indonesia; [3]Laboratory of Helminthology, Faculty of Veterinary Medicine of Bogor Agriculture Institute, Bogor 16144, Indonesia; [4]Laboratory of Pathology, Faculty of Veterinary Medicine of Bogor Agriculture Institute, Bogor 16144, Indonesia; [5]Laboratory of Parasitology, Faculty of Veterinary Medicine of Syiah Kuala University, Banda Aceh 23111, Indonesia; Coorresponding Author: d_darmawi@yahoo.com

Abstract. The nematode, *Ascaridia galli*, caused pathological changes in the gut, while the larval development takes place in the intestine epithelium. The aim of this research was to examine the effect of excretory/secretory product released by L_3 of *A. galli* to prevent intestine destructions based on villous compact in duodenum, jejunum, and ileum of laying hens. Excretory/secretory was prepared from metabolic of L_3 released in culture médium in flasks containing rosswell park memorial institute (RPMI) 1640 media, pH 6.8, without phenol red. Excretory/secretory product was concentrated with vivaspin 30.000 MWCO. The chickens vaccinated with 80 µg protein excretory/secretory in crude mixed with Fruend Adjuvant Complete and repeated three times with dose of each 60 µg mixed with Freund Adjuvant Incomplete with an interval of one week intra muscularly. The chickens were challenged with 1000 infective larvae *A. galli* one week later. After two weeks the chickens were operated. Villous compact in duodenum, jejunum, and ileum of laying hens were analyzed. The result showed that immunization was able to sustained significantly intestine villus compact, particularly in duodenum. This research concluded that the excretory/secretory product could protect villus in duodenum against parasitic diseases caused by *A. galli*.
Keywords: *Ascaridia galli*, excretory/secretory, hen, villi

Introduction

The pathogenesis of nematode infection, especially ascaridiosis, is probably a multistep process (Permin and Nansen, 1998) that includes adhesion, degradation, and invasion. Adhesion should be the primary step, followed by invasion and degradation of extracellular matrix protein and host cells. Here, we assumed that excretoyy/secretory of *A. galli* may be to degrade extracellular matrix protein, thereby facilitating mucosal penetration by the invasive stage. *A. galli* may likewise utilize excretory/secretory to facilitate larval invasion, migration within the host tissue, and modulation of host immune response mechanisms. Various authors have indicated that excretory/secretory played an important role in parasite survival or stimulating the host immune response. Evidence that during transition of L_3 – L_4 stage larvae of *Ascaris suum*, which are more often associated with molting (Rhoads *et al*. 1997), have a greater requirement for enzyme to facilitate penetration of host tissues during the extensive migration of the larval stages through the host (Rhoads *et al*. 2001). Product secreted by *Ostertagia ostertagi* may be useful target molecules for serodiagnosis designed to detect antibody and vaccination to stimulate host immune response (Cock *et al*., 1993).

The immediate impact of parasitic tissue penetration is free radical generation by the host's leucocytes and the accompanying intestinal pathology that results in physical irritation, hypertrophy of the tunica muscularis, decrease in villus height, intestinal cell sloughing and gut leakage, and goblet cell hyperplasia and exhaustion. Infestation of *A. galli* worms can also cause damage to the epithelia as a result of pressure atrophy of the villi, causing necrosis of the mucosal layer. Our early study showed that laying hens infected by *A. galli* could damage mucosal barrier implicated to decrease in villi surface area of intestine (Balqis, 2004). We have shown that excretory/secretory (ES) released by *A. galli* adult worm was able to generate intestine mucosal defence of laying hens base on prolipheration and hyperplasia of mucosal mast cells (Darmawi and Balqis, 2004). In addition, Balqis (2004) suggested that the ES would be of beneficial in strengthening the host defence mechanisms of intestine mucosa and the laying hens challenged with L_2, did not influence the pathological lesion of duodenum. This study was carried out to obserb the effect of excretory/secretory product released by larvae of *A. galli* regarding prevention of intestine destructions based on villus compact in duodenum, jejunum, and ileum of laying hens.

Materials and Methods

Animals and Experimental Design

Amount 12 heads laying hens (Isa brown) divided into four groups containing three of each. The first group, chickens were not immunized nor infected (control group). The second group, chickens were immunized (with 80 µg protein excretory/secretory in crude mixed with Fruend Adjuvant Complete and repeated three times with dose of each 60 µg mixed with Freund Adjuvant Incomplete with an interval of one week intra muscularly). The third groups, chickens were orally infected with 1000 infective larvae. The fourth group, chickens were immunized and infected. All groups of laying hens of 12 weeks old were studied. Infection with 1000 stage infective larvae of *A. galli* was performed as described (Balqis *et al*. 2011[b]). All chickens were housed under individual and given feed and water ad libitum before being killed on days 14 by slaughtered. Chickens were infected by a orally injection of 1000 *A. galli* larvae.

Parasite

Female adult worms were obtained from lumen of village chickens. The eggs in uteri of female adult worms were expelled, prepared and colected under stereomicroscope. The eggs were incubated in sterile aquadestilata at room temperature for 21- 30 days developed embrionated eggs (L_2) (Tiuria *et al*. 2003). *A. galli* larvae were recovered from intestines of 100 heads chickens 7 days after each oesophagus inoculation with 6000 infective larvae. Larvae recovered in this manner were cultured (5–10 ml^{-1}) in flasks containing RPMI 1640 media, pH 6.8, without phenol red and suplemented with 100 units ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin, 5 µg ml^{-1} gentamycin, and 0.25µg ml^{-1} kanamycin. Cultures were incubated at 37^0C in 5% CO_2 and excretory/secretory product of L_3 released in culture was collected after 3 days (Darmawi *et al*. 2006).

Excretory/Secretory A. galli

To prepare excretory/secretory product, culture medium *A. galli* larvae were centrifuged at 1000 g, temperature 4°C for 10 minutes. Supernatan was filtered with 0.45 µm Minisart® (Sartorius), poured and concentrated in vivaspin 30,000 MWCO at 1000 g, temperature 4°C for 10 minutes. Filtrate was washed in phosphate buffered saline (PBS) for three times (Balqis *et al*. 2007).

Histopathological Procedures

At two weeks post infection, chickens were slaughtered, the abdomen opened and small intestine taken for histopathological examination. This level of intestine was elected because it is the *A. galli* trophisms. Duodenum taken from around 10 cm from the pylorus, segments of jejunum and ileum were dissected, flushed with cold sterile saline solution, opened longitudinally, and placed, mucosa side up, onto small pieces of blotting paper. The tissue specimen was then fixed in 10% buffered formalin. Tissues were prepared for microtomy, cut in 8 µM. This process was performed for each chick using sterile instruments for each dissection. Fixed samples were dehydrated, cleared, and embedded in paraffin wax for subsequent histological analysis. Tissues were dehydrated in the ascending concentrations of ethanol (50%, 60%, 70%, 80%, 96% (1), 96% (2) and 100%). Later they were placed in xylem and were kept in the solution of paraffin and xylol saturated at +37°C. Then tissues were put in paraffin and beeswax solution at 56°C. Sections were then deparaffinized in Histolene and rehydrated in preparation for staining. Specimens were stained with haematoxylin and eosin (Balqis *et al*. 2007).

Microscopic Study

Slides were studied on Olympus camera attached microscope. Observations were recorded and microphotography was done for projection slides and photographs. Villous compact (villi/mm) of duodenum, jejunum, and ileum in laying hens were determined based on amount of villous at 10 grounds observing on 1 mm length of duodenum, jejunum, and ileum (Video measuring gauge IV – 560, FOR A company limited).

Results and Discussion

Amount of villous compact in duodenum of infected chickens decreased significantly ($P < 0.05$) compared with the other chickens group. However, differentiation amount of villous were not significant in jejunum and ileum (Table 1). Tiuria *et al*. 2003 described that decreasing amount of villous caused by helminthiasis contribute in nutrient absorb inflicting retarded growth. Other adverse effects of these parasite include heavy production losses and lowered egg production. In the case of the infected chickens, jejunum had distinct separate villi. Cellular infiltration was seen at mucosal surface and it was observed that in some cases, epithelial cells near border undergone autolysis and looked tattered compared to the proximal regions of small intestine. In the case of the infected chickens, the villi had more goblet cells and mucosal epithelium was rough, crypts were deep and mitotic figures were seen, suggesting the increased cell division (Balqis *et al*. 2011[b]).

Table 1. Villous compact (villi/mm) of duodenum, jejunum, and ileum in laying hens

Groups	Duodenum	Jejunum	Ileum
Control	5.67 ± 0.57[a]	6.00 ± 1.00 [a]	6.33 ± 1.52 [a]
Excretory/Secretory	6.33 ± 1.15[a]	5.33 ± 1.15[a]	5.33 ± 0.57[a]
1000 infective larvae	4.33 ± 1.15[b]	6.33 ± 1.38[a]	4.67 ± 2.89[a]
Excretory/Secretory and 1000 infective larvae	5.67 ± 0.57[a]	4.67 ± 0.57[a]	4.33 ± 1.15 [a]

Bahrami (2011) examined in horses showed that the treatment group received 6000 L3 multiple nematodes namely *H. contortus* (50%), *Ostertagia ostertagi* (25%), *Trichostrongylus axie* (12%), *Chabertia ovina* (8%), Cooperia (all most 5%)] orally marked hypertrophy and hyperplasia was observed in all regions of gastro intestinal tract of infected animals. Villi were broad and appeared to be flattened in distal regions of small intestine of infected animal in comparison to treated group of the lambs after eight weeks with the plant extraction of *S. striata*. Recently, Lukianova *et al*. (2012) revealed that the histological study of horse intestinal tissues, which were affected by *Strongylidae* larvae, showed local changes in the shape of connective tissue capsule around the cysts in the area of implantation of parasites, tissue oedema, vascular congestion and lymphocytic and eosinophilic infiltration in the wall of the small and large intestines. Analysis of the horse's small intestine microslides indicates that a weakly pronounced oedema of the villi, partial desquamation of surface epithelium with the formation of microerosion were marked in areas of mucosa without macroscopic signs of damage by helminthes.

The intestinal inflammatory response accompanying infection with gastrointestinal helminths is thought to be a contributory factor leading to the expulsion of the parasite. Hong *et al*. (1997) revealed that the intestinal pathologic findings at early stage infection, where crypt became mildly hyperplastic and villi were moderately athropied et 4 days post infection by *Centrocestus armatus* (fluke) in albino rats. The stroma of villi around the young flukes was edematous and infiltrated by imflammatory cells such as lymphocytes, plasma cells, and eosinophils. Our previous study showed that immunoglobulin *yolk* (IgY) stimulated by the excretory/secretory antigen of *A. galli* L_3 stage was able to recognized *A. galli* surface antigen on cuticle of adult worms so the excretory/secretory and IgY could be applied for immunodiagnostic (Balqis *et al*. 2011[a]). Balqis *et al*. (2011[b]) showed that laying hens vaccinated with excretory/secretory secreted by *A. galli* L_3 satage stimulate mucosal response against parasitic diseases caused by *A. galli* indicated by increasing significantly goblet cells in duodenum, jejunum and ileum of laying hens.

Conclusions

Vaccination was able to sustained significantly intestine villus compact, particularly in duodenum. This research concluded that the excretory/secretory product could protect villous in duodenum against parasitic diseases caused by *A. galli*.

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