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The Ability of Immunoglobulin Yolk Recognized the Antigen in the Tissue of *Ascaridia galli*

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ABSTRACT

Antigen-antibody reaction is an important tool for the analysis of localization of target molecules, including antigenic protein within worm tissues. The purpose of the present research was to demonstrate the ability of immunoglobulin yolk (IgY) anti-excretory/secretory recognized the antigen in the tissue of *Ascaridia galli* by mean of immunohistochemistry method. The excretory/secretory protein was procured from *A. galli* and concentrated by mean of vivaspin 30,000 MWCO. IgY was produced by egg yolks of immunized chickens with excretory/secretory, and purified using fast protein liquid chromatography (FPLC) method. *A. galli* adult worms were cut in transversal and longitudinal section of the center and anterior region. Slides were incubated with both primary IgY for overnight at 4 °C and secondary antibody rabbit anti-chicken IgY HRP-conjugate for one hour at room temperature. The slides were stained with 3-amino, 9-ethylcarbazole (AEC) chromogen, counterstained with Lillie Mayer Haematoxylin, and mounted in glyserin aqueous mount. Antigen-antibody reaction was investigated under a microscope. The result showed that antigen was appeared in the tissues such as cuticle, epicuticle, buccal cavity, and eggs inside the uterine of *A. galli*. This research concluded that IgY stimulated by the excretory/secretory was able to recognized the antigen scattered in the tissues of *A. galli* so the IgY could be applied for immunodiagnostic.

Key words: Ascaridia galli, excretory/secretory, IgY, immunohistochemistry

ABSTRAK

Reaksi antigen-antibodi adalah salah satu cara yang penting untuk menganalisis lokasi target molekul, termasuk protein antigen di dalam jaringan cacing. Tujuan riset ini adalah untuk menunjukkan kemampuan *immunoglobulin yolk* (IgY) anti-ekskretori/sekretori mengenal antigen di dalam jaringan cacing *Ascaridia galli* melalui metode imunohistokimia. Protein eksretori/sekretori diperoleh dari *A. galli* dan dipekatkan melalui vivaspin 30.000 MWCO. IgY dihasilkan oleh kuning telur dari ayam yang divaksinasi dengan ekskretori/sekretori, dan dimurnikan dengan menggunakan metode *fast protein liquid chromatography* (FPLC). Cacing *A. galli* dewasa dipotong secara melintang dan memanjang pada bagian tengah dan atas. *Slide* dieramkan dengan kedua antibodi, yaitu antibodi primer IgY selama satu malam pada temperatur 4 °C dan antibodi sekunder IgY *HRP-conjugate* selama satu jam pada temperatur kamar. *Slide* diwarnai dengan kromogen *3-amino, 9-ethylcarbazole* (AEC), dilatar-belakangi oleh *Lillie Mayer Haematoxylin*, dan direkatkan di dalam gliserin. Reaksi antigen-antibodi diamati di bawah mikroskop. Hasil menunjukkan bahwa antigen ditampilkan di dalam jaringan seperti kutikula, epikutikula, rongga mulut, dan telur di dalam uterus cacing *A. galli*. Riset ini menyimpulkan bahwa IgY yang dirangsang oleh ekskretori/sekretori mampu mengenal antigen yang tersebar di dalam jaringan *A. galli* sehingga IgY dapat diaplikasikan untuk imunodiagnostik.

Kata kunci: Ascaridia galli, ekskretori/sekretori, IgY, imunohistokimia

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INTRODUCTION

Ascaridiosis, caused by a nematode parasite so-called *Ascaridia galli*, is one of the major poultry intestinal roundworm infections. The disease is an important poultry health problem in many parts of the world, may give rise to serious economic problems in laying hens husbandry (Anwar & Zia-ur-Rahman, 2002). According to some reports, *A. galli* has a worldwide distribution wich predominates in temperate zones (Schou *et al*., 2003). *A. galli* is also found on most continents, but primarily in the tropical regions of Asia (Lalchhandama *et al*., 2009), and Africa (Siamba *et al*., 2007). Laying hens are usually infected by ingestion of feed and water that contain infective larvae. *A. galli* infection is prevalent in Aceh (Fahrimal & Raflesia, 2002) and some parts of Java (Zalizar *et al*., 2007; Balqis *et al*., 2009; Suharti *et al*., 2010).

The parasitological diagnosis of poultry ascaridiosis is often unreliable because the parasite's eggs are not found in the stool during the early phase of infection. Even when the worms have matured the diagnosis may still be difficult since eggs are only intermittently released. The classic diagnosis of ascaridiosis usually depends on the demonstration of *A. galli* eggs in the feces (Fahrimal & Raflesia, 2002; Suharti *et al*., 2010). However, this method as described by many investigators, is not sufficiently sensitive. Diagnosis of worms infection by mean of immunodiagnostic methods during prepatency utilizing excretory/secretory antigen in some serologic tests have already been reported. The immunodiagnostic tests could be utilized for the detection of disease even at the prepatent stages before eggs begin to appear in the feces. Prasad *et al*. (2008) suggested that early diagnosis of ascaridiosis is necessary for prompt treatment before irreparable damage to the intestine occurs. Thus, serological tests are the most dependable diagnostic methods (Rokni & Kia, 2005; Hassan & Aziz, 2010).

Serological techniques such as immunodiffusion test using excretory/secretory and somatic antigen of the worm for the detection of antibodies against this parasite are sensitive and have been exploited for its serodiagnosis. Karimi *et al*. (2008) reported that excretory/secretory and somatic antigen of *Ornithobilharzia turkestanicum* had strong cross reaction with each other in agar gel diffusion test. Smith *et al*. (2009) analyzed that excretory/secretory product identified in the larval secretome of *Teladorsagia circumcincta*, an important parasitic nematode of domestic small ruminants, were potentially involved in immunity so targets of local immunoglobulin A (IgA) responses in mucus from sheep rendered immune to infection.

Immunoglobulin yolk (IgY) antibody produced by chickens is considered to be a potent antibody for immunodiagnostic. These polyclonal and monospecific IgY antibodies are of higher-titer and specifically recognize recombinant *Helicobacter pylori* urease purified from *Escherichia coli* (Kazimierczuk *et al*., 2005). Futhermore, application of IgY was extended for immunotherapy (Dias da Silva & Tambourgi, 2010). Diraviyam *et al*. (2011) described that the specific activity of IgY antibodies against specific bacterial pathogens have been suggested as the mode of action of specific IgY to inhibit *E. coli* and *Salmonella enterica* growth. Previous investigation showed that passive immunization of chickens with anti-coccidia IgY antibodies provided protective immunity against coccidiosis caused by multiple species of *Eimeria* in broiler infection (Lee *et al*., 2009). The authors described generation of IgY antibodies directed against various antigens to tested antigen-antibody reaction. However the standardization of test under different conditions and with different types of antigens is essential before its practical application and utility under field condition. In this study, tissue sources of the antigen were investigated by immunohistochemistry in the body of adult worm sections using the IgY-anti excretory/secretory *A. galli* purified from chicken egg yolk of immunized laying hens.

MATERIALS AND METHODS

IgY Anti-Excretory/Secretory *Ascaridia galli* Production

Excretory/secretory crude protein was procured from *A. galli* cultured in flasks containing RPMI 1640 media. The protein was poured and concentrated by mean of vivaspin 30,000 MWCO, and quantities determined by Bradford method (λ= 280 nm) as described by Darmawi *et al*. (2009). Twowenty four-week old Isa brown chickens were vaccinated with the crude protein of excretory/secretory, applied intra muscularly with an initial dose of 80 μg. The immunizations were repeated three times with dose of each 60 μg with an interval of one week. The first immunizations were excretory/secretory protein mixed with Fruend Adjuvant Complete (Sigma, USA) and subsequently mixed with Freund Adjuvant Incomplete (Sigma, USA). The chicken's eggs were collected after ELISA test shown antibody titre increased significantly (Darmawi *et al*., 2008).

The antibody enrichment procedure was performed as described by Darmawi *et al*. (2010), a serial precipitations was used. Chicken IgY was precipitated from egg yolks by adding 1 volume of 40% polyethylene glycol (PEG) 8000 (Sigma, USA) in PBS to 3 volumes of egg yolk, followed by centrifugation at 13,000×g for 20 min. IgY was extracted from egg yolks by means of ammonium sulphate. The crude extraction suspension was mixed with 50% (V/V) saturated ammonium sulfate solution and stirred at 4 °C for 2 h. After centrifugation, the precipitate was collected and dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.4). Residual was removed with dialysis. The purified IgY was dialyzed against 10 mM PBS (pH 7.2) four times. IgY was purified using FPLC as suggested by Tangvarasittichai *et al*. (2009) with modifications. About 0.5 mL of extract was passed through a 5 x 0.5 cm (1 mL) column of thiophilic, 2-mercaptopyridine bond to sepharose high performance, Hi Trap™ IgY Purification (Amersham Pharmacia Biotech Inc., USA) connected to the FPLC (AKTA™ explorer 10S) with flow rate 2 mL/min.

Ascaridia galli Parasite

A. galli adult worms were procured from the intestine of freshly slaughtered chickens. They were brought to the laboratory from local restaurant. Worms were washed sometimes with phosphate buffered saline (PBS).

Tissue Preparation for Immunohistochemistry Protocol

The parasite tissues aimed for immunohistochemistry were fixed in Bouin's fixer for 24 h at room temperature and embedded in paraffin block until use. *A. galli* adult worms were preparated by mean of dehydration, clearing, infiltration, embedding, cutting, and staining. The tissues were blocked with paraffin, and saved in refrigerator in order to cut off the paraffin for easy cutting. Paraffin blocks of the worm were prepared in a sequence of 10% neutral formalin fixation, dehydration in graded alcohol series and paraffinization. The paraffin embedded tissues of *A. galli* adult worm were cut longitudinally and transversally using a microtome. Thin sections (3-5 µm) of the worm tissues were floated and warmed at 60 °C and coated on object glasses. The sections were deparaffinised with three changes of xylene (III, II, and I) for 3 min of each. The sections were rehydrated through graded alcohols, namely: 95%, 90%, 80%, and 70% for 3 min of each. The sections were rinsed (clearing) with diionized water for 15 min. The endogenous peroxidase activity was inactivated by incubating these sections in 3% H_2O_2 for 20 min and skim milk for 30 min at room temperature, and rinsed with three changes of diionized water and PBS for 5 min of each as described by Claeys *et al*. (2004) and Pokharel *et al*. (2006) with certain modifications.

After rinsing, the slides were reacted with primary antibody procured from purified IgY by mean of fast performans liquid chromatografi (FPLC) method. The slides were put on PBS-based in humidity chamber at 4 °C overnight, rinsed with three changes of PBS for 5 min of each. The slides were dropped with secondary antibody (IgY conjugate HRP rabbit anti-chicken, Promega, USA). Peroxides complex were reacted for 30 min at room temperature, and rinsed with three changes of PBS for 5 min of each. The reaction was colored by AEC chromogen (Sigma, USA) for 3 min at room temperature, and rinsed with three changes of PBS for 5 min of each. Finally, the tissue sections were counterstained with Lillie Mayer hematoxylin for 1 min, and rinsed with diionized water. The slides were attached with glyserin and covered cover glasses. Positively stained with AEC chromogen was investigated under a microscope (Olympus) with 40 objective magnifications and video micrometer (Video measuring gauge IV – 560, FOR A Company limited) as described by Claeys *et al*. (2004) and Pokharel *et al*. (2006) with certain modifications.

RESULTS AND DISCUSSION

The results of the present study showed that egg yolk antibody IgY had strong reaction with antigen some parts of the round worm's tissue of *A. galli* as seen in Figure 1. Excretory/secretory product released by *A. galli* contained antigenic substance(s) could stimulate specific antibody. This reflect that the antigenic materials are common between somatic and excretory/secretory products of parasite and there is no difference between the antigenicity of somatic and excretory/secretory antigens of *A. galli*. This hypothesis supported by many previously reports exist about the role of excretory/secretory released by nematode in generating immune response mechanisms. Prasad *et al*. (2008) suggested that the purified fraction of excretory/secretory antigen may be utilized for early diagnosis against *Haemonchus contortus* in sheep. Smith *et al*. (2009) analyzed of excretory/secretory products released by *Teladorsagia circumcincta*, where antigens that were targets of local IgA responses in mucus from sheep rendered immune to infection. The similar phenomenon observed by Hassan & Aziz (2010), excretory/secretory antigen of *Toxocara vitulorum* infective larvae was the most diagnostic antigen and was successful in the detection of high infection percentage of toxocariasis among buffalo calves. Excretory/secretory antigen has also been reported to be a better antigen for a serodiagnosis of clonorchiasis than crude antigen (Choi *et al*., 2003).

Excretory/secretory as described by many investigators, is more sufficiently sensitive and specific compared with somatic antigen. It has been shown that using the excretory/secretory antigens of worms by enzyme linked immunosorbant assay (ELISA) method could be

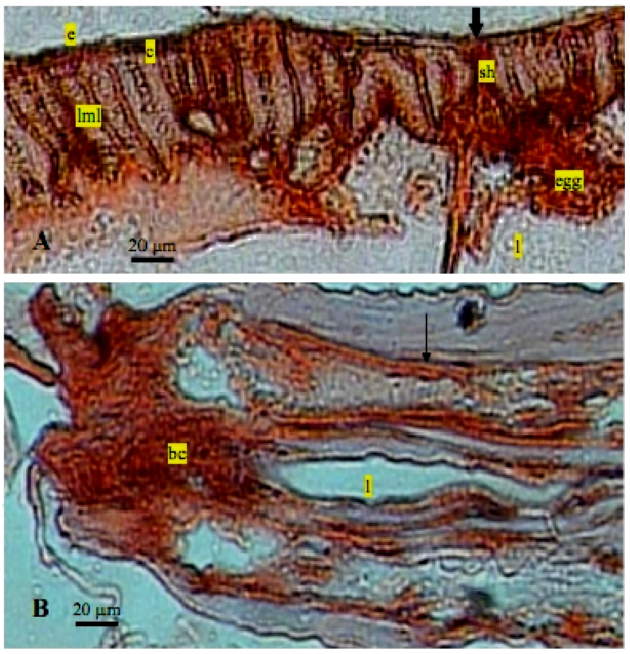

Figure 1. Positive reaction in AEC staining of *A.galli* adult worm. A= tranversal section of the distal region (20x). B= longitudinal section of the anterior region (10x). (c= cuticle; e= epicuticle; l= lumen; lml= longitudinal muscle layers; sh= syncytial hypodermis; egg= eggs inside uterine; bc= buccal cavity) (Scale bar: A= 200 nm; B= 5 µm.)

a good diagnostic tool for the illness. Rokni & Kia (2005) have been used the ELISA test that overall excretory/secretory antigen showed a more convincing diagnosis in comparison with somatic antigen of *Strongyloides stercoralis* in human intestinal nematode infection, although every interpretation of the results should be in accompany with clinical manifestations and a history of the disease. Karimi *et al*. (2008) prepared and compared the somatic and excretory/secretory antigens of *O. turkestanicum* in gel diffusion test. According to the results, somatic antigens showed strong reaction with antisera raised against excretory/secretory antigens and excretory/secretory antigens also showed strong reaction with antisera raised against somatic antigens. Antigenicity of *O. turkestanicum* excretory/secretory and somatic antigens is the same in gel diffusion test. Zheng *et al*. (2011) found that the components of the excretory/secretory products from a food-borne zoonotic parasite that resides in bile ducts and causes clonorchiasis, *Clonorchis sinensis* localizes to the intestinal cecum and vitellarium in adult worms. Hewitson *et al*. (2011) described that gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of venom allergen-like (VAL) proteins, which should allow functional testing of the various potentially immunomodulatory proteins. Rica *et al*. (2005) analyzed that amphidial glands, excretory/secretory gland cells, pharyngeal glands secreted proteins in adult females and males of *Syngamus trachea*, a cosmopolitan nematode parasite of the trachea of wild and domesticated galliformes and some passeriformes,

Excretory/secretory released by *A. galli* was able to trigger humoral immune responses by mean of IgY antibody formation. It is a well-known concept that IgY is the major antibody produced by laying hens. IgY is continually synthesized, secreted into the blood and transferred to the egg yolk, where it is accumulated. Various authors revealed that IgY specific antibody formation in egg yolk could be stimulated by *Helicobacter pylori* (Kazimierczuk *et al*., 2005), bacterial enteric (Chalghoumi *et al*., 2009; Diraviyam *et al*., 2011), and against *Eimeria acervulina* (Lee *et al*., 2009).

IgY antibodies from egg yolk chickens should be commonly used for immunodiagnostics regarding the advantages of the IgY. Dias da Silva & Tambourgi (2010) supported that application IgY can be developed in immunodiagnostic tests that provide more accurate results. IgY antibodies are therefore the easiest way to eliminate the errors that arise due reactions from the use of mammalian IgG. Previously, Hau & Hendriksen (2005) reported that the antibodies from the egg yolk have great potential for active implementation of the three Rs (replacing, reducing, and refining the use of laboratory animals to the extent possible) in polyclonal antibody production schemes. In the part of animal welfare, it is also ethically more attractive to produce chicken antibodies, as they are purified from the yolk. Unlike the production of mammalian IgG antibodies, IgY production does not require bleeding of animals. Dias da Silva & Tambourgi (2010) explained that the amount of IgY that can be obtained from a hen is also much larger than from e.g. a similar sized mammals. So, chicken egg yolk IgY antibodies offer a practicable alternative to mammalian serum antibodies because of their feasibility for large-scale commercial production and the relatively noninvasive methods used for their preparation.

When the IgY anti-excretory/secretory, reacting to epitopes, was used as a primary antibody. The appearance of dark brown color of AEC was considered as the basis for evaluation of positive staining in the parasite tissues. As seen in Figure 1, very strong immunostaining was observed in the epicuticle and cuticle followed by syncytial hypodermis. A part of scattered parenchymal cells in the worm were faintly stained. The positive immunohistochemical findings of IgY bindings suggested strongly that these antigenic proteins were produced at the cuticle cells and transported to the syncytial via connecting tubules or pore canals. The positive reaction in AEC staining to the antigenic proteins were especially strong et the distal margin of cuticle. This histologic finding suggested also that proteins might released outside of the worm.

IgY antibody purified from yolk of laying hens immunized with excretory/secretory antigen showed positive reaction with somatic antigen that appeared in the body of *A. galli*. Cuticle and epicuticle cells were positively stained with AEC chromogen. Good staining was observed in longitudinal muscle layers and syncytial hypodermis. The cuticle in all nematode parasites is an extracellular hydroskeleton that is relatively inert, structurally robust, and selectively permeable. Antigenic protein of *A. galli* were localized at egg inside the uterus of the worm when observed by immunohistochemistry using the polyclonal IgY antibodies reacting to these proteins. Polyclonal IgY anti-excretory/secretory antibodies in immunized chicken yolks were reacting to almost all structures of *A. galli* such as muscle, parenchymal cells, lining cells of excretory canal. Buccal cavity also reacted with these polyclonal antibodies. It is possible that excretory/secretory is synthesized from the oesophageal glands secreted into buccal cavity. Different stained ability of worm structures confirmed the excretory/secretory might represent from the some tissues of *A. galli*.

Immunohistochemistry provides the most direct method for identifying both the cellular and subcellular distribution of protein, including the protein antigen of worms. The sensitivity of the immunohistochemistry test against protein antigen that appeared in the somatic of worms have been conducted by some researches. Immunohistochemical techniques employed by Claeys *et al*. (2004) showed that the formation of the cuticle, microvilli in the embryonic intestine and adult nematodes, *Caenorhabditis elegans*. Pokharel *et al*. (2006) found that proteases were present in the adult female *Setaria cervi* tissues mainly localized in the body wall, outer body surface of the parasite, epicuticle, cuticle, syncytial hypodermis and longitudinal muscle layers. This enzyme were also distributed in the nerve cord region, intestine, growing embryos, uterus and mature eggs of the parasite.

IgY antibodies from egg yolk could be effective and efficiency useful in various immunoassays. Indeed, compared to the mammalian antibodies, IgY had a higher sensitivity, stability, and specificity in binding IgY-specific surface antigens or epitopes. Shin *et al*.

(2009) described that the specificity of polyclonal IgY antibody produced in chicken egg yolk was able to capture live organisms and has potential in the development of an immunocapture assay in a variety of clinical samples for *Mycobacterium avium* subsp. *paratuberculosis*, a chronic inflammatory bowel disease of both domestic and wild ruminants. An immunomagnetic bead ELISA using IgY against soluble egg extract as a capture antibody was applied to detect circulating antigens parasitic disease caused by trematode flatworms in serum of mice infected with *Schistosoma japonicum* (Lei *et al*., 2009). Furthermore, Lei *et al*. (2011) also described that the same method appeared to be sensitive and specific by using serum samples for diagnosis of schistosomiasis of persons with schistosomiasis. Recently, Lu *et al*. (2012) successfully developed a novel method of immunoassay based on IgY is an effective approach to discriminate between previous exposure and current infection of *S. japonicum* by mean of identification and profiling circulating antigens in sera of *S. japonicum* infected patients.

CONCLUSION

IgY stimulated by the excretory/secretory was able to recognized the antigen scattered in the tissues of *A. galli* so the IgY could be applied for immunodiagnostic.

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