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Tuesday, July 10th 2018
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Moderator: Dr. Susilowati Andajani, dr., MS

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Production of Rabbit Anti-Excretory/Secretory Product of *Fasciola Gigantica* Lombok Isolate Antibody

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Abstract: The major limiting factor in immunodiagnostic development for Fasciolosis detection is the absence of specific, monoclonal or polyclonal, antibodies against Fasciola antigens specifically *Fasciola gigantica*, which is the main species responsible for Fasciolosis in ruminants in Indonesia. The purpose of this study was to produce polyclonal antibody against excretory/secretory (ES) product of *F. gigantica* Lombok isolate using rabbit. Four months old rabbits were immunized subcutaneously with 0.5ml (300µg/ml) of the ES product emulsified with 0.5ml Freund's complete adjuvant and then Freund's incomplete adjuvant. Serum antibodies were harvested after two-times booster at 1-month interval and determined using enzyme linked-immunosorbent assay (ELISA) and Western blotting. Immunization results evaluated by ELISA show that rabbits can be used as a bioreactor to produce ES antibodies. The anti-ES antibody response could be detected 4 weeks post-immunization, followed by increased humoral response of rabbit after first booster. The optical density (OD_{450nm}) value in the ELISA increased from 0.4 before immunization to 1.184 and 1.392 depending on the type of blocking agent used. The Western blotting results confirm that the ES protein bands were only recognized by rabbit serum samples post-immunization and thus confirmed the ELISA test result.

1 INTRODUCTION

One of the strategies in early diagnosis of Fasciolosis caused by *Fasciola sp*. is the development of detection methods that can indicate the presence of disease before it develops into chronic through immunologic approaches based on antibody detection or antigen detection. However, the major limiting factor in the development of immunodiagnostic methods to detect Fasciolosis in livestock is the absence of specific, monoclonal or polyclonal, antibodies against *Fasciola sp*. antigens. Thus, the antibody production will be able to overcome the major inhibiting factor.

Fasciola sp. (liver fluke) that reside inside the infected host release a significant amount of excretory/secretory (ES) products (Spithill et al., 1999), and they play an important role in the avoidance of parasites from the host immune system (Morphew et al., 2007). A one-dimension electrophoresis gel analysis shows that ES products are composed of proteins of different molecular weight. Several studies have reported that geographic variation and different species of fluke will affect the components of the ES products produced. Sriasih et al. (2013) stated that one-dimension gel electrophoresis of the ES products isolated from *Fasciola gigantica* that infest Bali cattle in Lombok island showed protein bands with molecular weight between 7-25 kDa. The results of the study indicated that there is a difference in the components of the ES proteins of *F. gigantica* Lombok isolate with the results of previous studies reported by Meshgi et al. (2008) and Estuningsih et al. (2004).

Studies show that ES products have potential as an immunoprophylactic agent against Fasciolosis (Acosta et al., 2008; Jayaraj et al., 2009). Moreover, ES products are immunogenic so that are potentially used as vaccine candidates (Ortiz et al., 2000; Sethadavit et al., 2009), and are used for the diagnosis of Fasciola-specific antibodies in livestock (Sriasih et al., 2005; El Ridi et al., 2007; Kooshan et al., 2010). This indicates that the ES products from the Fasciola

sp. may be potentially used as an antigen to produce antibodies.

Antibody may be produced by inducing laboratory animals, such as rats, guinea pigs and rabbits, with an immunogenic antigen. Immune response induced by antigen after exposure can then be measured and determined using serological testing. Serologic tests developed today can be divided into two categories; primary binding and secondary binding tests. The primary binding test is a test that directly measures the antigenic bonding to antibodies including fluorescence antibody technique (FAT), radioimmunoassay (RIA) and Enzyme Linked Immunoassay (ELISA). The secondary binding test is a test that measures the results of antigen-antibody interaction in-vitro which includes agar gel precipitation est (AGPT), serum agglutination test and complement fixation test (FAT). However, the primary binding is more sensitive than the secondary binding test.

The purpose of this study was to produce polyclonal antibody against the ES product of *F. gigantica* Lombok isolate using rabbit and to determine the antibody response of rabbit to the ES product using the ELISA test, and Western blotting. The success in producing specific antibodies against specific antigens will increase sensitivity and specificity of immunodiagnostic test to perform early detection of Fasciolosis in livestock.

2 MATERIALS AND METHODS

2.1 Research Design

Two local rabbits (4 months old) were placed in a special cage and fed with pellets, fresh vegetables, and drinking water (ad-libitum). The rabbits were then immunized with 0.5ml (300µg / ml) of emulsified ES product with 0.5ml Freund's complete adjuvant (FCA). Sera were harvested and tested with the ELISA before initial immunization and after booster. The ELISA results were then confirmed by Western blot analysis.

2.2 Immunization and Serum Collection

Animal Ethics approval (Protocol No. 235/UN18.8/ETIK/2017) had been obtained for conducting this study. Immunization was carried out by subcutaneous injection at multiple sites (no more than 5) behind the neck and was dispersed under the skin by gentle rubbing. Four weeks after initial immunization with the ES product emulsified with the FCA, the rabbits were then immunized twice subcutaneously with 0.5ml (300 µg / ml) of the ES product mixed with 0.5ml Freund's incomplete adjuvant (FICA) at 1-month interval.

Blood sampling was performed by taking blood from the auricularis vein of the rabbits using a 3ml syringe. Blood were placed on a sterile tube and were then incubated at room temperature for 3 hours. After incubation, the serum can be obtained by centrifugation at 5000rpm at 4^{o}C for 15 min. The sera were then aliquoted into several eppendorf tubes and then stored at -20°C until further assays.

2.3 Measurement of Humoral Immune Response

Humoral immune response of each rabbit to the ES product was measured by ELISA technique, and then confirmed by Western blot. ELISA was performed according to Sriasih's procedure (Sriasih et al., 2005). The ELISA plates (96 wells) were coated with 50 µl of the ES product and incubated for 1 h at room temperature. After incubation, the ES product was removed and the wells were washed 5 times using phosphate buffer saline (PBS) containing 0.05% Tween 20. Blocking agent (100 µl PBS containing bovine serum albumin (BSA) or skim milk) or were added into each well and were then incubated for 1 h at room temperature. Following incubation, the plate was washed again (5 times washing cycling). Fifty microliters serum that had been diluted a hundred times were added into each well and then incubated for 1 hour at room temperature. After 5 times washing, 50µl anti-rabbitIg G horse-radish peroxidase conjugated were added and then incubated for 1 hour. One hundred microliters of substrate (ABTS in 100ml of citrate buffer) were added into the wells and incubated for 15 min room temperature. Optical density (OD) was then measured at 405nm wavelength using an ELISA reader machine.

2.4 Western Blotting Analysis

After electrophoresis, gels were equilibrated in transfer buffer for at least 10 min. Polyvinylidine difluoride (PVDF) membrane was pre-incubated in 100% methanol for 1 min then rinsed with several changes of water. After rinsing, the membrane and filter papers were also allowed to equilibrate for at least 15 min in transfer buffer. The transfer of proteins from the gel to the membrane was carried out at a constant voltage of 15 Volts for 35-40 min using a Trans-Blot® Semi-Dry (SD) electrophoretic transfer cell as per the manufacturer's instructions.

For immunostaining, the membranes were washed multiple times in distilled water and

immediately blocked with blocking buffer (Tris buffer saline pH 7.4 [20 mM Tris-HCl, 100 mM NaCl] containing 5% [w/v] skim milk and 0.1% [v/v] Tween-20) at room temperature for 1 hour. After washing twice in washing buffer (Tris buffer saline pH 7.4 [20 mM Tris-HCl, 100 mM NaCl] containing 0.1% [v/v] Tween-20), the membrane was cut into strips and further incubation of each strip was carried out in individual reservoirs. Each strip was incubated with 10 ml of diluted rabbit sera (1:500) for 1 hour at room temperature. Each strip was then washed for five 5-10 min cycles with washing buffer. After washing, diluted anti-rabbit IgG HRP (1:8000) was applied and incubated for 1 hour at room temperature. Strips were then washed as previously described. Immunodetection was developed by addition of TMB substrate.

2.5 Data Analysis

Humoral immune responses of each rabbit were recorded and analyzed using a simple statistical calculation (Mean ± Standard Deviation).

3 RESULTS AND DISCUSSION

The ES product used for rabbit immunization in this study was derived from the Lombok isolate of *F.gigantica* (Sriasih et al., 2013). The ES products were injected into the rabbits as a bioreactor to produce the ES antibodies because they have a distant genetic relationship with cattle, are easy to handle and easy to maintain. Rabbits are also laboratory animals that are widely used in various studies for the production of antibodies, tumorigenesis, nutrition, genetics, radiation research and anaphylactic research. The immune system of the rabbit will recognize and react to the antigen. The lymphocyte cells exposed as part of the body's defense system then will multiply and develop into plasma cells that produce antibodies. The antibodies formed are polyclonal antibodies with varied composition in serum, either as a result of repeated immunization, or due to variations that occur during an immune reaction (Tizard, 2004).

The ELISA test results of humoral rabbit response before immunization (baseline), 4 weeks after the initial immunization (FCA), 4 weeks after the second immunization (FICA1) and 2 weeks after the third immunization (FICA2) using two blocking agents, BSA and non-fat dry milk are presented in Table 1.

Table 1: The humoral response of the rabbits to the ES antigen on ELISA test.

	BSA Blocking		Non-fat dry milk blocking	
	Rabbit #1	Rabbit #2	Rabbit #1	Rabbit #2
Baseline	1.866±0.002	0.459±0.013	1.628±0.01	0.548±0.007
FCA	2.06±0.012	1.184±0.011	1.729±0.008	1.392±0.001
FICA1	2.135±0.005	1.928±0.007	1.772±0.003	1.901±0.002
FICA2	2.165±0.006	1.867±0.002	1.783±0.002	1.864±0.002

Data on Table 1 depict that only rabbit #2 showed a good immune response. The baseline accounted for an optical density value of 0.459 in the ELISA with BSA blocking and 0.548 with non-fat dry milk blocking. After immunization with the emulsified ES antigen with FCA, the optical density value increased to 1.184 (BSA) and 1.392 (non-fat dry milk). This may occur as a result of activation of the B cells memory whose work is stimulated by T cells to produce antibodies in large quantities (Goldsby et al., 2000; Abbas et al., 2007).

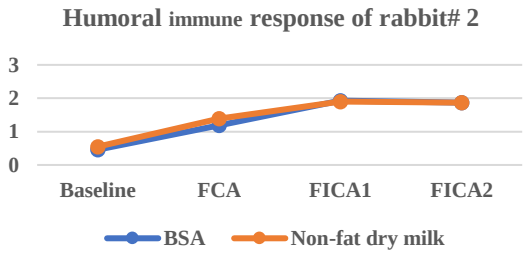

Figure 1: Polyclonal immune response of rabbit#2 anti-ES antibody blocked with different blocking agents.

The use of different blocking agents in this study (Figure 1) did not have a significant effect on the optical density values in the ELISA test. Both BSA and non-fat dry milk are regularly used blocking agents in the ELISA test. Milk contains a number of different proteins, and one of them is casein phosphoprotein. This phosphoprotein can cause a high background value due to the non-specific reaction to the phospho structure.

The Western blotting result (Figure 2) also corroborated the ELISA assay results and showed that the ES protein bands were only recognized by the rabbit serum (rabbit#2) after immunization. Cruse and Lewis (2002) suggest that the addition of adjuvants to the injected isolate serves to enhance the immunogenicity of the isolate. The presence of *Mycobacterium sp* in Freund's complete adjuvant in early immunization will stimulate B cells and T cells to produce an immune response. The primary immune

response of B cells is activated to proliferate and differentiate within antibody secretion cells and memory cells. Some antibody cells migrate and survive in the bone marrow for long periods. The second immunization (booster 1) and the third immunization (booster 2) with Freund's incomplete adjuvant will produce secondary immune response with higher concentration than the first immunization.

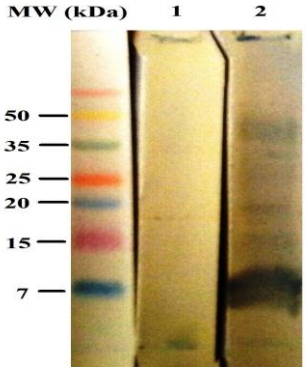

Figure 2: Western blot analysis of rabbit anti-ES polyclonal antibodies. MW = standar protein marker; 1 = rabbit pre-immune sera; 2 = rabbit sera collected after second booster.

The formation of rabbit antibodies is influenced by the antigenicity of the injected ES proteins. The principal feature of a substance or compound is determined by physicochemical limitation and degree of foreignness (Tizard, 2004). The physicochemical limitation of a substance or compound is that the size of the antigen molecule must be large, rigid and has a complex chemical structure (Kuby, 2007). The chemical structure of the ES proteins derived from the *F. gigantica* Lombok isolate that are large and complex will produce faster antibodies.

The results of this study indicate that rabbit can be used as a manufacturer to produce the ES antibodies. Antibodies formed 4 weeks post immunization, followed by increased humoral response of the rabbits after booster 1. Setyaningsih (2004) in her study using rabbits to produce polyclonal antibody against ES proteins isolated from *F.gigantica* buffalo isolate and goat isolate reported that antibodies anti-ES of *F. gigantica* goat isolate are formed faster than that of buffalo isolate. Antibodies anti-ES of *F. gigantica* goat isolate were formed at 4 weeks while antibodies anti-ES of *F. gigantica* buffalo isolate formed at 12 weeks. The time difference to elicit antibody-forming responses to the host may vary and is dependent on the immunogenicity, the form and stability of stimulants, animal species, injection routes, and the sensitivity of tests used to detect antibodies formation (Tizard, 2004). The host's response to the immunogens given is not only determined by the immunogenic physical properties but is also determined by host-related factors such as genetics, age, nutritional status and secondary effects derived from a disease.

4 CONCLUSIONS

Anti-ES polyclonal antibody specific to the ES antigens of *F. gigantica* Lombok isolate has been successfully produced in rabbit. The availability of the antibody will certainly facilitate the development of a better immunodiagnostic tests for controlling Fasciolosis.

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