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Antibody Titers in The Sheep which were Immunated Antigen of *Whole* Protein from Third Instar Larvae *Musca domestica*

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Abstract. This research aimed to know antibody titers in the sheep which were injected antigen of *whole* protein from third instar larvae *Musca domestica* by *Enzyme-Linked Immunosorbent Assay* (ELISA), that giving the antigen could cause increasing antibody titers in the sheep. A male sheep about two years old were immunated with *whole* protein from larvae *Musca domestica* which was added *Freund's Complete Adjuvant* and done three times injection again (booster) with range of time once-two weeks and addition of *Freund's Incomplete Adjuvant*. Taking sheep blood before and after immunitation was done to get serum as composition of ELISA test. Data which were got in this research, tabulated with descriptive analysis. The result of this research showed that there was a little of increasing antibody titers. The most titers at booster 1, then gradually had drastic decreasing. Based on the result of ELISA test can be concluded that antigen of *whole* protein from larvae *Musca domestica* can't stimulate immune response in the sheep.

Keyword : Antibody, Sheep, Immunated Antigen, Protein, *Musca domestica*

1. Introduction

Along with the development of the Indonesian population which is followed by the increasing number of market demand for animal-based nutritional needs, business is created to establish livestock, especially cattle farming. This is due to the desire of the community to meet the needs of consumers, especially milk and meat production. The development of cattle farming in Indonesia, which is currently starting to grow rapidly, is faced with various complex problems. There are several obstacles faced by farmers in their efforts to increase the production of livestock products, including problems with the occurrence of diseases, mismanagement, and the quality of feed used. One obstacle that is dangerous for farmers is the investment of parasites from fly larvae commonly called *myiasis*, although it rarely causes death but livestock productivity decreases. Flies of *M. domestica* are common insects with many species throughout the world. In the health sector is considered a pest because it is a mechanical vector of several diseases in humans and animals and can also cause *myiasis* [1].

Myiasis or *maggot* is an investment in fly larvae in humans and animals, in certain periods of life in living tissue or dead tissue, and the substance of body fluids. According to the location/damage caused, there are three types of *myiasis*: *cutaneus myiasis*, *body cavity myiasis* and *accidental myiasis*, while according to the nature of life the larvae are divided into *obligate myiasis* and *facultative myiasis*. The occurrence of *myiasis* begins with the arrival of adult flies that will place the eggs in the host body divided into primary, secondary and tertiary *myiasis* [2].

There are several insects that suck blood, tissue or tissue fluid from mammals for their life processes. These insects are often parasites or vectors of viral, protozoa and worm diseases in humans, livestock and wildlife [3; 4]. *M. domestica* flies include flies that cause tertiary *myiasis* that will worsen tissue damage that occurs in the host, *myiasis* that occurs includes *facultative myiasis*, because

these fly larvae can still live in places outside the animal's body. *Musca domestica* larvae can also be found in cases of *intestinal myiasis* that occur in humans [5]. Wounds caused by the bite of *Boophilus microplus* ticks as a cause of *myiasis* also invite a number of flies that live around livestock such as *Musca domestica* [6]. Several cases of nail *myiasis* found in Bogor reported that besides obligate fly larvae namely *Chrysomya bezziana* and *Booponus intonsus* there were also *Sarcophaga dux* and *Musca domestica* [7].

The strategy used to control insects in the livestock industry is currently being improved because the acceleration and development of insect flies is quite extensive, while insecticides can cause chemical residues on livestock products. Biological control of *myiasis* by releasing male flies that have been sterilized with radiation has been successfully carried out in Papua New Guinea, but this method is considered too expensive and requires basic information about the bioecology of flies [8], therefore an alternative to controlling *myasis* is sought vaccination.

Vaccination can be done using antigens comes from the intestines of flies *Lucilia cuprina* [9]. The intestine is coated with a semipermiable peritropic membrane (MP) consisting of layers of chitin, proteoglycan and protein. This membrane serves to facilitate the digestive process and protect intestinal epithelial cells from invasion of microorganisms and cause host immunity, therefore this MP protein can be used as a vaccine antigen. The vaccinated sheep using crude extracts of MP larvae protein can affect the immune response of the larvae, while the MP protein that has been purified when injected in sheep gives an immune response by inhibiting the development of larvae of *Lucilia cuprina*. Research on *Lucilia cuprina* flies has been done to see immune response after administration of antigens in the form of larvae [10].

Another possible approach is to vaccinate the host in order to fight the investment of insects with antigens from the insect's own body parts. Insect larvae are considered to be the easiest and most possible insect stage to be used in research. The body of the insect consists of chitin, proteoglycan and protein. In this study, purification of fly protein of *M. domestica* fly as antigen was then injected into sheep to determine antibody titers in sheep. Protein is the best antigen, almost all proteins with a molecular weight greater than 10,000 Dalton (Da) are antigenic [11], the greater the molecular weight the higher the immunological response.

Previous research shows that protein *M. domestica* fly larvae are antigenic and can cause immune response in rabbits [12]. The research was carried out by dot blot test which showed a strong antigen-antibody bond between the fly larvae of *M. domestica* and serum from rabbits.

The aim of this study was to examine the immune response of sheep to *M. domestica* larvae and find out how the antibody titers of sheep that had previously been injected with larvae protein, using the ELISA test. It is hoped that the results of this study can develop a vaccine for *cutaneous myasis* in sheep against *M. domestica* larvae which can ultimately reduce the severity of this *cutaneous myasis* process. In addition, the application of the results of this study can also be used as a basic reference in making an appropriate and protective *myiasis* vaccine material so as to overcome the problem of *myiasis* optimally.

2. Experimental Methods

2.1 Research Site

The research was carried out at the Veterinary Parasitology Department of the Airlangga University Veterinary Medicine Department, the Basic Medical Sciences Department of the Airlangga University Faculty of Veterinary Medicine, the Laboratory of Veterinary Molecular Biology, Airlangga University Veterinary Medicine, and the Airlangga Tropical Disease Center (TDC).

2.2 Research Materials and Tools

The ingredients needed in this study are: 1) In the *rearing* stage of flies and the collection of *M. domestica* larvae are flies in the form of 10% sugar water, vitamins and milk, laying chicken feces media; 2) At the extraction stage of *whole* protein, *M. domestica* fly larvae were 100 fly larvae of *M. domestica*, PBS, ice cubes, salt; 3) At the stage of examining the *whole* protein content of *M. domestica* fly larvae include samples extracted from *whole* protein larvae, Biuret reagents, protein standards, aquadest; 4) At the stage of immunization and blood collection are the *whole* protein antigen III larvae of flies *M. domestica*, PBS, *Freund's Complete Adjuvant*, *Freund's Incomplete Adjuvant*, Aquabidest, cotton, 70% alcohol, antibiotics; 5) The ELISA stage includes antigens

(proteins) from third instar larvae of *M. domestica* fly, buffer washing (PBS Tween-20), 4% Creamer in PBS I, serum samples from sheep, secondary antibodies (*conjugate anti sheep*), substrate (Alkaline phosphatase), stopper solution (NaOH 1 N).

The tools used for each stage of the research are as follows: 1) In the *rearing* stage of flies and the collection of *M. domestica* larvae, the cage is 40x40x40 cm, plastic cups are given a cotton cap, a device for laying eggs and larvae; 2) Tools at the extraction stage of *whole* protein larvae of *M. domestica* flies are test tubes, sonicators, glasses, eppendorf tubes; 3) At the stage of examining the *whole* protein content of *M. domestica* fly larvae, the test tube, pipette, spectrophotometer with OD_{540}; 4) At the stage of immunization and blood collection namely needle, sterile syringe, centrifuge tube, eppendorf tube, refrigerator 4°C; 5) Tools at the ELISA optimization stage include microplate, multichannel micropipette, eppendorf pipette, measuring cup, erlenmeyer, glass tube, electric scales, 37°C incubator, refrigerator, Aluminum foil, 405 nm ELISA reader.

2.3 Research Procedure

2.3.1 Rearing Flies M. domestica

Male and female adult flies are placed in flies (gauze) measuring 40x40x40 cm, this research is carried out using a laboratory population that has complete breeding in one generation in the laboratory. Male and female flies are placed in a fly cage so that they can mate naturally. Adult flies will be fed in the form of 10% sugar water, vitamins and milk, which have been prepared in plastic cups that are given a cotton cover until marriage occurs and the gravid female flies are ready to lay eggs. The egg is placed on the egg laying device that has been prepared in the cage.

2.3.2 Collection of M. domestica fly larvae

The eggs of flies that are produced from the marriage of male and female flies are kept in a fly cage. The resulting egg is separated in a separate place. The eggs that have hatched into the 1st instar larvae are separated and placed in separate containers containing layer chicken laying media. Identification of larvae is carried out using an identification key [13]. After becoming third instar larvae then put in a tube and stored in a refrigerator at 4°C. The larvae will later be used to supply ingredients in vaccination experiments.

2.3.3 Whole Protein Extraction of fly larvae of M. domestica

A total of 100 third instar larvae of *M. domestica* flies were put into a test tube which was filled with PBS 2 ml then destroyed by sonication using sonicator for 4 minutes and resting 2 minutes 10 times, with a vibration frequency of 20 kHz (20 thousand revolutions per second) When the test tube is sealed it is immersed in a glass of ice cubes and salt, this is intended to reduce the heat that arises when being authenticated, after the sample has been transferred in the eppendorf tube.

2.3.4 Examination of Whole Protein Levels of Flies Larvae of M. domestica

The *whole* supernatant resulting from sonication was collected and examined for protein content by Biuret method. Calculation of total protein was carried out by spectrophotometer at OD_{540}, and the results obtained were 17.06 µg/µl The calculation of the total protein is then used to determine the dose of immunization and antigen dilution at the indirect ELISA test. Supernatant is taken and stored at -20°C, and is ready to be fractionated with protein.

2.3.5 Immunization in Sheep

Immunization is performed on one ram with a body weight of 30 kg. In this study the dose of *whole* protein larvae antigen used was 1500 µg. The dosage was obtained by converting the dose given to rabbits with a weight of 2 kg which is equal to 100 µg in the previous study [12]. The results of the calculation of the total larval protein by spectrophotometer at OD540 is 17.06 µg/µl. Sheep are immunized with 88 µl of *whole* protein *M. domestica* fly larvae dissolved in PBS to 1 ml then added with *Freund's Complete Adjuvant* (1:1). Blood collection is carried out before the first injection as a control.

Injections are performed on the sheep subcutaneously in the neck area with different sides each time injected. The immunization application is carried out subcutaneously in the hope that through this method protein depots can be released gradually. Injection is carried out with 3 booster times, which is

two weeks after the immunization and booster are carried out two weeks later with the same dose accompanied by the addition of *Freund's Incomplete Adjuvant*. Booster is done to increase antibody formation and avoid hypersensitivity reactions.

Blood collection in sheep is taken through the jugular vein in the neck. Place fresh blood that has been taken in a 15 ml centrifuge tube then let stand for a while while tilting at an angle of 45º to 180º. Separate the serum by centrifuging at a speed of 5000 rpm for 10 minutes. Take the supernatant carefully and if there is resuspension, do centrifugation again. Serum collection is done to see the antibody response after immunization. The collected serum is stored in the freezer (-20 ºC) until the ELISA test is carried out.

2.3.6 Measurement of Antibody Titers by ELISA Test

This test is used to measure the levels of antibody and antigen titers by measuring the absorbent value through *Optical Density* (OD). The obtained *anti-whole* protein larvae were tested by an indirect ELISA technique using *conjugate anti sheep* that had been labeled with the enzyme Alkaline phosphatase. The ELISA test is done by coating the ELISA plate which has a concentration of 1 µg/ml with antigen, then reacted with antibodies, followed by visualization with Peroxidase or Alkaline phosphatase substrate [14]. Serum *anti-whole* protein larvae are read in ELISA reader with a wavelength of 405 nm [15].

2.3.7 Data processing

The data obtained in the ELISA test, then tabulated descriptively.

3. Results and Discussion

3.1 Rearing Flies and Collection of Larva M. domestica

Rearing male and female flies of *M. domestica* have been carried out in the laboratory and produced eggs in a few days. Fly eggs are hatched until larvae are obtained, starting from the first instar larvae to third instar larvae, then some larvae are collected for extraction of *whole* larvae, others are rearing to adulthood. *Rearing* flies is done by collecting flies of *M. domestica* to be kept. Maintenance of these flies is intended to obtain III instar larvae *whole* protein is used as a source of antigen to be used in the ELISA test, after a few days gravid female flies put their eggs in the faecal media and the eggs hatch into I instar larvae to III instar larvae. In this study, third instar larvae were used because they had a large body size so as to facilitate identification and handling. The collection of III instar larvae for the extraction of *whole* protein was done by using tweezers one by one, collected in petri dishes and washed with *Phosphate Buffer Saline* (PBS) three times until clean.

A good larval preservation process is by killing it in hot water and then storing it in 70-80% ethanol, this method can protect larval proteins and prevent larvae from turning black [16], when using formalin can cause larval tissues to become brittle not recommended for molecular analysis purposes except for the preparation of histological preparations [17]. Clean larvae are then placed in tubes and stored in a refrigerator.

3.2 Determination of Whole Protein Level of Larva M. domestica

The extraction results of *whole* protein larvae of *M. domestica* were measured by spectrophotometer with OD_{540}, it was known that the protein content was 17.06 µg/µl. A total of 100 third instar larvae of *M. domestica* collected were extracted using sonicator with a vibration frequency of 20 kHz. The samples that have been completed are transferred to the eppendorf tube, then the *whole* supernatant is collected and the protein content is examined by the Biuret method. Calculation of total protein was measured by a spectrophotometer with OD_{540}, and it can be seen that the protein content was 17.06 µg/µl. Measurement of protein content is intended to find out how much protein will be injected in experimental animals.

3.3 Measurement of Sheep Antibody Titers

ELISA test results of sheep antigen immunized by fly larvae of *M. domestica* can be seen in **Table 1**.

Table 1. Sheep ELISA Test Results Immunized by Fly Flies *M. domestica*

Antigen dilution (times)	Absorbance at λ405 nm					
	Control	Immunization	Booster 1	Booster 2	Booster 3	Blank
0	1,936	1,975	2,037	1,888	1,940	0,003
1	2,090	2,073	2,161	2,045	2,078	0,000
2	2,040	1,988	2,030	2,001	1,948	0,001
3	2,028	1,979	2,098	1,914	1,909	0,004
4	1,982	2,087	2,144	2,017	1,937	-0,001
5	1,966	2,004	1,936	1,954	1,975	-0,003
6	1,918	2,018	2,044	2,078	1,907	-0,003
Total N	7	7	7	7	7	7
Mean	1,99429	2,01771	2,0642	1,98671	1,98671	0
Std deviation	6,131E-02	4,5187E-02	7,7064E-02	7,7064E-02	6,9928E-02	0

The test limited to first 100 cases

The results of observation were the value of *Optical Density* (OD) of antibody titers of sheep with *indirect* ELISA test and the mean value can be seen in **Table 1 and 2**.

Table 2. Average *Optical Density* Value 405 nm Sheep Antibody Titers

Treatment	Average ± SD
Control	1,9943 ± 0,06131
Vaccination	2,0177 ± 0,04519
Booster 1	2,0643 ± 0,07706
Booster 2	1,9867 ± 0,06993
Booster 3	1,9563 ± 0,05850

In the table above shows that in vaccination with *whole* protein antigens of fly larvae of *M. domestica*, the increase in antibody titers produced was relatively small. The highest titers at the 1st booster, then gradually experienced a sharp decline.

Based on the results of *optical density* titers of *anti-whole* protein larvae antibodies by ELISA test, it was obtained data that on 2 weeks after vaccination with *whole* protein larvae of *M. domestica* flies added by *Freund's Complete Adjuvant,* sheep showed an increase in antibody titers from 1,9943 to 2,0177. If antigen that enters the body first time the immune response appears called the primary immune response [18]. The primary immune response is characterized by the appearance of IgM a few days after exposure. The time between antigen exposure and the appearance of IgM is called the Iag phase. IgM levels reach their peak after approximately 7 days. Six to seven days after exposure, IgG can be detected in the serum, while IgM begins to decrease [18; 19].

At 2 weeks after the 1st booster accompanied by the addition of *Freund's Incomplete Adjuvant*, antibody titers in sheep appeared to increase from 2,0177 to 2,0643. Increase in *optical density* antibodies

this is due to a response secondary [18; 20]. The secondary response formed has several characteristics, namely: 1) Immunoglobulin formation takes place more quickly and for a longer time, 2) Immunoglobulin reaches high optical density, 3) Immunoglobulin consists of IgG. So with the first, second and so on secondary responses given through a booster, memory cells will rapidly proliferate to form higher and longer *optical density* antibodies.

At 2 weeks after the 2nd booster, antibody titers decreased from 2,0643 to 1,9867. This is because the formation of antibodies does not last indefinitely, control mechanism that controls and stops excessive antibody formation [19]. Some of these control mechanisms are: reduced antigen content, regulation by idiotype and suppression by suppressor T cells.

Antibody titers are decreasing from 1,9867 to 1,9563, at 2 weeks after the 3rd booster. This is because the specific cells for which the antigen is concerned increase and the effector cells react to get rid of the antigen. After antibodies are formed, antigens are destroyed or neutralized by antibodies so

that only immunocytes with high receptor affinity alone can recognize antigens, thus immunocyte activity decreases. This decrease in activity is regulated by a decrease in the number of antigens, also caused by the antibody itself which can provide negative feedback [19]. So one day the formation of antibodies will decrease after the highest optical density is obtained.

The titer ELISA test has increased and will decrease with time [21]. It is said that antibody titers can have varying values from one study to another, this is influenced by differences in health and nutrition (nutrition) of animals and weather conditions of the experimental area.

The increase in titers depends on the immune response of the animal. Factors that can optimize the immune response to the incoming antigen are the nature of the immunogen, adjuvant, animal chosen, method of injection and dose of antigen given. Factors that can also increase the immune response to incoming antigens in addition to the five factors above are the period between the first immunization in FCA with booster immunization in the first FIA, and the period between the first FIA booster with booster in the next FIA [20]. The administration of adjuvants is intended to strengthen the response of the appearance of antibodies, because the antigens that enter the body will be secreted slowly, drop by drop so that they have a long period of time in the body [22].

Another factor that can influence the antibody's responsibility is the antigen that is injected. The antigen complexity affects the immune response produced, especially the molecular weight with the amino acid composition contained. The more complex and large the molecular weight the higher the immunological response. Factors that influence immunogenicity of a substance include acidity, molecular size, and immunogen complexity [11]. Another influential factor is the dose of antigen that is given so that it can trigger antibodies. Protein concentrations were measured by spectrophotometer with samples that had been authenticated. In the measurement of protein by spectrophotometer the damaged protein fraction and the cleanliness of the sample determine the measurement results so that the unrefined samples from other particles can give a biased result than expected.

4. Conclusion

The ELISA test results showed that immunization of sheep with larval *whole* protein antigens had only a slight increase in antibody titers. The highest titers were at the 1st booster, then gradually experienced a sharp decline, thus it can be concluded that *whole* protein antigens of *M. domestica* larvae have not been able to trigger an immune response in sheep.

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