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Immune Response (Serum Globulin) in BALB/c Mice after Hookworm Egg Protein Immunization as the Initial Stage of Developing Laboratory Diagnostics: An *In Vivo* Approach

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Abstract

BACKGROUND: Hookworm infestation is still high and requires practical laboratory diagnosis with high sensitivity and specificity. Meanwhile, there are several limitations associated with the existing method; hence, a new method is essentially needed. Furthermore, the principle of immunological reactions needs to be developed by identifying the extent of hookworm eggs suspension immune responses. The BALB/c mouse is among the most widely used inbred models used in biomedical research and is particularly utilized in immunology and infectious disease research.

AIM: This study aims to determine whether the protein concentration of hookworm eggs stimulates antibodies formation (proteins) in the serum of BALB/c mice.

METHODS: This is an experimental study with a post-test only control design approach. Egg protein was isolated by removing the contents using a mini drill to immunize BALB/c mice, while the antibody response was observed by spectrophotometer and agglutination methods.

RESULTS: The Chi-square and Post hoc statistical tests showed a significance $p \leq 0.001$ indicating a relationship between hookworm egg protein and agglutination results. The higher the antibody level, the more visible the agglutination and vice versa.

CONCLUSION: These results are expected to form a basis for developing more practical and efficient diagnostic methods based on antigen-antibody reactions.

Introduction

Worm infestation is one of the most common diseases that spread and infect people worldwide. In 2015, the World Health Organization (WHO) stated that 24% of the world's population suffers from worms. Sub-Saharan Africa, America, China, and East Asia have the largest incidence rates. Meanwhile, one of the causes of high worm infestation is the low quality of environmental sanitation [1], which leads to malnutrition, physical, mental, cognitive growth, and intellectual decline in children, as well as decreased immunity, disability, and death [2], [3].

Soil-transmitted helminth (STH) is a group of soil-borne worms which consist of four species, namely *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (Necator americanus and Ancylostoma duodenale) [4]. The prevalence of this STH group is still high ranging from 45–65% in Indonesia. The most prevalent is hookworm with an incidence of 32% in 1000 population [5], [6].

Helminthiasis diagnosis is established by laboratory examinations using conventional methods, including direct, indirect, and microscopic [7]. In the direct method, reagents such as eosin, Lugol, and NaCl are used to observe the worm eggs clearly and properly. Meanwhile, in the indirect method, the duration of flotation and sedimentation is often used as a variable to obtain a false positive/negative result. Moreover, expertise is required in identifying worm eggs by laboratory personnel. The limitations are minimized using an easy examination method with high sensitivity, specificity, as well as positive and negative predictive value [8].

Method development needs to be carried out using the principle of antigen and antibody reactions. This method requires antibodies as reagents at an early stage to establish a diagnosis. Meanwhile, hookworm eggs contain immunogenic proteins with similar structures at all stages. Antibodies are obtained by immunizing worm

egg protein in experimental animals [9]. The BALB/c mouse is among the most widely used inbred models used in biomedical research and is particularly utilized in immunology and infectious disease research. Their ability to produce plasma cell tumors within soft tissue is important in the production of monoclonal antibodies (mAbs). Furthermore, BALB/c mice showed the best response to polyclonal antibodies formation compared to other types [10] [11], the higher the antigenic level, the stronger the response [12] [13]. The content of antibodies is observed by reacting the serum of immunized mice with hookworm egg protein using agglutination and spectrophotometry methods. Agglutination is one of the immunogenicity test methods that induce an antigen-antibody reaction in the particles form [14]. Meanwhile, spectrophotometry was used to measure the concentration of immunoglobulin in the serum of BALB/c mice [15]. Immunoglobulins (IgG, IgM, IgA, IgE, and IgD) are present in serum gamma globulin which is one of protein fractions [16], [17]. Furthermore, globulin is a component of serum protein that indicates antibody expression [18], [19]. The antibody concentration in the serum is important to determine the extent of hookworm egg protein immune response for the development of laboratory test methods to establish worm infestations.

Materials and Methods

This is an experimental study with *post-test only controls design* approach, consisting of 4 groups, namely, 1 negative control and 3 test groups (P1, P2, and P3) each using five experimental animals. The outputs examined include hookworm egg protein and globulin levels from the BALB/c mice serum.

The stages are as follows:

The making of hookworm egg protein

Fecal samples that were only positive for hookworm eggs were screened using the microscopic method (Tables 1 and 2). Meanwhile, worm eggs isolation was carried out by modified flotation and sedimentation methods. Positive samples were separated using the saturated salt flotation method, because the number of samples required was large. This process was carried out using a separating funnel to easily remove dirt deposits. The supernatant was collected for repeated centrifugation with physiological NaCl solution and filtered using a gauze with a pore diameter of 300 mess.

The hookworm egg protein components were obtained by crushing the eggshells in a mixer using a mini drill for 10 min. The mixing was controlled microscopically to observe the egg grains, meanwhile, residual eggs when found were destroyed repeatedly. Furthermore, the sample was examined for protein content with a spectrophotometer to determine the absorbance and protein concentration (Figure 1).

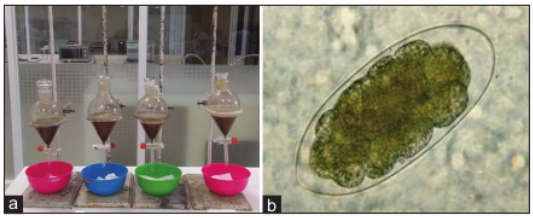

Figure 1: Modified methods of flotation, sedimentation, and microscopic examination of stool samples

The examination of hookworm egg protein levels

Hookworm egg protein suspensions were prepared in concentrations of 5%, 10%, and 15%, while the protein content was measured using the spectrophotometer method by making a standard curve from a protein standard solution, measuring the absorbance of samples at each concentration, as well as a negative control of the stool suspension, and input data on a standard curve. The X-axis was protein concentration while Y-axis was the absorbance; hence, the formula for the equation of the line was obtained, while the protein content was measured by entering the absorbance value.

Selection of experimental animals

The experimental animals selected were female BALB/c mice aged 6–8 weeks weighing about 18–20 g, because BALB/c mice have a very good response to immunization, BALB strain mice are more able to survive twice as strong as the CBA/CBA2 strain and C3H/HeJ. The immune status of the immunized individual can also determine the outcome of the immunization procedure. The age of mice used by young adults is good for the production of polyclonal antibodies (pAb). Conventionally, female animals have been used most often in pAb production. Females are generally more docile for handling purposes, and less aggressive in social interactions, and can therefore be grouped more successfully. While there is some evidence that androgens can slightly dampen antibody responses, there is no major scientific reason not to use male animals.

The protein immunization in experimental animals

BALB/C mice were injected with hookworm egg protein in treatments 1st (P1), 2nd (P2), and 3rd (P3) with concentrations of P1 (5%), P2 (10%), and (15%). Meanwhile, the control was injected with negative stool samples. *Boosters* were carried out on the 7th, 14th, and 21st, while, on the 30th day, blood harvest was carried out. Furthermore, this study used variations in the concentration of injection based on the recommended dose for mice which

are 5 ug per 100 ul injection. However, because the antigen used is in liquid form, the concentration was made directly into 5% and an additional variation of 10% and 15%.

Blood collection

Mice blood samples were obtained from the conjunctiva after 35 days using a capillary tube. It was drawn with minimum stress for mice, that is, by administering anesthesia before sampling to reduce stress and pain, by veterinary staff. Furthermore, blood collection was carried out under warm conditions to ensure smooth blood flow, no sudden noise which might trigger stress, and the sampling site was kept clean to avoid blood contamination. To obtain the antibody, the sample was placed in a plain vacutainer tube, frozen, and then centrifuged at 10,000 rpm for 5 min. The supernatant formed was collected and placed into a sterile tube and then stored at −20°C.

The spectrophotometric test

The protein globulin level was obtained by calculating the difference between total protein and serum albumin which was read by the photometer method. Globulins make up 40% of the total protein, the remainder consisting of 60% albumin.

Globulin formula (g/dl): Total protein – Albumin

Each solution mixture was homogenized and then incubated at 37° for 10 min, the sample absorbance was read to the blank at a wavelength of 546 nm.

Agglutination test

Mice serum from the control, as well as treatments 1, 2, and 3, were used as samples to check for the presence of antibody which is formed by the principle of agglutination. An antigen in this test is the hookworm egg protein concentration, meanwhile, the volume of each stage is 20 ul of both mice serum and antigen. Agglutination appeared in the form of grains on the slide and was observed for 2 min.

Table 1: Total protein procedure

	Blank	Standard	Sample
Aquadest	20 ul	-	-
Lar Standard	-	20 ul	-
Sample	-	-	20 ul
Monoreagent (4R1:1R2)	1000 ul	1000 ul	

Ethical clearance

This study was conducted after obtaining *ethical clearance* from the ethics committee of FKM UNIMUS Semarang No.423/KEPK-FKM/UNIMUS/2020. The Head of the Clinical Pathology Laboratory, Muhammadiyah Semarang University, approved this study after receiving notification of *ethical clearance* result.

Table 2: Albumin procedure

	Blank	Standard	Sample
Aquadest	10 ul	-	-
Lar standard	-	10 ul	-
Sample	-	-	10 ul
Reagent	1000 ul	1000 ul	1000 ul

Results

Analysis of worm egg protein levels

The pure fecal suspension samples were examined for the number of hookworm eggs to predict the density. In each 20 µl, there were 3–4 eggs/field of view with a magnification of 100 times. This produced an image indicating that each milliliter of the pure sample contains 1000 hookworm eggs.

Examination of hookworm egg protein levels using the spectrophotometer method obtained the following data (Table 3):

Table 3: Hookworm egg protein level based on the concentration (%)

Hookworm egg protein concentration	Protein content (ug/ul)
Negative stool suspension	0.420
5%	39.305
10%	58.694
15%	72.679

The protein content of hookworm eggs is directly proportional to the concentration, hence, the higher the concentration, the higher the protein content. In the negative stool suspension, almost no protein content was found.

Analysis of serum globulin protein levels in mice

The examination of globulin protein levels using a spectrophotometric device on five mice serum per each treatment group after 35 days of immunization using worm egg protein produced the following results (Figure 2):

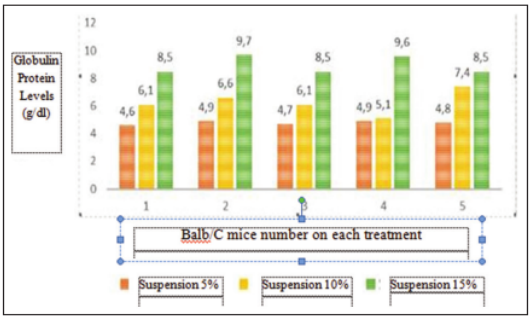

Figure 2: Graph of serum globulin protein levels in mice after immunization with hookworm egg protein

Based on Figure 3, the highest and lowest globulin protein content were found in the 15%

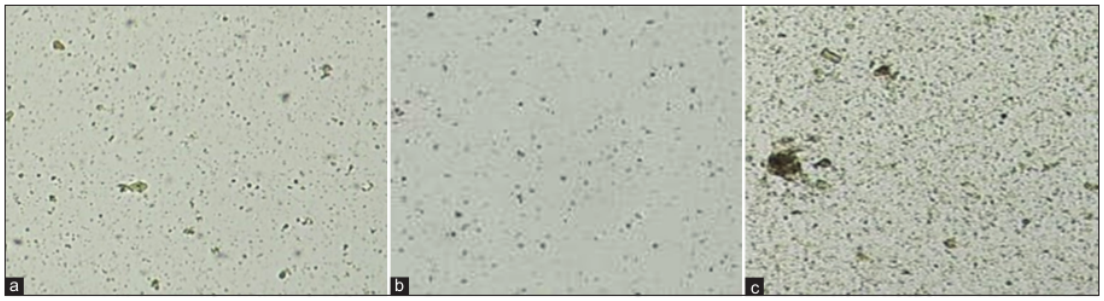

Figure 3: (a-c) Agglutination test results of mice serum and hookworm egg protein, where a and b showed negative results while c showed positive results

Table 4: Antibody concentration of egg serum BALB/c mice after immunization of antigen variation by spectrophotometric method

Stool Suspension Concentration	Hookworm egg protein content/Antigen (ug/ul)	Serum Protein Levels in BALB/c mice after immunization)/antibodies (ug/ul)
5%	39.305	43.167
10%	58.694	68.802
15%	72.679	110. 713
50%	100.086	-
100%	167.090	-

hookworm egg protein suspension. The protein concentration is directly proportional to globulin protein levels in the serum of BALB/C mice.

The antibody concentration in ug/ul based on the injection of antigen (hookworm egg protein content) is illustrated in the following Table 4:

Based on Table 4, the higher the dose given, the higher the antibody expression produced.

Table 5: BALB/c Mice serum agglutination test results with hookworm egg protein concentration

Hookworm egg protein concentration (Ag) (%)	BALB/c mice serum after immunization with hookworm egg protein					
	5%		10%		15%	
	Agl	#Agl	Agl	#Agl	Agl	#Agl
5% (39.305 ug/ul)	5	0	5	0	5	0
10% (58.694 ug/ul)	5	0	5	0	5	0
15% (72.679 ug/ul)	0	5	0	5	5	0
50% (100.086 ug/ul)	0	5	0	5	5	0
100% (167.090 ug/ul)	0	5	0	5	2	3

Information: Agl: The amount of agglutination, #Agl: The amount of agglutination does not occur.

Antigen and antibody which reacted with the same concentration all formed agglutination. The serum of Balb/C mice was also reacted with antigen concentrations of 50% and 100%. The result showed that when the antibody concentration was lower than the antigen, agglutination was faint, negative, or totally absent (Table 5).

Based on the Chi-square statistical test, $p \leq 0.001$ was obtained, which indicates that there was a significant difference in the agglutination results of mice serum with 5%, 10%, and 15% fecal suspension (Table 6). To determine the differences, the *Post hoc* test was carried out as follows in Table 7.

Table 6: Statistical test results of agglutination of mice serum proteins to hookworm egg protein concentration

	Value	Df	(2-sided)
Pearson Chi-square	69.871[a]	14	0.000
Likelihood ratio	92.376	14	0.000
Linear-by-Linear Association	0.108	1	0.742
N of valid cases	75		

Based on the *Post hoc* test, p-value between each group was below 0.05, this indicates that there were significant differences in concentrations of 5%, 10%, and 15% with serum antibody levels in BALB/c mice.

The results showed that hookworm egg suspension contains numerous proteins and the levels increase according to the percentage of the suspension. Hookworm egg suspension immunized in BALB/c mice also indicated an increase in globulin levels. This was further supported by the agglutination result which showed that there were significant differences in concentrations of 5%, 10%, and 15% with serum antibody levels in BALB/c mice.

The ability of an immunogen to stimulate antibody formation depends on the type, mode of entry, the receiving individual, and the sensitivity of the method used for detection [20], [21], the greater the immunogenic protein the better. This is evident from the increase in protein as one of the dominant components in antibodies. Furthermore, the results showed that each concentration of hookworm egg protein stimulates immune response formation characterized by increased levels of protein globulins in the experimental animals' serum. This increase activates components of the body's defenses such as lymphoid organs such as the spleen, lymph nodes, and lymphoid tissue associated with the intestines, including the tonsils where various antibodies are produced by stimulating B lymphocytes (plasma cells) [22], [23].

The modulating effect of worm infection on the immune system occurs due to changes in the balance of T-helper 1/T-helper 2 (Th1/Th2) to Th2 cells (Th2 polarized) [24]. Acute infection with intestinal worms stimulates the host immune response known as *Th2 response* [25]. This immune response polarization is characterized by an increase in Th2-specific *cytokines* such as *interleukin-4* (IL-4), *interleukin-5* (IL-5), *interleukin-13* (IL-13), as well as immunoglobulin E (IgE) [26], [27], [28] as demonstrated in the levels of

Table 7: Post hoc statistical test results of serum agglutination of BALB/c mice to hookworm egg protein concentration

Multiple comparisons						
Globulin		erroni level				
(I) Stool Suspension	(J) Stool suspension	Mean Difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Stool Suspension 5%	Stool suspension 10%	−1.4800*	0.3861	0.007	−2.553	−0.407
	Stool suspension 15%	−4.1800*	0.3861	0.001	-5.253	-3.107
Stool Suspension 10%	Stool suspension 5%	1.4800*	0.3861	0.007	0.407	2.553
	Stool suspension 15%	−2.7000*	0.3861	0.001	-3.773	-1.627
Stool Suspension 15%	Stool suspension 5%	4.1800*	0.3861	0.001	3.107	5.253
	Stool suspension 10%	2.7000*	0.3861	0.001	1.627	3.773

*The mean difference is significant at the 0.05 level.

the gamma globulin fraction in the total protein globulin. The results showed that the higher the concentration of hookworm egg protein, the higher the globulin response.

High levels of globulins are used as material to develop immune response reactions for a more practical and efficient serological-based hookworm egg examination method.

The presence of hookworm egg protein is an early indication used as the most potent immunogen component in the macromolecular proteins form, such as polysaccharides, or other synthetic polymers including *polyvinylpyrrolidone (PVP)*. Meanwhile, the configuration of the antigen-antibody molecule only produces antibodies that are specific to one type of antigen [29], [30]. The greater the immunogenic protein the better, meanwhile, protein increases in an organism as one of the dominant components in antibodies [31].

Agglutination test is one of the methods used to prove the presence of antigen and antibody reactions. One of the methods used is direct agglutination [32], which requires that the antigen and antibody react directly, with the antigen in the form of a particle or cell, hence, when it reacts with a specific antibody, clumps are formed [33].

Conclusion

The statistical tests showed that variations in the protein concentration of hookworm eggs produced antibodies indicated by the increase in serum protein of BALB/c mice, the occurrence of agglutination reactions, and the difference in agglutination results. These results are applicable as a reference for further studies to develop a new simple method for detecting hookworm using serological methods.

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