Sensitivity and Specificity of ELISA Using Excretory/Secretory of *Fasciola Gigantica* For Detection Fasciolosis In Cattles

Ima Malawati[1], Made Sriasih[1] and Djoko Kisworo[1]
[1]Laboratorium Mikrobiologi dan Bioteknologi Fakultas Peternakan Universitas Mataram, Jalan Majapahit No. 62 Telp/Fax (0370) 633603/640592 Mataram.
Email: Ima.Malawati27@gmail.com

ABSTRACT

Fasciolosis is one of the diseases caused by *Fasciola hepatica* and *Fasciola gigantica* which attacks livestock especially ruminants. This disease can be acute and chronic and causes a big loss to farmers due to a decrease in cattle's weight gain as well as death. Generally, the disease could be determined by egg examination on faeces. However, this method poses some drawbacks, such as the excretion of eggs that is irregular, and the number of eggs excreted in faeces is very low, thus affecting the result of diagnosis. Another drawback is inability of eggs examination method to detect infection on pre-paten period (8-10 weeks post infection) and it's low sensitivity due to eggs production that is relatively limited on pre-paten period. Alternatively, Fasciolosis could be detected using serologic tests such as ELISA test. This study aimed to determine sensitivity and spesificity of ELISA in detecting Fasciolosis on cattles using Excretory Secretory (ES) component of *F.gigantica.* The ELISA results showed that all sera samples tested (n = 16) using 3 different antigens gave a sensitivity of 100% while its specificity could not be determined as all sera samples tested showing positive OD 405nm value (above cut off value) though eggs examinations on faeces gave a negative result.

.

Keywords: *F. gigantica*, *Fasciolosis,* ES Antigen, ELISA, Sensitivity

INTRODUCTION

Fasciolosis is a parasitic disease caused by trematoda digenetic worms. *Fasciola hepatica* and *Fasciola gigantica* that commonly attack ruminant livestock and result in economic losses due to decreased production of meat, milk, wool, even death and the rejected liver. Epidemiologic case of Fasciolosis is a cosmopolitan livestock disease because of its distribution in countries that raise ruminant animals (Mitchell, 2007). Control of Fasciolosis can be done through prevention programs and treatment of livestock. The success of the control program depends on the diagnosis being performed. Conventional diagnosis of Fasciolosis is performed by examining the worm eggs in the feces sample. This method has several deficiencies such as low sensitivity and it can show false negative results because worm eggs are not found in the prepatent period. According to Anderson et al. (1999), from 72 cows containing Fasciola sp. in the liver showed a false negative result on stool examination of 34.72%.

However, this method also has several advantages, like the needed cost is not much, the process is simple and it is more practical for some people. In addition, the method of examination

of worm eggs in feces is a gold standard which is usually done to detect various diseases before the existence of more accurate new methods (Awad et al., 2009). An alternative approach to overcome the difficulties in diagnosing Fasciolosis is by serodiagnosis, antibody tests against antigens specific to Fasciola spp. in execretory secretory (ES) liquid of *F.hepatica* worm in addition to the discovery of the adult worm in the liver's livestock. Serodiagnosis is performed by Enzyme Linked Immunosorbent Assay (ELISA), which had been developed and used to diagnose *F. hepatica* infection in cattle and sheep (Farrel et al., 1981).

Various research results indicate that ES fluid isolated from *F.hepatica* is highly potential for use in ELISA assays for the detection of Fasciolosis in cattle (Sobhon et al., 1996). Nevertheless, Fasciolosis in Indonesia and other tropical countries are mainly due to the infestation of *F. gigantica* (Estuningsih, 2006). The diagnostic approach for Fasciolosis detection in cattle in Indonesia based on the causative agent *F. gigantica* is still very limited and inadequate (Estuningsih, 2006). Therefore, it is necessary to study the development of Fasciolosis detection using *F. gigantica* worm component like ES liquid. This study was conducted to determine the sensitivity and specificity of ELISA using ES of *F. gigantica* worm liquid component as an effort to detect Fasciolosis disease in cattle.

MATERIALS AND METHODS

ES liquid collection

Mix the *F.gigantica* with 10 ml of PBS and incubated for 20 minutes. Regurgitant 1 which generally contains blood, bile, and dirt was removed and then add new PBS as much as 10 ml. This process was repeated up to 3 times and the last, incubated in PBS for overnight at room temperature. Centrifuged at 2500rpm at 4^0C for 15 min and filtered through a 0.22 μm filter. Stored at -20^0C before being used. The protein concentrations present in ES fluids were measured using a spectrophotometer at a 595nm wavelength.

ES fluid fractionation

PEG_{6000}, sodium sulphate and diluted ES liquid were taken at a ratio of 1: 1: 1, 3 ml of ES liquid, 3 ml of PEG_{6000} and 3 ml of sodium sulphate, mix it slowly for 30 minutes. Centrifuged at 1500g for 20 minutes. Next, the sample according to fraction was separated and inserted into eppendorf, then the sample was dialysis.

ELISA (Enzyme-linked Immunosorbent Assay)

ELISA was performed by following Sriasih's procedure, et al. (2005). The ELISA plates (96 wells) were coated with 50 μl ES antigen, 50 μl fraction I ES antigen and 50 μl fraction II ES antigen (coating) and were incubated overnight at 4^0C. After incubation, ES liquid and ES fractions I and fraction II were discarded and the wellbore was washed 5 times using PBS containing 0.05% Tween 20. In each hole, 100 μl PBS was added containing 2% BSA and incubated during 30 minutes at room temperature. The washing process was repeated as before, then adding 50 μl serum FBS that had been diluted a hundred times as a negative control and 50 μl serum sample at each well. After being washed, 100 μl rabbit anti-bovine IgG horse-radish peroxidase conjugate was added to the wellbore and then was incubated for 1 hour at room temperature. After incubation, the liquid in the well was removed, then 100 μl of substrate (ABTS in 100 ml of citrate buffer) was added and incubated for 30 minutes at room temperature.

Then 100 µl stop solution was added to each well if the desired color was sufficient for further measurement of Optical Density value at 405nm wavelength using ELISA reader machine.

RESULTS AND DISCUSSION

The result of fractionation shows the existence of two components (fraction) namely fraction I and fraction II. The fractions were then dialyzed with membrane cellulose tubing for 24 hours at 4°C using PBS to remove PEG_{6000} and sodium sulfate so that it could be used as an antigen in the test. The protein concentrations of both fractions were measured using spectrophotometer (Bradford method). The result of measurement of protein concentration at 595nm wavelength compared with BSA standard showed that the fraction protein concentration I was 250 µg / ml while fraction II was 100 µg / ml. The concentration of ES liquid before fractionation was 2000 µg / ml. To know the protein profile of ES fluid and its fractions, SDS-PAGE was performed. The result of characterization with acrylamide gel is presented in Figure 1.

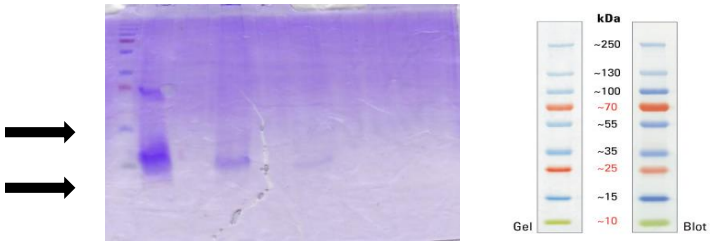

Figure 1. Analysis of ES antigen profiles using SDS-PAGE. (A) Marker, (B) Original ES fluid, (C) Fraction I, (D) Fraction II, (E) Diluted Faction I, (F) Diluted Fraction II, (G) Standard Marker

The Figure 3 data shows that the major protein constituent of ES (profile B) was a protein with a molecular weight between 14-25 kDa. The protein profile of fraction II and diluted fraction II show the same profile that was at 14 kDa molecular weight. The protein profile of fraction I and diluted fraction I was not apparent in the gel (smear). The use of Brilliant Blue Coomassie dyes may have an effect on the appearance of protein bands on the gel. To get more sensitive coloring, silver staining silver can be used. Soulsby (1986) states that the originally *F. gigantica* antigen from cattle had 20 patterns of polypeptide bands in the molecular weight range of 14-156 kDa. The results of this study are in line with the results from Soulsby (1986) study although protein above the 25 kDa molecular weight was not identified in the results of this study.

In this study, the concentration of antigen used was 50 µg/ml. The coating of the antigen by dilution was a sufficient concentration, because according to Stewart et al. (1990) the concentration of a coated antigen on a microplate surface ranges from 1-10 µg / ml. The first type of antigen used for the ELISA test was ES fluid prior to fractionation. As for the second antigen, it used fraction I ES fluid and fraction II of ES liquid were served as the third antigen. In all three tests, the positive determinant detection limit of F. gigantica ES antigen was detected, as it was seen from the cut off value of each test. The cut off value of each test was calculated from the average negative control absorbance value added with three times from the standard deviation value.

The negative control used was FBS (Fetal Bovine Serum) while for blank used PBS-Tween 20 without added serum samples. The average absorbance value of test results greater

than the cut off value was a positive test result. Conversely, the average absorbance value of test results that was smaller than the cut-off value was a negative test result.The cut off value of ELISA for the first antigen was 0.046849 (Fig. 2) obtained by summing the absorbance value by 3 times from the standard deviation. The cut off value of the second antigen ELISA (Figure 3) was 0.046184 and the cut off value of the third antigen ELISA (Figure 4) was 0.027743. ELISA test results used microplate which was coated with ES antigen to measure antibody response in cattle sample serum are presented in Figure 2, Figure 3 and Figure 4.

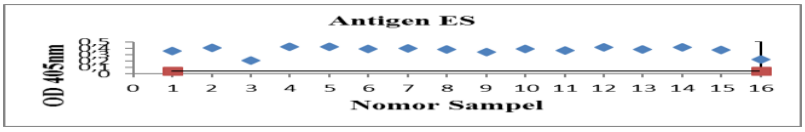

Figure 2. The absorbance value (OD) of ELISA using the original ES liquid

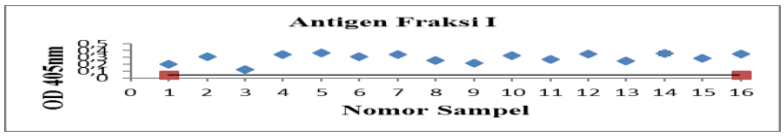

Figure 3. The absorbance value (OD) of ELISA using fraction I antigen

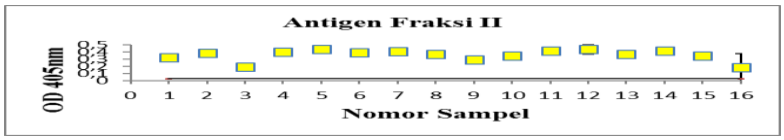

Figure 4. The absorbance value (OD) of ELISA using fraction II antigen

The graph in Figure 2 shows that the highest absorbance value was in sample number 5 which is 0, 4255 and the lowest absorbance value is in sample number 3 that was 0,207. While the graph in Figure 5 shows that the highest absorbance value shown in sample number 5 which is 0,564 and the lowest absorbance value was in sample number 3 that is 0,1215. The highest absorbance value shown in Figure 2-4 is the sample number 12 which is 0.4375 while the lowest absorbance value shown in sample number 16 is 0.1825. The average absorbance value described in the three graphs above is 0.37 to 0.43.

Table 1. Standardized ELISA Test of all three antigens based on egg examination in the feces.

		Examination of eggs in feces		
		Positive	Negative	Total
ELISA Result	Positive	10	6	16
	Negative	0	0	0
	Total	10	6	16

The data in Table 1 shows that the sensitivity of ELISA test using ES antigen is 100%, as well as antigen fractions I and fraction II. Nevertheless, the specificity value of the ELISA test in this study could not be determined because all test samples had a positive OD value (above the threshold value) even though the results of the worm egg examination showed negative results. Other studies had reported that ELISA test results with serum blood had high sensitivity ranging from 85% -98% (Estuningsih et al., 2004). Spithill et al. (1999) explains that many factors affect

the sensitivity of a test, including the type of antigen and purity of antigen used. This type of antigen is also closely related to the environmental factors in which the parasite originated.

CONCLUSIONS

The results of this study indicate that the sensitivity of ELISA using ES liquid component is 100%. However, the ELISA specificity value could not be determined. The results of this study indicate that serological tests using ES fluid components could be used for Fasciolosis detection in cattle.

REFERENCES

Anderson, N., T.T. Luong, N.G. Vo, K.L. Bui, P.M. Smooker and T.W. Spithill. 1999. The sensitivity and spesificity of two methods for detecting Fasciola infections in cattle. Vet. Parasitol. 83: 15-24

Awad WS, Ibrahim AK, Salib FA. 2009. Using indirect ELISA to assess different antigens for the serodiagnosis of Fasciola gigantica infection in cattle, sheep, and donkeys. Res Vet Sci 86:466-471.

Estuningsih SE. 2006. Diagnosis of Fasciola gigantica infection in cattle using capture ELISA assay for detecting antigen in faeces. JITV 11(3):229-234.

Estuningsih SE, Widjajanti S, Adiwinata G, Piedrahita D. 2004. Detection of coproantigen by sandwich ELISA in sheep experimentally infected with Fasciola gigantica. Trop Biomed 21(2):51-56.

Farrell, C.J., D.T. Shen and R.B. Webcott. 1981. An enzyme-linked immunosorbent assay for diagnosis of Fasciola hepatica infection in cattle. Am. J. Vet. Res. 42: 237-240.

Mitchell GBB. 2007. Liver Fluke. Edisi ke-4. London: Blackwell.

Sobhon, P.S. Anantavara, T. Dangprasert, A. Meepool, C. Wanichanon, V. Viyanant, S. Upatham, T. Kusamram, T. Chompoonchan, S. Thammasart, P. Prasittirat. 1996. Fasciola gigantica: Identification of Adult Antigens, Their Tissue Source and Possible Origins. J. Sci. Soc. Thailand.

Soulsby EJL. 1986. *Helminths, Arthopods, and Protozoa of Domesticated* Animal Seventh Editon. London: Bailliere Tindall.

Spithill, T.W., P.M. Smooker, D.B. Copeman. 1999. Fasciola gigantica: epidemiology, control, immunology and moleculer biology. In: Dalton, J.P. (Ed), Fasciolosis. CAB International, Wallingford, pp.465-525.

Stewart I, Wood D, Jenkins J, Jinna A, Dodge B, Acaster M, Said B, Burns R, Gridley S, Brenner J, Gibson V. 1990. Immunochemical Techniques a Practical Workshop. Hatfield Herts: Hatfield Polytechnic