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Identification of Somatic Antigens of Adult *Fasciola gigantica* Isolated from Bali Cattle

Made Sriasih[1*] and Ahmad Munjizun[2]

[1]*Faculty of Animal Science University of Mataram, Jl Majapahit 62 Mataram 83125, Lombok, NTB, Indonesia*
[2]*Graduate Student, Department of Animal Science, North Carolina State University, USA*

*Corresponding author's Email: madesriasihphd@unram.ac.id; ORCID: https://orcid.org/0000-0002-7497-8146

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ABSTRACT

In most tropical countries, such as Indonesia, fasciolosis is generally caused by *Fasciola gigantica* known as tropical liver fluke. However, most fasciolosis serodiagnostic tests have been developed solely for diagnosing fasciolosis caused by *Fasciola hepatica* (non-tropical liver fluke), and very few have been specifically designed for *F. gigantica*. The aim of this study was to determine the profile of antigenic proteins from the somatic extract of *F. gigantica* isolated from Bali cattle (*Bos javanicus*). The liver flukes were collected from a slaughtering house in Mataram, Indonesia. The somatic extracts were prepared by homogenizing in buffers containing 0.05 M NaCl, 0.02 M PMSF, and 0.05% Triton X-100. The characterization of the somatic extract proteins was performed using one-dimension gel electrophoresis and followed by Western blotting to determine the profile of its antigenic proteins. There were 14 bands of the somatic extracts with an estimated molecular weight ranging from 8 to105 8 kDa shown on the gel electrophoresis. The results of the Western blot show that there were five prominent protein bands. Three out of five prominent antigenic proteins with molecular weights of 8, 27, and 33 kDa are promising to enrich the existence of antigens that have immunodiagnostic value for fasciolosis. Therefore, further studies are required to examine more deeply the potency of those three antigenic somatic proteins of *F. gigantica*.

Keywords: Bali cattle, *F. gigantica*, Immunodiagnostic, Somatic extract, Western Blot

INTRODUCTION

Fasciola gigantica and *Fasciola hepatica*, known as liver fluke, are important parasites of class Trematoda that cause a zoonotic parasitic disease, termed fasciolosis, in humans and animals. In definitive hosts, such as cattle, sheep, goats, and buffaloes, these flukes enter orally and then migrate to the liver through the peritoneal cavity. Fasciolosis have affected 30-80% of cattle herds in developed countries (Charlier et al., 2014). Moreover, climate change and increased livestock movement have led to a wider range of liver fluke infections in livestock (Howell & Williams, 2020). In most tropical countries, including Indonesia, the disease caused by *F. gigantica* is detrimental with varying prevalence rates. In Indonesia, the prevalence of fasciolosis in ruminants varies from 40 to 95% (Estuningsih et al., 2004; Manus and Dalton, 2006; Astiti and Panjaitan, 2012). Worldwide, economic losses caused by *Fasciola* infection cost at least three billion dollars annually resulting from the loss of weight, decreased fertility, decreased milk and wool production, increased mortality, and high costs for treating infected animals as well as liver damage (Spithill et al., 1999; Abunna et al., 2010; Nyirenda et al., 2019; Arias-Pacheco et al., 2020). This condition is exacerbated by the emergence of resistance to the anthelmintic triclabendazole which is often given as a fasciolosis therapy (Fairweather, 2005).

Fasciolosis incidence in humans has a positive correlation with the incidence of fasciolosis in livestock, and therefore, fasciolosis is a current public concern. This concern is not only because of its high prevalence and economic losses caused by infecting various types of livestock (Schweizer et al., 2005), but also due to its zoonotic nature which can infect humans through ingestion of infective metacercariae (Mas-Coma et al., 2005). World Health Organization (WHO) reported that human fasciolosis has spread in about 70 countries around the world with the number of cases nearly 2.4 million (WHO, 2007). Given its enormous impact on public health, it is necessary to undertake rapid and precise disease control efforts in both livestock and humans.

Disease control can be in the form of prevention and/or treatment in infected hosts. One of the main keys that determines the success of disease control is a correct diagnosis. Traditionally, *Fasciola* infection in animals is diagnosed by examining the presence of eggs in the feces. However, this method is considered less effective in detecting the parasite at the prepatent stage as eggs will only be produced 12 to 14 weeks after infection (Anderson et al., 1999). In addition, other factors, such as the age of the host, fecal consistency, and the amount of fecal specimen tested, can affect the sensitivity of detection based on egg fecal counting (Rojas et al., 2014).

Serodiagnostic methods, such as enzyme-linked immunosorbent assay (ELISA), offer better sensitivity and specificity than that of traditional methods for the early diagnosis of *Fasciola* infection (Rojas et al. 2014; Naeemipour

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et al., 2016, Naqvi et al., 2019). Serodiagnostic methods, however, require a number of potent antigens that can recognize the infection during its prepatent period. A variety of *Fasciola* antigenic components derived from whole-worm (somatic) extract and excretory/secretory (E/S) products, as well as recombinant proteins, have been evaluated and used to develop serodiagnostic tests for ruminants. Amongst those types of antigenic components, the metabolic antigens released in the E/S material of adult parasites remain the main source of potential antigens (Aguayo et al., 2019). Moreover, most of the serodiagnostic tests have been developed solely for diagnosing fasciolosis caused by *F. hepatica*, and very few have been specifically designed for *F. gigantica* (Kelly et al., 2019).

Considering that the main cause of fasciolosis in tropical countries, including Indonesia, is *F. gigantica*, it is necessary to explore its potential antigenic components for early detection. A recent study carried out by Dar et al. (2019) on various types of antigenic proteins of *F. gigantica* isolated from sheep showed that both somatic and E/S fractions were good sources of antigen. They found that protein bands with molecular weights of 38 and 44 kDa in the somatic fraction, and protein with sizes of 27 and 33 kDa in E/S fraction were very promising to be used for *Fasciola* detection in sheep. This finding indicates that studies addressing the potential of somatic antigens are also promising to enrich the existence of antigens that have immunodiagnostic value for diagnosis as well as vaccine development. The present study was carried out to determine antigenic components of somatic extract of adult *F. gigantica* isolated from Bali cattle with promising diagnostic value using Western blotting.

MATERIALS AND METHODS

Ethical approval

The process of collecting sera from calves in this study was carried out carefully and in accordance with national guidelines (Animal Health Division, Ministry of Agriculture, Indonesia) for blood collection.

Collection of worms

Adult worms of *F. gigantica* were collected from the liver of Bali cattle (*Bos javanicus*) at an abattoir in Majeluk, Mataram, Indonesia March to April 2020. The liver was excised and the adult worms were removed and placed in sterile 0.1 M phosphate-buffered saline (PBS, Sigma-Aldrich) pH 7.4 at 37°C. Identification of the collected worms as *F. gigantica* was perfomed based on visual gross inspection (brown gray in color, body shape resembles leaves, flat dorso-ventral, does not have a clear shoulder shape and longer body length than *F. hepatica*) and morphometric measurements (1.6-3.6 cm x 0.3-0.8 cm) (Oktaviana et al., 2019). The worms were then washed five times with PBS to remove any traces of blood and bile, transported under cold condition immediately to Microbiology and Biotechnology Laboratory of Animal Science Faculty, University of Mataram, Indonesia, and stored at -80°C.

Preparation of somatic extracts

Somatic extracts were prepared according to the method described by Wijffels et al. (1992). Two *F. gigantica* adult worms were placed and grounded on a mortar, then homogenized using 800 µl buffer containing 0.05 M NaCl, 0.02 M PMSF, and 0.05% Triton X-100. The worms were then crushed vigorously using a mortar and pestle to the smallest size possible. The resulting mixture was centrifuged at 10,000 rpm for 30 minutes. The supernatant was then collected and stored at -20°C for further assay.

Sodium dodecyl sulphate polyacrilamid gel electrophoresis

The *F. gigantica* somatic proteins were separated according to their molecular weight by Sodium Dodecyl Sulphate-Polyacrilamid Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). The SDS-PAGE was performed under reducing conditions with 12% separating and 6% stacking gel mixture in Mini Protean II electrophoresis apparatus (Bio-Rad Laboratories, Inc., USA). Prior to loading the gel, the somatic proteins were mixed (1:1) with loading buffer (100 mM Tris-HCL pH 7.0, 4% SDS, 100% mercaptoethanol, 0.2 bromophenol blue, and 20% glycerol). The proteins were then heated in a water bath at 100°C for 5 minutes. The gel was run at 100 V, 40 mA for approximately 2 hours, and 5 µl of Broad-Way Dual pre-stained protein marker (Intron Technology, China) was included on each gel as a size reference. After running completely, the gel was stained using Coomassie brilliant blue dye and let stand for 1 hour. The stained gel was washed in 150 ml acetic acid with gentle shaking for 20 minutes until the protein bands on the gel could be seen.

Western blotting

The immunoblotting technique was performed according to the method of Towbin et al. (1979). After electrophoresis, the gel was equilibrated in transfer buffer for at least 20 minutes. Nitrocellulose (NC) membrane was pre-incubated in dH_2O for 2 minutes then was allowed to equilibrate in the transfer buffer for 10 minutes. The transfer of proteins from the gel to the membrane was carried out at a constant voltage of 5 V, 0.1 A for 45 minutes using a Trans-

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Blot® SD Semi-Dry electrophoretic transfer cell (Bio-Rad Laboratories, Inc., USA) as per the manufacturer's instructions. The transfer was confirmed by staining the membrane with 0.2% (w/v) Ponceau S in 30% (v/v) trichloroacetic acid for 10 minutes with gentle shaking. The membrane was washed with distilled water until protein bands could be easily visualized.

Following the washing, the membrane was immediately blocked with blocking buffer made from 5% skim milk in Tris buffer saline-Tween 20 (TBST, pH 7.4 containing 0.1% Tween 20) at 4°C overnight. After washing twice in washing buffer (TBST) 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 sera (1:100) for 1 hour at room temperature or 4°C overnight. The sera were taken from cattle confirmed fasciolosis positive or negative (based on the presence of eggs in their fecal samples) so that the antigenicity of the protein on the Western blotting can be determined. Each strip was then washed for five 5-10-minute cycles with washing buffer. After washing, diluted anti-bovine IgG-HRP (Sigma- Aldrich) 1:8000 was applied and incubated for 1 hour at room temperature, and followed by washing step as previously described. Immunodetection for each strip was carried out by adding 1.5 ml of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich) substrate as recommended by the manufacturer. The strips were then dried for further analysis.

Molecular weight determination

The molecular weight (MW) of the somatic protein bands both in SDS-PAGE and Western blot was determined by comparing the migration rate of the protein against the known standard migration rate of the standard protein markers. The relative mobility (Rf value) of both the standard marker bands and each of the dominant bands from the sample was calculated (Rf = migration distance of band ÷ migration distance of dye front). The protein MW in each band was calculated by substituting the Rf value to the regression equation obtained from two standard marker bands flanking the intended band (y=ax+b). Finally, the MW was determined by calculating the anti-log of y value (10y).

RESULTS AND DISCUSSION

Somatic protein profiles of *F. gigantica* isolated from Bali cattle

Protein profile characterization is the first step that must be taken to determine protein candidates that can be used as a basis for vaccine production, medicinal development, or development of diagnoses in overcoming various diseases, including fasciolosis, that has spread sporadically both in Indonesia and throughout the world. There have been many methods used to separate protein molecules in a sample (Allam et al., 2002), including SDS-PAGE. The SDS-PAGE is a technique that is still widely used to estimate the number of polypeptides and the complexity of the protein in samples or purified samples (Garfin, 2003). The working principle of SDS-PAGE is the separation of protein molecules that occurs due to differences in the speed of each protein in which smaller proteins migrate faster than the larger proteins.

The results of the characterization of the somatic protein components of the *F. gigantica* isolated from Bali cattle are presented in Figure 1. Based on the SDS-PAGE results as presented in Figure 1, the somatic protein profiles in lanes 1 to 3 show that after the separation of proteins based on their MW, the protein bands look very thick so that it is difficult to distinguish between one band and the other one. The thickness of the protein bands illustrates the amount of protein with similar MW in each band. The best dilution that shows clear protein separation in Figure 1 is in lane 4 with 1:2 dilution. The SDS-PAGE results in lane 5 show the presence of 14 dominant bands (band number 1 to 14) of *F. gigantica* somatic protein extract.

Table 1 shows the MW of the dominant proteins resulting from SDS-PAGE gel electrophoresis. There are 14 protein bands with estimated MW of 8, 16, 17, 21, 27, 30, 33, 37, 43, 52, 58, 73, 89 and 105 kDa. El-Rahimy et al. (2012) compared the antigen components between *F. gigantica* and *F. hepatica* and showed that there were differences in the molecular weight of 13 types of protein as evident from the SDS-PAGE results between *F. gigantica* and *F. hepatica*. The size of the proteins, however, only ranged from 9.1 to 35.7 kDa (El-Rahimy et al., 2012). The most dominant protein in the study performed by El-Rahimy et al. (2012) were proteins with MW of 29.3, 26, and 19 kDa. The SDS-PAGE results from the somatic extract in this study indicated the presence of protein bands with molecular weight up to 89 kDa with the most dominant proteins being proteins with the MW of 8, 16, 17, 27, to 37 kDa. These differences may be related to the influence of age of infection which is correlated to age and species of flukes (*F. gigantica* and *F. hepatica)* (Estuningsih and Widjajanti, 1999), the effect of host species (Mas-Coma et al. 2005), and the influence of different geographic locations of research objects (Sobhon et al., 1996; Meshgi et al., 2008). Estuningsih and Widjajanti (1999) compared protein profiles of *F. gigantica* of five different ages (juvenile, 3 weeks, 6 weeks, 9 weeks, and adult). They found that a number of protein bands found in adult flukes samples were not present in the other-age sample groups. Differences in protein profiles have also been associated with host species. De Vera et al. (2009) compared protein profile of *Fasciola sp*. between two different host, cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*). They found that although the worms between the two host species shared some common protein bands, a number of bands were found to be bubaline specific for both *F. hepatica* and *F. gigantica*. Species-related differences in protein bands of the fluke samples was also reported by Lee et al. (1992), who infected cattle, llama, rats, and mice with *F. hepatica*. They found that the variation was higher in E/S samples than the whole body prtoein, suggesting that the

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differences may be associated with different metabolism in responsse to different host species (Lee et al., 1992; De Vera et al., 2009). In regards with different geographic locations, there were some differences in nucleotide sequences of internal transcribed spacers (ITS-1 and ITS-2) of *Fasciola sp.* ribosomal DNA from various countries and this may relate to the different origins of the trematodes (Itagaki et al., 2005; Alasaad et al., 2007; Ali et al., 2008; Farjallah et al., 2009).

Another important factor to consider is the calculation of molecular weights for each protein band in the gel, which is relative to the molecular weight of the protein marker. It is notable that the logs of the marker do not show a completely linear curve (Figure 2). Therefore, the calculation of the protein band molecular weights would be more accurate by generating the linear regression from each two adjacent marker bands. The intended protein bands that fall between these known bands, hence, can be obtained using the respective formula. Meanwhile, the molecular weights that are obtained from the linear regression of the whole marker bands would result in the less accurate calculation since the log MW of all protein bands are forced to be linear. The calculation method and the accuracy in determining the band migration distance in the gel would affect the results, in which similar proteins could possibly be written in slightly different molecular weights. This can be solved by further study through the sequencing process.

Antigenic protein profile of *F. gigantica* somatic extract based on Western blot analysis

Western blotting is a technique that is widely used to detect protein expression in a cell or tissue extract (Kurien & Scofield, 2006). The principle of the method which consists of several stages is to determine the presence or absence, size, and modification, or degradation of a target protein and as far as possible can determine the quality of the protein itself (Taylor et al., 2013). This technique measures the protein level in a biological sample through antibody binding to the specific protein desired. Western blot can detect a specific protein in materials containing a number of proteins and can provide information about that specific protein (Dechend et al., 2006; Sakudo et al. al., 2006).

Nine Bali cattle serum samples consisting of six fasciolosis-positive samples and three fasciolosis-negative samples (based on the results of examining the presence or absence of eggs in the feces) were used in the current study to determine the reactions between the somatic antigens and specific antibodies in the serum samples. The somatic antigenic protein profile of the *F. gigantica* using the Western blot method is presented in Figure 3. The results of the Western blot showed that there were 5 prominent proteins. The molecular weight of each protein band from the Western blot results is presented in Table 2.

Data from Figure 3 show that proteins with MW of 70 and 47 kDa appeared only on the NC membrane incubated with negative samples (lane 9). Proteins with MW of 33 and 27 kDa were present in nearly all membranes incubated with fasciolosis-positive serum samples but were also recognized by two negative samples (lanes 8 and 9). This phenomenon may indicate that these proteins are potential for further investigation using a larger population of positive and negative samples. The protein with an MW of 8 kDa only appeared in three of the six positive samples.

The immunoblotting results in the current study indicated that the antigenic protein was represented by a protein band with an MW of 8 kDa. The 8 kDa protein bands were recognized by 50% of bovine serum infected with fasciolosis and did not appear on the NC membrane that was probed with negative samples. The absence of that antigenic protein on the other three positive samples was probably due to the different responses of each individual to an antigen (Mas-Coma et al., 2005).

The results of the present study are very interesting since an antigenic protein with a size of 8 kDa is rarely recognized as a specific antigen in various experiments. In line with the findings of the current study, Kim et al. (2003) stated that the use of 8 kDa protein from crude extract fractionation of *F. hepatica* in detecting fasciolosis gave good results without any cross-reaction with other trematode infections. Of the five positive serum samples used in their study (Kim et al., 2003), four of them were recognized as an antigen with a molecular weight of 8 kDa and none appeared in the negative samples. The results of the study carried out in our research are similar to that of De-Almeida et al. (2007) on the evaluation of the immune response of patients infected with fasciolosis. The findings indicated that the antigen with a molecular weight of 8 kDa was the dominant molecule recognized by the patient's antibody.

The proteins with molecular weights of 33 and 27 kDa appeared in all positive samples (100%) but were also recognized by two negative samples (Figure 3), indicating that these proteins were still potential as antigenic proteins in our study. This is because the determination of the fasciolosis-positive and -negative samples used was based on the presence or absence of worm eggs in the feces, which was less sensitive than the serological method. Worm eggs would not be found in the feces of fasciolosis-infected livestock if the worms present in the liver are not yet sexually mature although specific antibodies are already formed. Some researchers claimed that proteins with MW of 33 kDa and 27 kDa are specific antigens. Gonenc et al. (2003) suggested that a protein with a molecular weight of 33 kDa was the most potential antigen for diagnosis. Rivera-Marrero et al. (1988) also reported that the protein band weighing 25-30 kDa of the ES antigen was a specific protein for acute and chronic fasciolosis in rabbits, cattle, and sheep. Sampaio-Silva et al. (1996) stated that *F. hepatica* with molecular weights of 25 and 27 kDa were the most sensitive antigens to serum infected with fasciolosis. Mohamed et al. (2004) again emphasized that the antigens that were immunoreactive against the serum of patients infected with fasciolosis were 25-29 kDa and 12 kDa, which were purified from crude extracts of *F. hepatica*. Farghaly et al. (2009) reported that the diagnosis using the Western blot method using *Fasciola* E/S antigens showed that in all fasciolosis infected patients, an antigen with MW of 27 kDa gave the highest specificity, sensitivity, and accuracy to detect *Fasciola* antibodies.

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The use of somatic extracts as an antigen source for serological diagnosis has been developed by previous researchers. Farghaly et al. (2009) affirmed that the use of somatic extracts of *F. hepatica* in the Falcon assay screening test (FAST)-ELISA method showed sensitivity, specificity, positive and negative predictive values of 92.5%, 86.7%, 89%, 82.2%, and 94.5%, respectively. Another fasciolosis study showed that the use of a somatic antigen to determine the IgG-ELISA response demonstrated a sensitivity of 100% with a specificity of 96.4%, with positive and negative predictive values of 97.8% and 100%, respectively (Rokni et al. 2003). The results of these studies indicate that the use of somatic extracts as an antigen source for the diagnosis of fasciolosis is very feasible to develop.

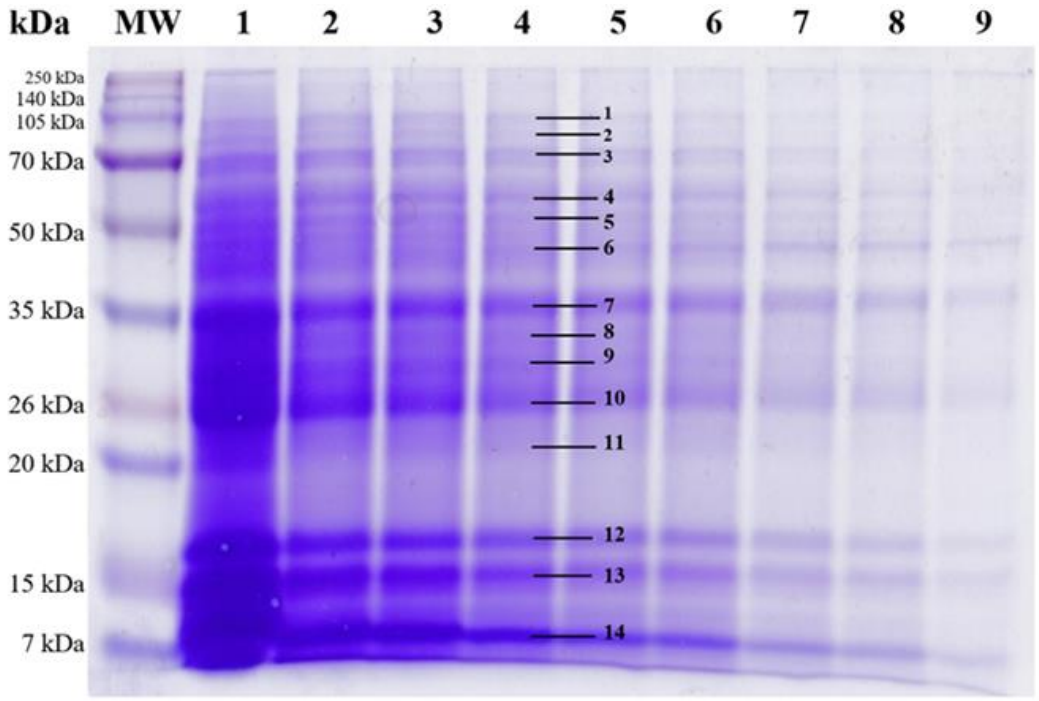

Figure 1. Characterization of the *F. gigantica* somatic proteins using sodium dodecyl sulphate polyacrilamid gel electrophoresis. Lane molecular weight (MW): Protein standard marker. Lane 1-2: Somatic protein extract without dilution. Lane 3: Somatic protein extract diluted 1x in phosphate-buffered saline. Lane 4: Somatic protein extract diluted 2x in phosphate-buffered saline. Lane 5: Somatic protein extract diluted 3x in phosphate-buffered saline. Lane 6: Somatic protein extract diluted 5x in phosphate-buffered saline. Lane 7: Somatic protein extract diluted 10x in phosphate-buffered saline. Lane 8: Somatic protein extract diluted 20x in PBS. Lane 9: Somatic protein extract diluted 40x in phosphate-buffered saline.

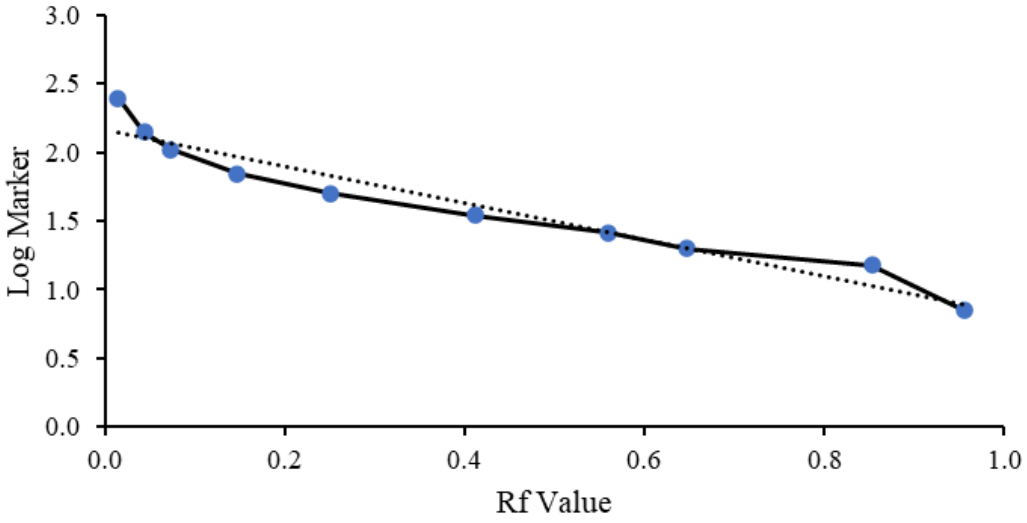

Figure 2. Standard protein molecular weight curves in sodium dodecyl sulphate polyacrilamid gel electrophoresis. The log molecular weight of the intended protein bands should fall within the black gridlines.

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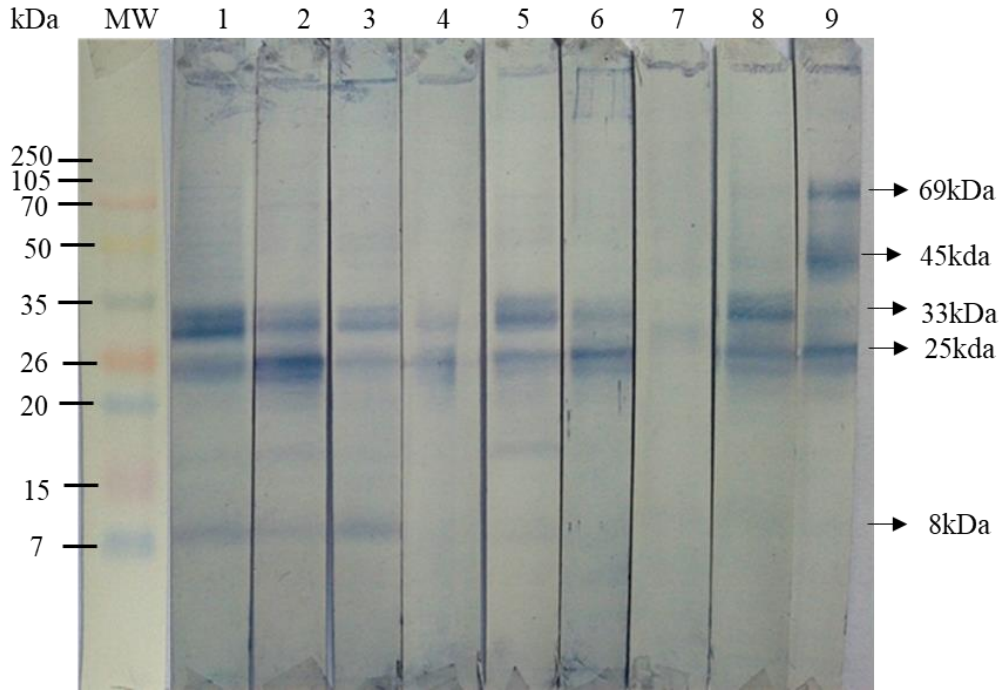

Figure 3. Western blot analysis of somatic extract of adult *F. gigantica* isolated from Bali cattle. Lane MW: Protein standard marker. Lane 1-6: Probed with fasciolosis positive sera. Lane 7-9: Probed with fasciolosis negative sera.

Table 1. Calculation of the molecular weight of sodium dodecyl sulphate polyacrilamid gel electrophoresis results based on the regression value.

Band number	Distance (mm)	Rf (x)	Slope (a)	Intercept (b)	Y (ax+b)	MW (10^Y) (kDa)
1	5	0.074	-2.3948	2.1973	2.02	105
2	7	0.103	-2.3948	2.1973	1.95	89
3	9	0.132	-1.4195	2.0539	1.87	73
4	14	0.206	-1.4195	2.0539	1.76	58
5	16	0.235	-1.4195	2.0539	1.72	52
6	20	0.294	-1.4195	2.0539	1.64	43
7	26	0.382	-0.9576	1.9384	1.57	37
8	30	0.441	-0.9576	1.9384	1.52	33
9	33	0.485	-0.8778	1.9055	1.48	30
10	37	0.544	-0.8778	1.9055	1.43	27
11	43	0.632	-1.2914	2.1366	1.32	21
12	52	0.765	-0.6068	1.6937	1.23	17
13	56	0.824	-0.6068	1.6937	1.19	16
14	64	0.941	-3.2154	3.9186	0.89	8

Note: mm: Millimeter, Rf: Relative mobility, MW: Molecular weight.

Table 2. The molecular weight of each protein band from the Western blot.

No.	Distance (mm)	Rf (x)	Slope (a)	Intercept (b)	Y ax+b	MW (10^Y) (kDa)
1	17	0.262	-1.8997	2.3419	1.8450554	70
2	23	0.354	-1.6781	2.2669	1.6731108	47
3	29	0.446	-1.3985	2.1465	1.5225538	33
4	33	0.508	-1.4813	2.1898	1.4377554	27
5	52	0.8	-3.5858	3.7689	0.90026	8

Note: mm = millimeter; Rf = relative mobility; MW = molecular weight

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CONCLUSION

Identification of protein from the somatic extracts of *F. gigantica* isolated from Bali cattle on one-dimension gel electrophoresis showed that there are 14 protein bands with MW ranging from 105 to 8 kDa. The Western blot results determined that three out of five prominent antigenic proteins with MW of 8 kDa, 27 kDa, and 33 kDa are promising to enrich the existence of antigens that have immunodiagnostic value for fasciolosis. Therefore, further studies are required to examine more deeply the potency of those three antigenic somatic proteins of *F. gigantica.*

DECLARATIONS

Authors' contribution

Made Sriasih and Ahmad Munjizun are equally contributed to designing, analyzing, and writing the manuscript. All authors read and approved the final manuscript.

Competing interests

All authors have declared no conflict of interests.

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