



***Blastocystis* sp. : Evaluation of polyclonal antibody prepared from crude protein for serological diagnosis using Rabbit serum**

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ABSTRACT

The diagnosis of *Blastocystis* infection is still based on the clinical sign which is not specific and there is no available serologic test for it. This study aimed to evaluate the polyclonal antibody prepared from crude protein of *Blastocystis* for the development of the *Blastocystis* serological test. Crude protein was extracted from the yeast of *Blastocystis* sp, then inoculated into rabbits to produce the antibody of crude protein. The serum of rabbits would be collected before and after immunization to compare the antibody titer. The profile of crude protein was analyzed using SDS-Page. The rabbit serum was analyzed using ELISA and Western Blot. The SDS-Page result showed bands in 100 kDa, 90 kDa, 70 kDa, 60 kDa, 58 kDa, 50 kDa, 40 kDa, 35 kDa, 30 kDa and 27 kDa. The ELISA assay showed that there was an increase in antibody titer of crude protein after immunization. Western Blot showed that three proteins (30 kDa, 40 kDa and 50 kDa) having immunogenicity characteristic. It is concluded that protein 30 kDa, 40 kDa and 50 kDa prepared from the crude protein of *Blastocystis* sp. can be used for developing a serologic test for *Blastocystis* infection.

Introduction

Blastocystis is an intestinal parasite in humans and a wide range of animals (cattle, pigs, horses, birds, amphibians and reptiles). The pathogenicity of *Blastocystis* is undetermined because in some *Blastocystis* infection cases showed no clinical signs. However, several studies reported that the infection of *Blastocystis* could cause irritable bowel, constipation, urticaria and imbalance of gut flora (Barbosa *et al.*, 2017, Li *et al.*, 2018; Sadaf *et al.*, 2013). The human who often makes contact with *Blastocystis*-infected animals is prone to get infected by *Blastocystis*. Therefore, *Blastocystis* has the potential to be zoonosis disease (Li *et al.*, 2018).

The infection of *Blastocystis* sp generally occurs through the fecal-oral route from drinking water (Skotarczak, 2018) and food contamination (Ahmed *et al.*, 2018). It is often found in

immunocompromised patients such as people with HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) or cancer. A higher risk of *Blastocystis* sp. infection was found in people with close animal contact (Wawrzyniak *et al.*, 2013).

The study about *Blastocystis* in cattle had been done in several countries. (Badparva *et al.*, 2015; Wen *et al.*, 2018) reported 19 samples taken in Iran are positive *Blastocystis* with details STS (47.36%), ST3 (10.53%) and ST6 (10.53%). Yoshikawa *et al.* (2004) also found 3 of 11 samples taken from Japan infected with ST3 and ST6. The prevalence of *Blastocystis* is predicted at 1.5% - 10% in developed countries and 30% - 76% in developing countries (Barbosa *et al.*, 2017).

Diagnose of *Blastocystis* infection is only based on fecal examination. This method is ineffective because of the difference in parasite morphology and size

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under the microscope. *Blastocystis* has many forms of stadia (vacuolar, granular, amoeboid, avacuolar and multivacuolar) and each of them has a different size (Hemalatha et al., 2014). The environment condition can also affect parasite morphology. The high oxygen levels can cause the *medusa's head* form and if the parasite culture is stored in a long period, it can cause *chestnut burr* form (Parija and Jeremiah, 2013).

The serologic test is one of the diagnostic methods which utilize the reaction between antigen and antibody. Antigen, a protein part of virus, bacteria or parasite, is a foreign body to the host immune cell and can induce an immune response to produce antibodies. There are two proteins in *Blastocystis* that are responsible for the infection process, crude protein and excretory-secretory antigen (ESA) protein. The crude protein can induce the immune response of the host and can also act as an immunomodulator (Gomes et al., 2015).

The study about antigen protein and antibody profile of anti *Blastocystis* in animals especially in cattle has not been reported. Therefore, this study aimed to evaluate the crude protein of *Blastocystis* sp. in preparing for the development of the *Blastocystis* serological test.

Materials and Methods

Ethical approval

Ethical approval for this study was obtained from the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universitas Airlangga (approval number: 1.KE.063.01.2018).

Parasite identification

The fecal samples were collected from several local dairy farms in Bangkalan, Madura. The samples then were inspected under a microscope using the floating method to identify *Blastocystis*. The *Blastocystis*-positive samples were cultured in yeast extract (Mohammed et al., 2015) and stored for PCR assay as a confirmation of the *Blastocystis* culture. The primer used in this study was genus primer of *Blastocystis*, b11400 FORC (5'-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3') as the forward primer and b11710 REVC (5'-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3') as the reverse primer, a positive result would show a band at 310 bp (Badparva et al., 2015). PCR was performed in 94°C in 5 minutes for pre-denaturation and followed by 35 cycles of 94°C denaturation for 2 minutes, 50°C annealing for 14 seconds and 72 °C elongation for 60 seconds and continued with post-elongation with 72 °C in 5 minutes.

Crude protein Extraction

The *Blastocystis*-positive culture was centrifuged in 10000 rpm for 10 minutes. The pellets were washed in 3 ml PBS and centrifuged in 10000 rpm for 10 minutes, this step is repeated 3 times. The pellets then were crushed using the sonication method. Once the pellets were sonicated, it was ready for protein analysis.

Excretory Secretory Protein Profile

The profile of crude protein was analyzed with SDS PAGE. The separating gel and stacking gel was made from adding acrylamide, Tris HCl, APS, SDS, aquadest and temed. The concentration of acrylamide in separating gel was 12% and 4% in stacking gel. The separating solution had been poured into gel dock, then stacking gel was poured after separating gel clotting. The electrophoresis assay was set at 120 V, 40 mA in 100 minutes.

The buffer solution, marker and ES protein were added into the well after the electrophoresis. The gel then transferred into the staining solution (Coomassie Blue) and shaken in 37 °C for 24 hours. The stained gel then was washed with a destaining solution containing methanol, acetic acid and aquadest until the protein bands could be identified. The destaining process was stopped by adding 10% acetic acid then the gel was stored in a distilled water solution.

Immunization in experimental animal and serum collection

This experimental study used 2 male New Zealand rabbits (*Oryctolagus cuniculus*), 12-16 weeks old. 250 µg of Crude protein dissolved in PBS and homogeneous emulsified with Freund's Complete Adjuvant in ratio 1:1 for first immunization. Immunization was done 4 times with a predetermined period time. In booster immunization, the antigen was emulsified in Freund's Incomplete Adjuvant. The first immunization was done after adaptation, the booster was done in 14, 28 and 42 days after the first immunization.

The serum was collected from experimental animals before immunization, 7 days after the first booster and 14 days after the third booster. Serum was analyzed using ELISA assay to identify the optical density (OD) using an anti-rabbit (IgG) as a secondary antibody. The profile of antibody is characterized using Western Blot assay.

ELISA test

A microplate was coated with 100uL (10ug/uL) crude protein and incubated at 4°C overnight. After 3 times of washing with washing buffer (200uL/well), the blocking solution added into the well (200uL/well) and incubated at 37 °C for 1-2

hours and washed in the same way. Diluted sera samples (1:10) were added into the well and incubated at 37°C in 1 hour. The conjugate goat anti-rabbit IgG (1:500) was added into sera samples then incubated at another 37°C for 1 hour. The samples then were washed 5 times and added a pNPP substrate incubating at 37°C in 1 hour. The reaction was stopped using 50uL NAOH 1N then the OD was determined using a microplate reader with a wavelength of 450 nm.

Western blot test

SDS-PAGE result (contain crude protein) and whatman (Merck™, UK) was soaked into PBST (PBS with 0.05% tween-20) solution. The nitrocellulose membrane was prepared by drenching it into methanol, then into the buffer transfer solution. That four layers were arranged from the bottom to top, started with whatman, nitrocellulose membrane, gel and whatman. The crude protein was transferred into the nitrocellulose membrane using a transbloter at 20V in 15 hours. The membrane was colored with Ponceau for 5 minutes then rinsed with distilled water. The nitrocellulose membrane was soaked into a 5% blot solution (5% creamer in PBST) while being agitated, then washed using PBST. The membrane that contains crude proteins had been incubated into the rabbit serum (1:200) overnight in 4°C and was washed with TBS. After overnight incubation, secondary antibody (goat anti-rabbit IgG) was soaked into the nitrocellulose membrane (1:200 in TBS) for 60 minutes in room temperature then washed with PBST and soaked into 1 mL western blue until bluish-colored protein bands appear modification from Suwanti (1996).

Data analysis

The result of SDS PAGE and Western blot will be presented in descriptive. The comparative data of ELISA will be analyzed using ANOVA and turkey HSD method. Statistical analysis used in this study evaluated using SPSS 23.0 program for windows.

Results

Crude protein profile

The polymorphic band with 310 bp size has been successfully presented in the electrophoresis result using primer b11400 FORC (5'-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3') as the forward primer and b11710 REVC (5'-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3) as the reverse primer, this related to the study that was done by Badparva et al. (2015). The PCR result confirmed that the parasite used in this study is *Blastocystis* sp. The SDS PAGE result of the crude protein shows 10

bands with the size of molecule 100 kDa, 90 kDa, 70 kDa, 60 kDa, 58 kDa, 50 kDa, 40 kDa, 35 kDa, 30 kDa and 27 kDa. Figure 1 depicts the SDS PAGE result of *Blastocystis* crude protein.

Elisa test result of the rabbit blood serum before and after immunization shows an increase of optical density (OD) IgG. There is a significant difference ($p \leq 0.05$) of OD value between before immunization and after the booster, also there is a significant difference ($p \leq 0.05$) of OD value between the second booster and third booster. The following table (Table 1) shows the average OD value of rabbit blood serum, before and after immunization. The table shows that the highest OD value is collected after 14 days after the third booster (2.466) and the lowest OD value is collected before immunization (0.043) statistically significant ($p \leq 0.05$).

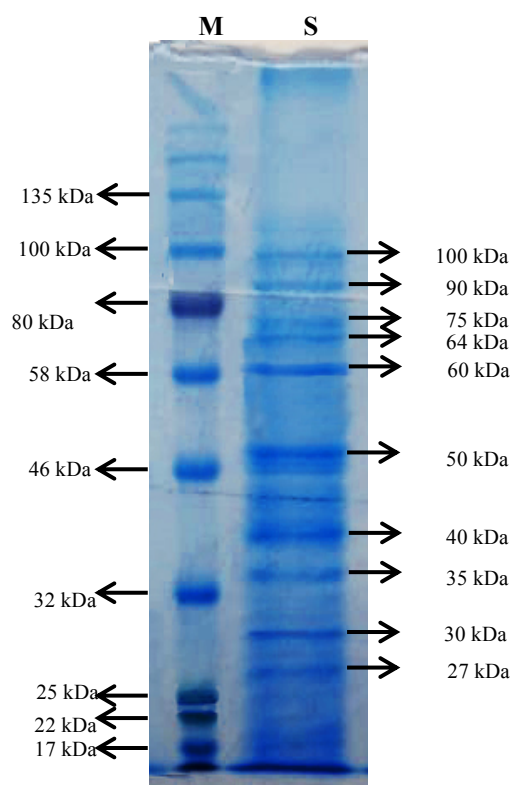


Figure1. Crude protein profile of *Blastocystis* sp in SDS-PAGE (M: Marker; S: Sample).

Table 1. Average of Optical Density Value of Experimental Animal Blood Serum ($\bar{X} \pm SD$) Immunized by crude Protein.

Time of Serum Collection	Average of Optical Density Value of Experimental Blood Serum ($\bar{X} \pm SD$) Immunized by crude Protein
Before immunization	0.043 ^c $\pm$ 0.0014
7 days after 2 nd booster	2.243 ^b $\pm$ 0.0781
14 days after 3 rd booster	2.466 ^a $\pm$ 0.1806

Different superscript shows a significant difference in proportions ($p \leq 0.05$)

Polyclonal antibody profile

Immunoblotting was done by reacting the crude protein of *Blastocystis* sp. with polyclonal antibody prepared from the same crude protein. Three proteins, 30 kDa, 40 kDa and 50 kDa, corresponded to the antigen meaning these proteins have the immunogenicity characteristic.

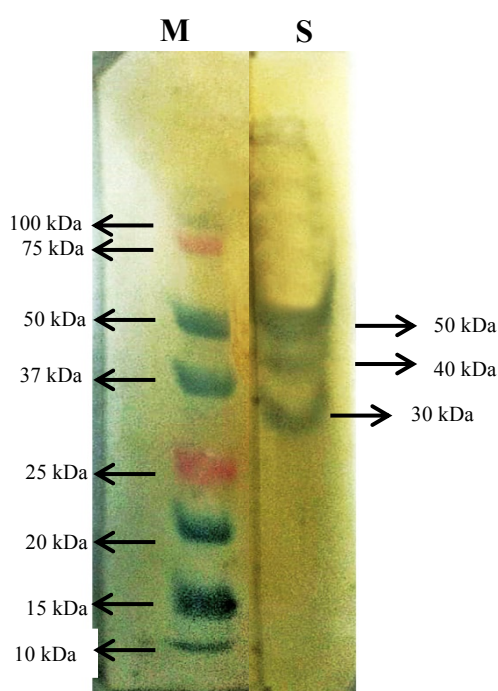


Figure 2. Immunoblotting reaction crude protein of *Blastocystis* sp with antibody anti crude, M = marker; S = antigen with Rabbit's Serum.

Discussion

The fecal examination is ineffective in diagnosing the *Blastocystis* infection due to the diverse size and form of the parasite. Moreover, the environment condition has an impact on the parasite morphology that could hinder the examination result. Serological test may be an another option as a diagnostic tool.

However, there is little information of the antigens and antibodies of the *Blastocystis* sp. in animals. Understanding this information can be a valuable information for development of serological test for *Blastocystis*.

Blastocystis has two proteins that play major role during infection process, they are crude protein and excretory-secretory antigen (ESA) protein. In *Taenia solium*, the crude protein can induce the immune response of the host and can also act as an immunomodulator (Gomes et al., 2015). In this study, the focus will be on evaluation of the crude protein of *Blastocystis* sp. The result of SDS PAGE found that the crude protein of *Blastocystis* contains 10 proteins which are 100 kDa, 90 kDa, 75 kDa, 64 kDa, 58 kDa, 50 kDa, 40 kDa, 35 kDa, 30 kDa and 27 kDa. Some of the protein is also found in the human-infected *Blastocystis* (Mansour et al., 1995, Hanaa et al., 2016).

Nagel et al. (2015) reported that a wide range of proteins (from 17 kDa protein to 150 kDa protein) had been successfully been collected from *Blastocystis* including 27 kDa protein which is presumed to be as a cysteine protease. Meanwhile, 30 kDa protein has a similarity with cysteine protease of legumain type. Other proteins such as 35 kDa protein is a cathepsin B and 40 kDa protein is a recombinant legumain (Nourrisson et al., 2016).

50 kDa protein is assumed to be a Matrix Metalloproteinase-9 (MMP-9), a member of zinc-binding matrix metalloproteinases that degrade extracellular matrix namely gelatin (gelatinase B), collagen and elastin (Nagel et al., 2015). The rest of the proteins which are 64 kDa protein, 75 kDa protein, 90 kDa protein and 100 kDa protein, belongs to the protease family. Rajamanikam et al (2013) demonstrated that the activity of protease was increasing in the amoeboid form of *Blastocystis* sp. with the size of a molecule ranging from 60 kDa to 100 kDa.

ELISA results show that there is a difference in OD between before immunization, after the second booster and after the third booster. This concludes that the antigen from soluble and ESA are immunogenic protein. Immunogenic protein can induce an immune response by activating B cells to produce antibodies and T cells to release cytokines. Protein is a potent immunogen structured by several amino acids and has a specific epitope. The size of the protein molecule also affects the ability of a protein to induce an immune response. Protein with a high size of molecule tends to have a variety of epitopes (Baratawidjaja and Rengganis, 2010).

Western blot results show there are three reactions, marked with the size of molecule 30 kDa, 40 kDa and 50 kDa, between antigen and polyclonal antibody collected from the immunization of *Blastocystis* crude protein. This shows that these three proteins are immunogenic proteins. 30 kDa protein is a specific protein to the infection of *Blastocystis* sp. This protein correlates to the cysteine protease from the legumain type. On the parasite, cysteine protease serves as a surface antigen responsible in the entry-exit of the parasite from the host (Verma et al., 2016). 40 kDa protein is a recombinant legumain that accounted for the inflammation of the host cell. Legumain is a protease lysosome located on the cell surface, cytoplasm and central vacuole (Wu et al., 2010). 50 kDa protein is a matrix metalloproteinase-9 (MMP-9). This protein has a significant role in the parasite invasion and corresponds to the immune system altering interleukin (IL) 8 activity (Nagel et al., 2015).

Conclusions

The results of this study showed that the crude protein of *Blastocystis* sp. contains 10 proteins and among them, three proteins (30 kDa, 40 kDa and 50 kDa) exhibited an immunogenicity response. Therefore, these three proteins might be used as a potential protein for developing a serological test of *Blastocystis* infection.

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Declarations of interest

The author(s) declare that there is no conflict of interest with regards to the research, authorship and/or publication of this article.

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