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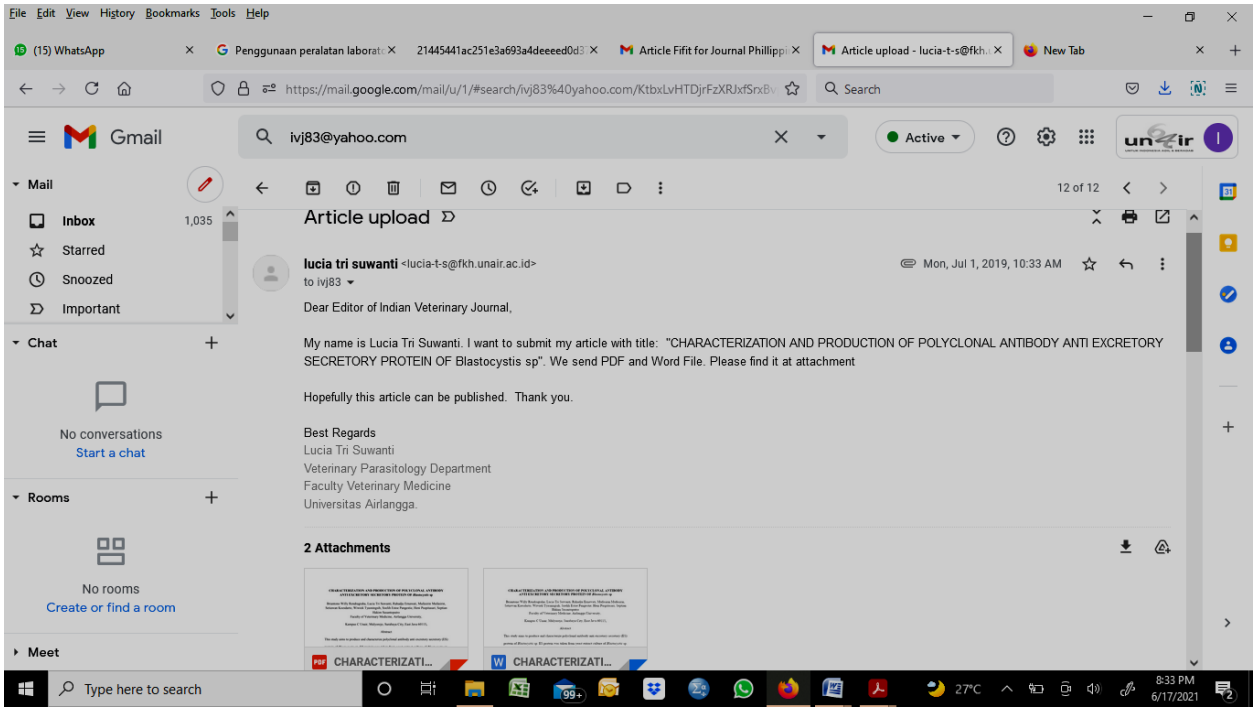
Article: “CHARACTERIZATION AND PRODUCTION OF POLYCLONAL ANTIBODY ANTI EXCRETORY SECRETORY PROTEIN OF Blastocystis sp"



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CHARACTERIZATION AND PRODUCTION OF POLYCLONAL ANTIBODY ANTI EXCRETORY SECRETORY PROTEIN OF *Blastocystis* sp

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Abstract

This study aims to produce and characterize polyclonal antibody anti excretory secretory (ES) protein of *Blastocystis* sp. ES protein was taken from yeast extract culture of *Blastocystis* sp. ES protein profile was analyzed using SDS-Page. Rabbits was immunized with ES protein, than the serum analyzed using ELISA and Western Blot. SDS-Page result showed band in 40 and 50 kDa. The ES protein immunized into the Rabbits, antibody tittre analyzed with indirect ELISA method and the result showed significant difference between before and after immunization. Western Blot result showed two band in 40 and 50 kDa also, the result confirmed that 40 and 50 kDa protein from ES protein of *Blastocystis* sp has immunogenicity characteristic.

Key words: *Blastocystis* sp, Excretory Secretory Protein, Polyclonal Antibody.

Blastocystis sp is a non-motile extracellular protist in digestive tract that widely prevalence in many countries and associated with non-specific gastrointestinal symptoms such as diarrhea, nausea, vomiting, abdominal pain, irritable bowel syndrome, and urticaria (Ajjampur *and* Tan., 2016). This parasite can infect human (Roberts *et al.*, 2014) and various animals such as such as amphibian, reptiles, bird, and mamals (Alfellani *et al.*, 2013).

The infection of *Blastocystis* sp generally occurs through 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

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Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) or cancer. A higher risk of *Blastocystis* sp infection was found in people close to animal contact (Wawrzyniak et al., 2013).

Pathogenic status of *Blastocystis* sp was debated in the literature to determine whether this microorganism is pathogenic or commensal organism, but the recent literature shows *Blastocystis* sp is an emerging pathogenic organism that can be found in symptomatic and asymptomatic patients (Wawrzyniak et al., loc cit).

In parasite organism, Excretory Secretory (ES) protein has important role as virulence factor and it can affect and control host immune system during infection, otherwise ES protein is an important component that help the parasite survive in the host and in the environment. ES protein is a component that can be recognized by the host immune system, it makes ES protein can be used as a biomarker to detect the presence of parasite and status of the infection in different infectious disease (Gomez *et al.,* 2015). In vitro studies showed that excretory secretory products in culture filtrate led to pathogenic mechanism such as apoptosis, degradation of intestine membran that increased intestinal permeability, and inflamation (Ajjampur *and* Tan., loc cit).

This study aims to characterize and produce polyclonal antibody anti excretory secretory protein of *Blastocystis* sp.

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MATERIALS AND METHODS

Blastocystis sp was isolated from beef cattle in Bangkalan, Madura. Cattle feces was microscopically examined with native method and sugar flotation method. Positive sample was cultured using yeast extract media with ratio of sample and media 1:9 (Mohammed *et al.,* 2015). The growing culture was confirmed with PCR examination using primers Bb11400 FORC (5`-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3`) and b11710 REVC (5`-

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TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3). PCR was performed in 94^{o}C in 5 minutes for pre-denaturation, and followed by 35 cycle of 94^{o}C denaturation for 2 minutes, 50^{o}C annaeling for 14 seconds and 72^{o}C elongation for 60 seconds and continued with post-elongation in 72^{o}C in 5 minutes. Positive result would show band at 310 bp (Badparva *et al.*, 2015).

Positive sample that growing in yeast extract media was centrifuged with 10.000 rpm for 10 minutes, the supernatant was removed. Pellets that contain *Blastocystis* sp was washed 3 times with 3 mL PBS and centrifuged at 10.000 rpm for 10 minutes. The pellets were dissolved in PBS and incubated in 37^{o}C overnight, than centrifuged at 10,000 rpm for 10 minutes, the supernatant is Excretory Secretory (ES) protein. The concentration of excretory secretory protein was measured with spectophotometer (NanoDrop, ND-1000,).

The profile of ES Protein was analyzed with SDS-Page. Separating gel and stacking gel were made from adding acrylamide, Tris HCl, APS, SDS, aquadest, and temet. The concentration of acrylamid in separating gel was 12% and in stacking gel was 4%. Separating solution had been poured into geldock, then stacking gel was poured after separating gel cloting. The gel put into electrophoresis chamber (Bio-Rad) and electrophorized at 120 V, 40mA in 100 minutes. After elecrophorized, buffer solution was added then marker (Bio-Rad) and ES protein was loaded into the well. The gel put in staining solution (Comassie Blue) then shaken in 37^{o}C for 24 hours and washed with a destaining solution that contain methanol, acetid acid, and aquadest. After the protein bands appeared, the reaction was stopped by adding 10% acetic acid then stored in distilled water solution and documented (modification from Nagel *et al*., 2015).

This experimental study used 2 male New Zealand rabbits (*Oryctolagus cuniculus*), 12-16 weeks old. Rabbits were adapted for 2 weeks. 250 µg of ES protein dissolved in PBS and homogeneous emulsified with Freund's Complete Adjuvant in ratio 1:1 for first

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immunization. Immunization was done 4 times. In booster immunization, antigen was emulsified in Freund's Incomplete Adjuvant (Naisiri *et al.,* 2017). First immunization was done after adaptation period, booster immunization was done in 14, 28, and 42 days after first immunization.

The serum was collected from experimental animals before immunization, 7 days after 3rd immunization and 14 days after 4th immunization. A microplate was coated with 100uL (10ug/uL) ES protein and incubated at 4°C overnight. After 3 times of washing with washing buffer (200uL/well), 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 sample (1:10) was added into the well and incubated at 37 °C in 1 hour. After washing, conjugate goat anti rabbit IgG (Invitrogen) (1:500) was added and incubated at 37 °C in 1 hour, then washed 5 times and added pNPP substrate and incubated at 37 °C in 1 hour. Reaction was stopped using 50uL NAOH 1N then the OD was determined using microplate reader (iMark™, BIO-RAD) with wavelength of 450 nm.

SDS-PAGE result (contain ES protein) and whatman were soaked into PBST (PBS with 0.05% tween-20) solution. Nitrocellulose membrane had been soaked into methanol, then soaked into buffer transfer solution. That three components were arranged from the bottom up start form whatman, nitrocellulose membrane (porablot NCL), gel, and whatman. The ES protein was transfered into nitrocellulose membrane using transbloter (Trans-Blot® Turbo™ Transfer System) at 20V in 15 hours. After transfering, the membrane was colored with Ponceau (Merck) for 5 minutes then rinsed with distilled water. Nitrocellulose membrane was soaked into 5% bloto solution (5% creamer in PBST) while agitated, then washed using PBST. Membrane that contain ES proteins had been incubated into rabbit`s serum (1:200) overnight in 4°C and washed with TBS. After overnight of incubation, secondary antibody (Goat anti Rabbit IgG, invitrogen) was soaked into the nitrocellulose

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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 Nagel *et al*., loc cit).

RESULTS AND DISCUSSION

Blastocystis sp that identified microscopically from cattle was cultured with yeast extract media, after 3 days, the organism reproduced and reached a number $5x10^9$/ml. Based on PCR method using Bb11400 FORC (5`-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3`) and b11710 REVC (5`-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3) primers, the organism was *Blastocystis* sp. The sample showed positive result with band in 310 bp (Figure 1) as discribe in Badparva *et al* (loc cit).

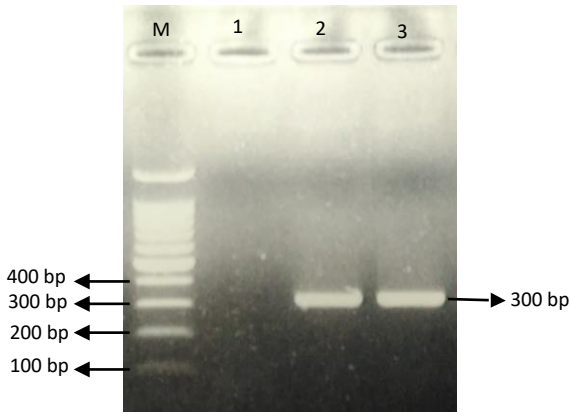

Figure 1. PCR result, M = marker; 1 = Negative control; 2 = Sample; 3 = Positive result

ES protein which obtained was 6.63 mg/ml in concentration. SDS PAGE of ES protein of *Blastocystis* sp showed that there were two protein bands with molecular weight; 40 kDa and 50 kDa (Figure 2). Fadl *et al* (2016) in their research identified 22 proteins from whole protein of *Blastocystis*, with range from 12 kDa to 200 kDa, include 40 kDa and 50 kDa. According to Nagel *et al.* (loc cit), the 50 kDa is Matrix metalloproteinase-9 (MMP-9), member of zinc-binding matrix metalloproteinases (MMP) which degrades extracellular matrix including gellatin (gelatinase B), collagen and elastin. They explained that MMP-9 exist as pro-enzymes around 92 kDa with an active form around 84 kDa and turncuted

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around 50 kDa and it has important role in tissue invasion and has corelation with immune system, including potentiation of interleukin (IL)-8. Nourrisson *et al*. (2016) reported that rocombinant legumain had a size ~40 kDa. Legumain is a lysosomal protease that localizes on cell surface, cytoplasm, and central vacuole, this protein has several functions such as mediated pro-survival function for surface molecule and inflamation reaction in host cell (Wu *et al.,* 2009).

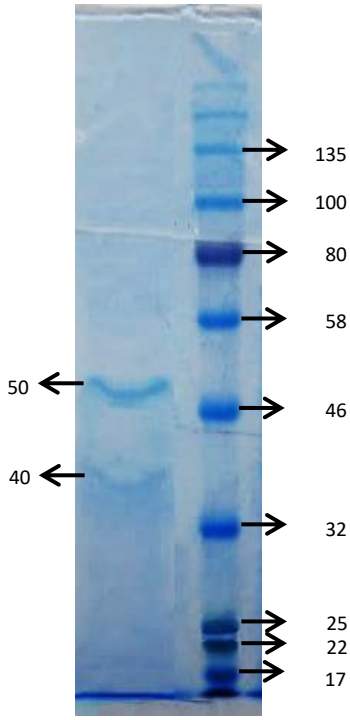

Figure 2. Profile excretory secretory protein of *Blastocystis* sp in SDS-PAGE

Antibody production was verified by indirect ELISA to detect titer antibody after immunization based on value of optical density (OD). The OD data showed significantly difference between before and after immunization (Table 1). Significant difference of OD Value indicates that ES protein of *Blastocystis* sp was antigenic.

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Table 1. Average of Optical Density Value of Experimental Animal Blood Serum (X ± SD) Immunized by ES Protein

Time of Serum Collection	Average of Optical Density Value of Experimental Animal Blood Serum (X ± SD) Immunized by ES Protein
Before vaccination	$0.0415^{c} \pm 0.0007$
7 days after 3rd immunization	$1.6217^{b} \pm 0.2216$
14 days after 4th immunization	$2.2232^{a} \pm 0.0300$

In blotting product, antibody was reacted with 40 kDa and 50 kDa proteins (Figure 3). It means 40 kDa and 50 kDa proteins have antigenic characteristic. Based on Western Blott reaction, whole proteins of *Blastocystis* sp with blastocystosis patient serum, Nagel *et al* (loc cit) reported that 50 kDa protein was found in *Blastocytis* sp subtype 1, 2, 3, 4, 5, 7, and 8.

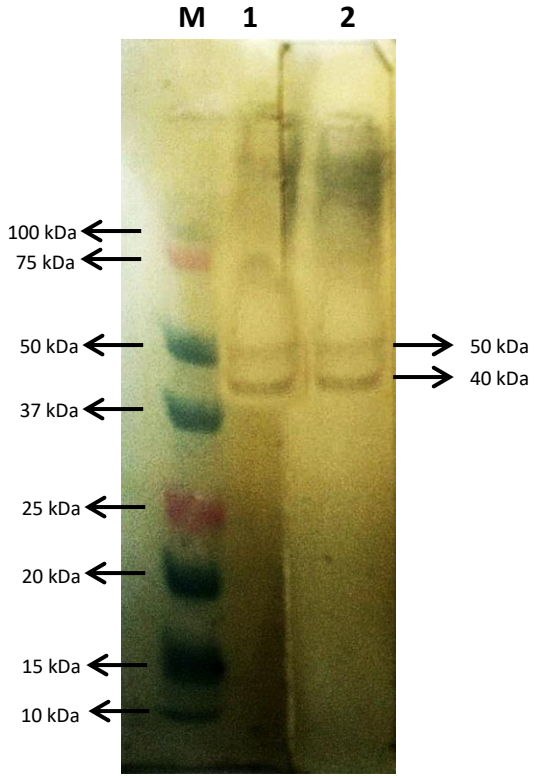

Figure 3. Immunobloting reaction exretory secretory protein of *Blastocystis* sp with antibody anti ES, M = marker; 1 = antigen with Rabbit`s Serum I; 2 = antigen with Rabbit`s Serum II

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Summary

ES protein of *Blastocystis* sp, both 40 kDa and 50 kDa are antigenic protein and can be promoted as kit diagnostic to detect serum blastocytosis patient and Antibody anti ES can be promoted as kit diagnostic to detect copro antigen.

Acknowledgement

The authors thankful to the Rector, Dean of Faculty of Veterinary Medicine, and Institute of Tropical Disease Universitas Airlangga

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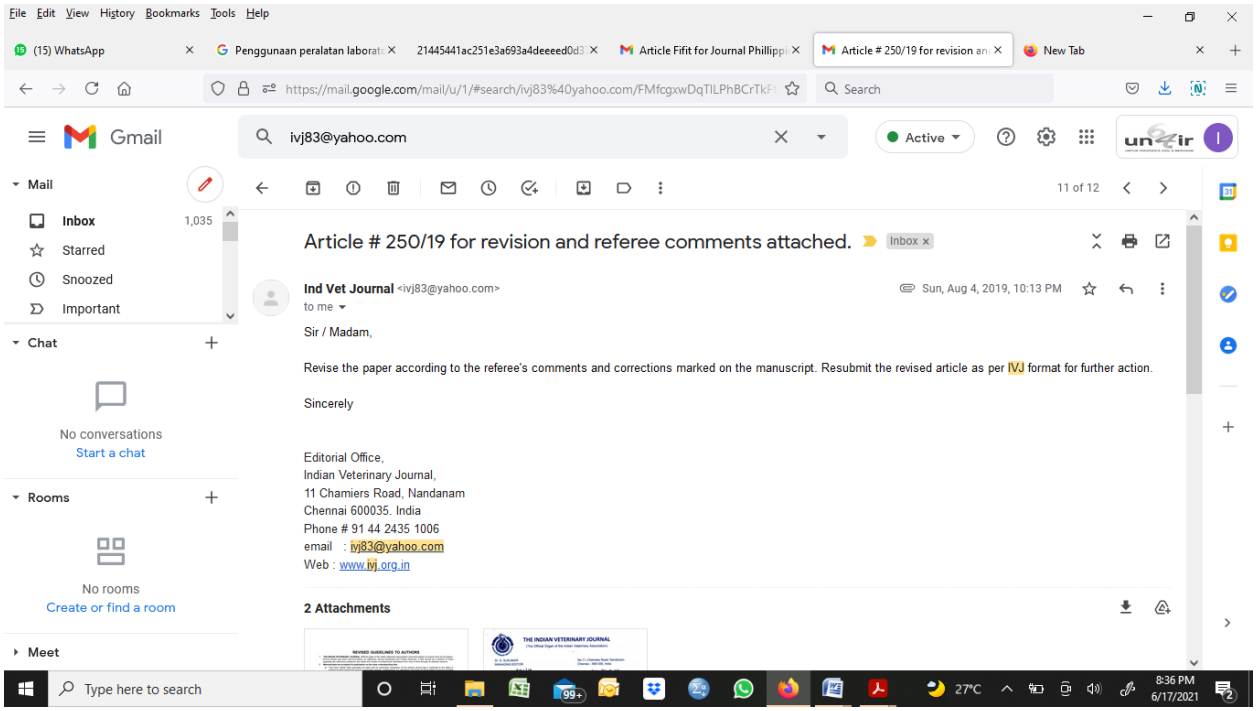

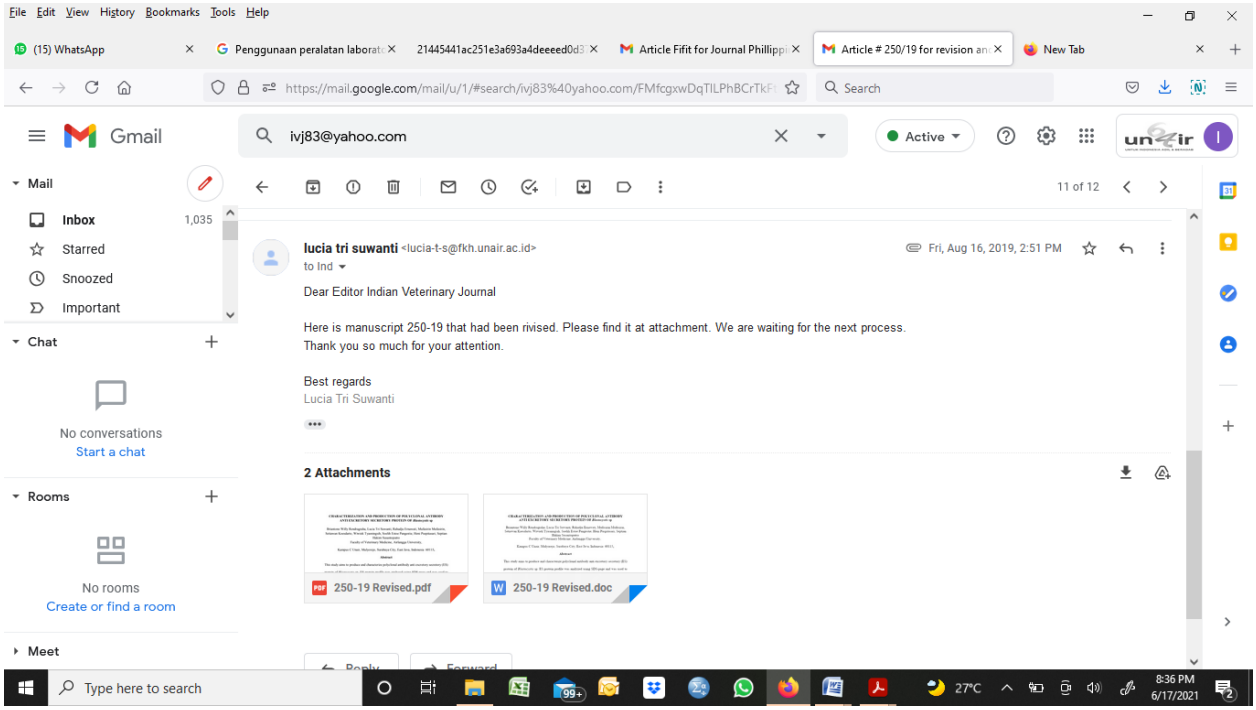

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Abstract

This study aims to produce and characterize polyclonal antibody anti excretory secretory (ES) protein of *Blastocystis* sp. ES protein profile was analyzed using SDS-page and was used to immunize 2 rabbits. Rabbit's serum were analyzed using indirect ELISA and Western Blot. The result showed that molecular weight of ES protein of *Blastocystis* sp was 40 and 50 kDa and the protein was immunogenic. Both ES protein and antibody anti ES of *Blastocystis* sp can be promoted as kit diagnostic.

Key words: *Blastocystis* sp, Excretory Secretory Protein, Polyclonal Antibody.

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Blastocystis sp is a protozoan parasite that widely prevalence in many countries and it cause gastrointestinal symptoms such as diarrhea, nausea, vomiting, abdominal pain, irritable bowel syndrome, and urticaria (Ajjampur *and* Tan., 2016). This parasite can infect human (Roberts *et al.,* 2014) and various animals such as amphibian, reptiles, bird, and mammals (Alfellani *et al.*, 2013).

Excretory secretory (ES) protein of parasites have important role as virulence factor, it can affect and control host immune system during infection and it can be used as a biomarker to detect the presence of parasite and status of the infection in infectious disease (Gomez *et al.,* 2015). This recent study aims to characterize and produce polyclonal antibody anti ES protein of *Blastocystis* sp.

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MATERIALS AND METHODS

Blastocystis sp was isolated from feces of beef cattle in Bangkalan, Madura. The sample was cultured using yeast extract media (Mohammed *et al.*, 2015). The growing culture was confirmed with PCR examination using primers b11400 FORC (5`-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3`) and b11710 REVC (5`-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3) from Badparva *et al* (2014).

Blastocystis sp was harvested from media and centrifuged with 10.000 rpm for 10 minutes. Pellets was washed 3 times with PBS and incubated in 37^{o}C overnight. The supernatant was as ES protein. The profile of ES protein was analyzed with SDS-Page and visualized with a digital camera.

Two male New Zealand rabbits were immunized with 250 µg of ES protein. Immunization was done 4 times. The serum was collected before and 7 days after immunization Antibody production was verified by indirect ELISA based on value of optical density (OD) and Western Blot. The OD value between groups was analyzed using one-way ANOVA.

RESULTS AND DISCUSSION

Blastocystis sp that identified microscopically from feces of cattle was cultured with yeast extract media, after 3 days, the organism reproduced and reached a number $5x10^{9}$/ml. Based on PCR, the sample showed positive result with band in 310 bp (Figure 1) as describe in Badparva *et al* (*loc cit*).

SDS PAGE of ES protein of *Blastocystis* sp showed that there were two protein bands with molecular weight; 40 kDa and 50 kDa (Figure 2). Fadl *et al* (2016) in their research identified 22 proteins from whole protein of *Blastocystis*, with range from 12 kDa to 200 kDa, include 40 kDa and 50 kDa. According to Nagel *et al.* (*2015*), the 50 kDa is Matrix

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metalloproteinase-9 (MMP-9), member of zinc-binding matrix metalloproteinases (MMP) which degrades extracellular matrix including gellatin (gelatinase B), collagen and elastin. They explained that MMP-9 exist as pro-enzymes around 92 kDa with an active form around 84 kDa and turncuted around 50 kDa and it has important role in tissue invasion and has corelation with immune system, including potentiation of interleukin (IL)-8. Nourrisson *et al*. (2016) reported that rocombinant legumain had a size ~40 kDa. Legumain is a lysosomal protease that localizes on cell surface, cytoplasm, and central vacuole, this protein has several functions such as mediated pro-survival function for surface molecule and inflamation reaction in host cell (Wu *et al.*, 2009).

Antibody in rabbit's serum increased significantly after immunization with ES protein. Before immunization the OD was $0.0415^{c} \pm 0.0007$, while 7 days after 3rd and 4th immunization, respectively, $1.6217^{b} \pm 0.2216$, and $2.2232^{a} \pm 0.0300$. It indicated that ES protein of *Blastocystis* sp was immunogenic.

In blotting product, antibody was reacted with 40 kDa and 50 kDa proteins (Figure 3). It means 40 kDa and 50 kDa proteins were immunogenic. Nagel *et al* (*loc cit*) also reported that 50 kDa protein reacted with blastocystosis patient serum and 50 kDa protein was found in *Blastocytis* sp subtype 1, 2, 3, 4, 5, 7, and 8.

Summary

Molecular weight of ES protein of *Blastocystis* sp is 40 kDa and 50 kDa and both of protein are immunogenic. ES protein of *Blastocystis* sp can be promoted as kit diagnostic to detect serum blastocytosis patient and antibody anti ES as kit diagnostic to detect coproantigen.

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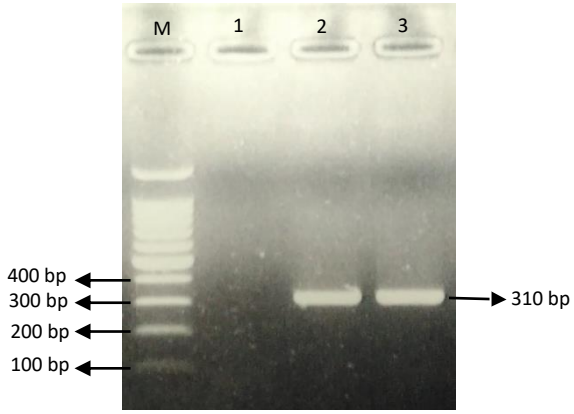


Figure 1. PCR Product of *Blastocystis* sp. , M = marker (Promega); 1 = Negative control; 2,3 = positive culure

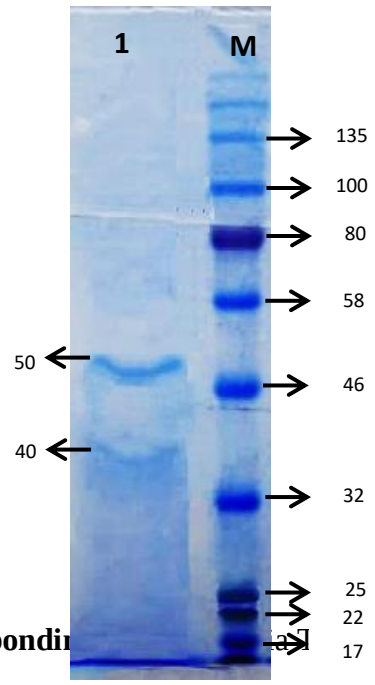


Figure 2. Profile of excretory secretory protein of *Blastocystis* sp in SDS-PAGE, M = marker; 1 = ES protein of *Blastocystis* sp

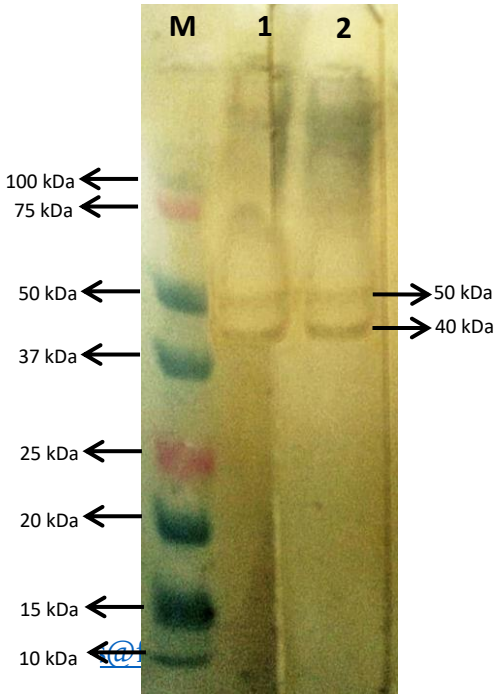
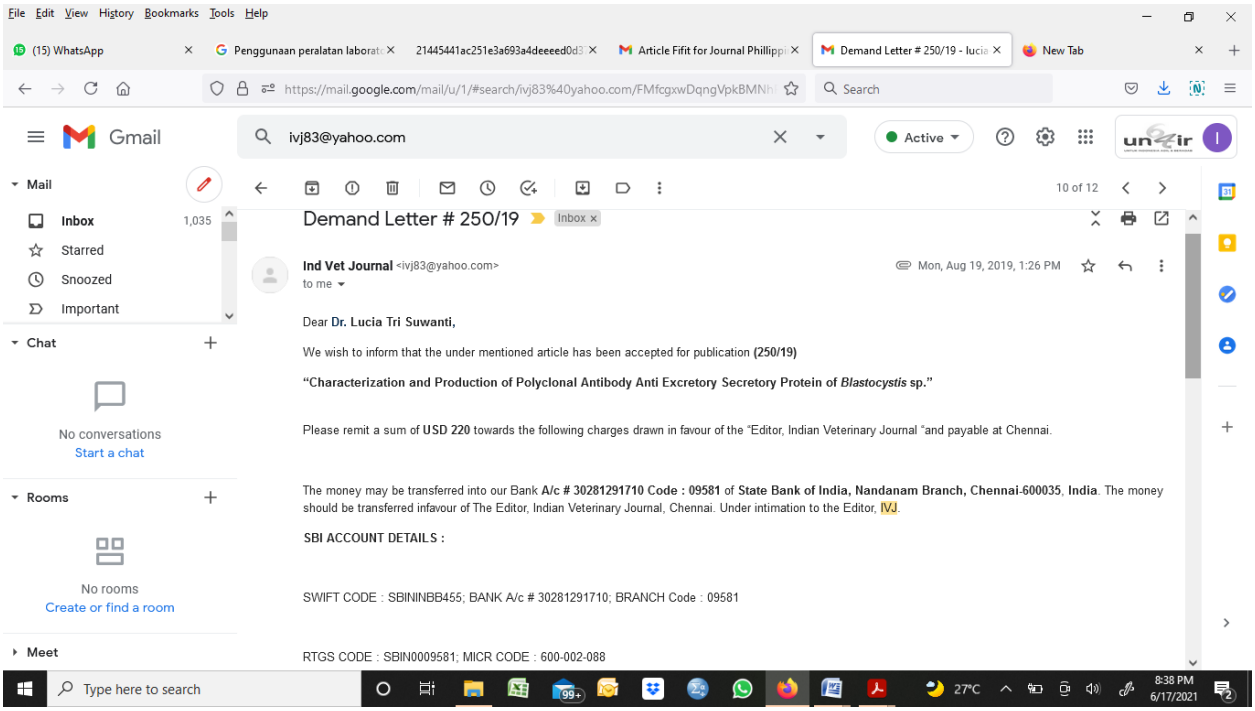
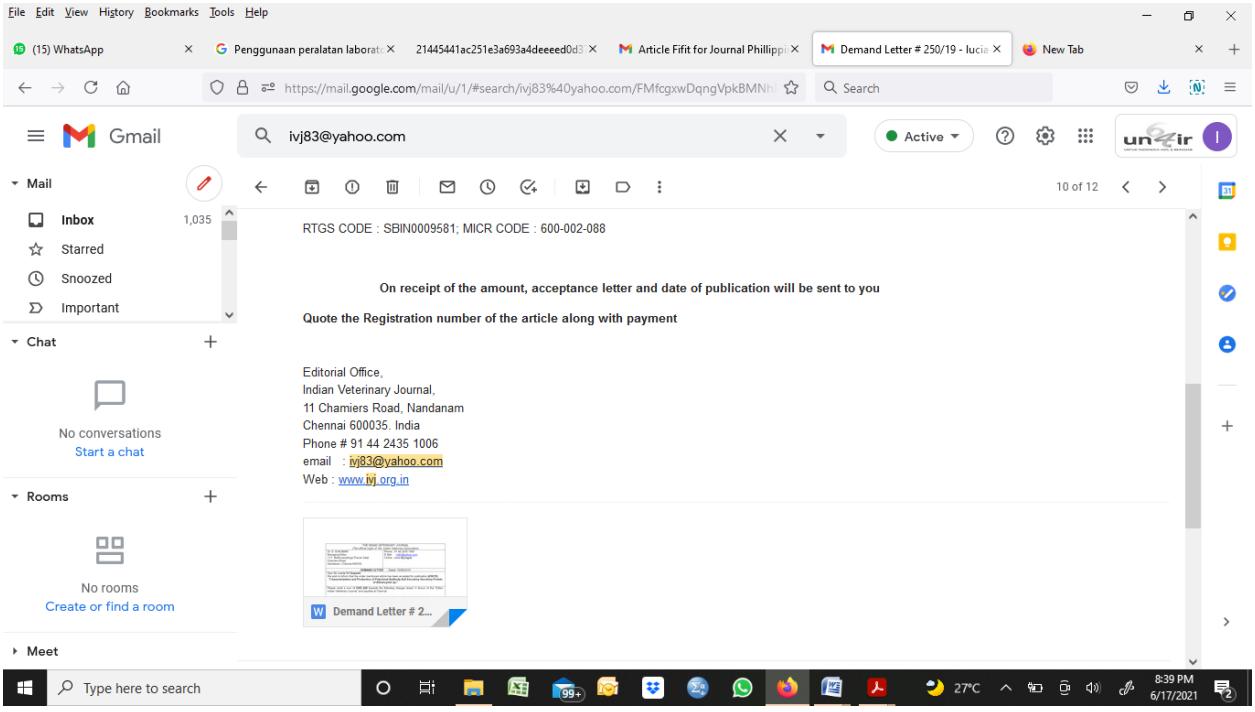


Figure 3. Immunobloting of exretory secretory protein of *Blastocystis* sp with antibody anti ES, M = marker; 1,2 = antigen with Rabbit`s Serum 1;2

***Correspondi** anti, email





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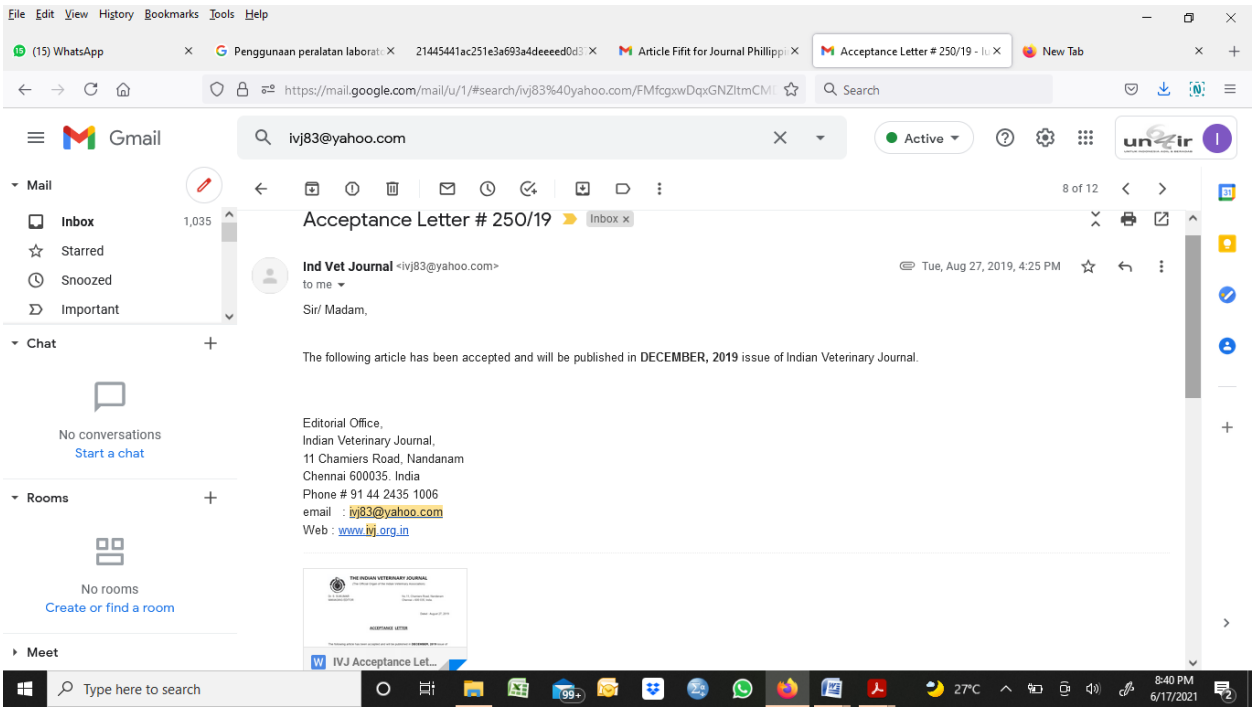

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