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ABSTRACT

The study aimed to explore the antigenic proteins in third-stage *Anisakis* spp. larvae from mackerel tuna. This research used 300 *Anisakis* spp. larvae from 110 mackerel tuna fish from Brondong, Lamongan, East Java, Indonesia. The research was carried out in several stages: isolation and identification of *Anisakis* spp.; isolation of soluble proteins and measurement by spectrophotometry; whole-protein characterization with SDS-PAGE; rabbit immunization for polyclonal antibody production; antibody titer measurement with indirect ELISA; and antigenic protein characterization via Western blot. Spectrophotometry results of the larvae showed homogenate protein level of 6833.3 µg/ml. Protein analysis of whole worm extract of the larvae using SDS-PAGE yielded 21 bands, with molecular weights between 12 kDa-238 kDa. Further, protein characterization via Western blot produced six antigenic protein bands: 64 kDa, 45 kDa, 38 kDa, 30 kDa, 27 kDa and 23 kDa. In conclusion, *Anisakis* spp. larvae in mackerel tuna contain six types of antigenic proteins, which might play a role in hypersensitivity or allergic reactions.

Key words: *Anisakis* spp., antigenic protein, *Euthynnus* sp., third-stage larvae

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INTRODUCTION

Mackerel tuna (*Euthynnus* sp.) has high economic value, which makes it a potential export. Its protein content, 26.2 mg/100g, is considered quite high, and it is rich in omega-3 fatty acids, making it favorable for general human consumption. Anisakiasis, a zoonotic disease, is caused by *Anisakis* spp. infection due to consumption of raw fish carrying third-stage *Anisakis simplex* larvae (Audicana *et al*., 2002; Armentia *et al*., 2006). Fish, squids, and crustaceans serve as intermediate hosts where the parasitic nematode larvae can grow. Sea mammals, such as seals, sea lions, dolphins, and whales consume smaller fish and crustaceans, which then provide a favorable environment for the larvae to mature. The larvae cannot propagate in humans, but they can cause gastrointestinal infection and allergies (Foti *et al*., 2002; Do *et al*., 2010). These parasites can also live in the digestive system and human lungs, and disrupt infected organs. Examination via scanning electron microscope by Sumarsono (2016) showed that *Anisakis* spp. larvae in mackerel from Kranji auction, Lamongan were identified as *Anisakis*

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larvae type 1. The larvae are described as white, 15-25 mm in length, and have a boring tooth at the anterior part and a mucron at the posterior part. *Anisakis* larvae type 1 includes *A. simplex*, *A. pegreffii*, *Anisakis* sp., *A. typica*, *A. ziphidarum*, and *A. nascettii* (Murata *et al*., 2011).

The first cases of anisakiasis in humans were reported in Netherlands, Japan, and other countries in Europe that saw an increase in raw fish consumption (Mattiucci *et al*., 2011). In Japan, more than 2000 anisakiasis cases are reported yearly (Umehara *et al*., 2008). Such high incidence in humans necessitates proper serological detection of *Anisakis* spp. larvae antigen. However, diagnostic kits for anisakiasis are currently unavailable in Indonesia, thus the need to conduct preliminary research in exploring antigenic proteins from third-stage *Anisakis* spp. larvae.

This research is the first to conduct polyclonal antibody production in rabbits, which can then be used as secondary antibodies for western blot reactivity tests that would produce antigenic proteins. The aim of this study is to explore the antigenic proteins from third-stage *Anisakis* spp. larvae isolated from mackerel tuna (*Euthynnus* sp.) from fish auction in Brondong, Lamongan, East Java. The result of antigenic protein profile can be used for the development of serological diagnostic method for anisakiasis.

MATERIALS AND METHODS

Ethical approval

The research was conducted appropriately, following the ethics in using experimental animals, and has been approved by the Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga, No: 666-KE on 1st March 2017.

Isolation and Identification of third-stage *Anisakis* spp. larvae

Third-stage *Anisakis* spp. larvae were isolated from mackerel tuna collected from a fish auction in Brondong, Lamongan, East Java, Indonesia between January to March 2017. Three hundred third-stage larvae were isolated from the digestive organs of 110 mackerel tuna. Larvae were identified according to Murata *et al*. (2011). Larvae were stained using Semichen-Acetic Carmine method (Gökçen, 2008) and examined under the microscope at 100× magnification. The larva is white, 13-25 mm, has a ventro-lateral mouth and an anterior boring tooth (Soulsby, 1986; Murata *et al*., 2011; Muttaqin and Abdulgani, 2013). Larvae were crushed until homogenenous, put into a tube with PBS, and the solution was sonicated at 30 to 40 kHz (3 × 30 sec at 60 sec interval), and protein concentrate was measured with a spectrophotometer.

Whole protein characterization via SDS-PAGE

SDS-PAGE was done to analyze the type of protein from whole worm extract (WWE) of third-stage *Anisakis* spp larvae. This was performed based on the manufacturer's instruction (BIO-RAD). Materials used were electrophoresis buffer solution (Sigma, Germany), Laemli buffer solution (Merck, USA), 12% separating gel solution (Sigma, Germany), 5% stacking gel solution (Sigma, Germany), samples of homogenate *Anisakis* spp., and the gel stained with commasie brilliant blue (Amresco, USA).

Polyclonal antibody production

Three rabbits were used in producing polyclonal antibody. Rabbits were treated under the ethical clearance set by the Ethical Commission of Veterinary Medicine, Universitas Airlangga. Each rabbit was injected subcutaneously with 500 µg of protein soluble third-stage *Anisakis* spp. larvae and added with equal amount of complete Freund's adjuvant (Sigma, Germany). Booster was done four times with 2-week interval. Five hundred µg of the protein soluble was added with equal amount of incomplete Freund's adjuvant (Sigma, Germany) (Lastuti *et al*., 2017; Lastuti *et al*., 2018). Two weeks after the last booster, 5 ml blood was extracted from the rabbits' auricular vein and centrifuged at 3,000 rpm for 5 min to obtain the serum, which was then used for antibody titers via indirect ELISA.

Indirect ELISA test

This procedure used the following components: carbonate buffer (50 mM, pH 9.6); washing buffer (NaCl-Tween 20); blocking buffer (skim milk or creamer was dissolved in 1% PBS with 0.05% Tween-20); conjugate IgG anti-rabbit labelled as alkaline phosphatase (Novus Biologicals); p-NPP substrate; and 3N NaOH. The test involved the following steps: 1) Coating antigen: antigen of third-stage *Anisakis* spp. larvae was diluted in carbonate buffer (50 mM, pH 9.6) to concentration of 1 µg/100 µl and placed in a 100 µl well. This was incubated for 1 h at room temperature or stored overnight at 4ºC, then washed thrice with NaCl-Tween 20. 2) Blocking: each well was filled with 200 µl blocking buffer (skim milk or creamer dissolved in 1% PBS with 0.05% Tween-20), incubated for 1 h at room temperature (37°C) and washed thrice with NaCl-Tween 20. 3) Serum incubation: serum of anti-*Anisakis* spp. was diluted with blocking buffer with a ratio of 1:100, then 100 µl of the serum was placed in each well, incubated and washed with the same conditions in step 2. 4) Conjugate incubation: anti-rabbit conjugate IgG (Novus Biologicals) was diluted with blocking buffer with a ratio of 1:1,000, and 100 µl of the conjugate was placed in each well, incubated and washed with the same conditions in step 2. 5) Substrate incubation: 100 µl of p-NPP substrate was inserted per well and incubated for 30 min at room temperature in a dark room. Then 50 µl of 3N NaOH was added per well to stop the reaction. Finally, samples were read at 405 nm using an ELISA reader (Rantam, 2003). Polyclonal antibodies obtained with positive OD values were then used for characterization by Western blot.

Antigenic protein characterization via Western blot

Western Blot was performed to determine the antigenic protein from third-stage *Anisakis* spp. larvae. This test used the following: homogenate antigen of *Anisakis* spp.; polyclonal antibody of *Anisakis* spp. (antibody serum from rabbits immunized with homogenate antigen); conjugate IgG anti-rabbit labeled with alkaline phosphatase (Novus Biologicals); commasie brilliant blue (Amresco, USA); substrate (BCIP/NBT) dye (Rantam, 2003). Antigen molecular weight was calculated using regression analysis, along with the Rf (retardation factor) value of the bands that appeared after SDS-PAGE and Western blot (Rantam, 2003). Rf value was obtained by calculating the result of the division between the distance of the protein band movement from the starting place (the band distance) and the distance of the color movement from the starting point (gel length) (Rantam, 2003).

RESULTS AND DISCUSSION

Third-stage *Anisakis* spp. larvae were collected from the digestive organs of mackerel tuna. The larva collected was white in color, 13-25 mm, had a ventriculus and a long esophagus with nerve ring based on Murata *et al*. (2011). Larvae were examined with semichen-acetic carmine staining and observed under a light microscope (Nikon E-100, Japan) at 100× magnification. Boring tooth at the anterior part and mucron at the posterior part are evident (Fig.1).

Three hundred *Anisakis* spp. larvae were crushed and suspended in 1.3 ml of PBS, producing 2.3 ml homogenate. The protein value of the larvae homogenate was 6,833.3 µg/ml. Blood sera from rabbits where tested with indirect ELISA to measure the OD value or antibody titer, which was read using an ELISA reader set at 405 nm. Highest OD was obtained from the fourth booster at 1.092, with 0.165 as the control serum value. Tested serum was positive, since the OD value was higher than twice the cut-off value (COV) for negative control, qualifying it for Western blot assay (Rodero *et al*., 2002). WWE protein analysis of the larvae using SDS-PAGE yielded 21 bands, with molecular weights 238 kDa, 172 kDa, 129 kDa, 114 kDa, 96 kDa, 87 kDa, 75 kDa, 64 kDa, 54 kDa, 51 kDa, 48 kDa, 45 kDa, 41 kDa, 38 kDa, 36 kDa, 34 kDa, 30 kDa, 27 kDa, 23 kDa, 18 kDa, and 12 kDa (Fig. 2)

Protein characterization of *Anisakis* spp. larvae via Western blot yielded a total of six antigenic protein bands, with molecular weights of 64 kDa, 45 kDa, 38 kDa, 30 kDa, 27 kDa, and 23 kDa (Fig. 2).

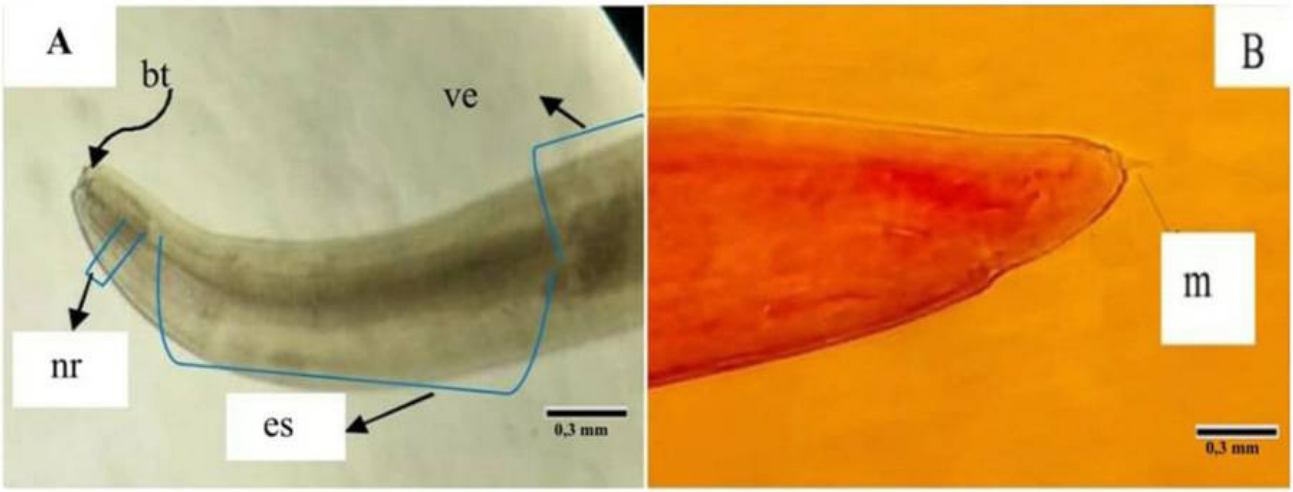

Fig. 1. Microscopic appearance of third-stage *Anisakis* spp. larva isolated from mackerel tuna fish under a light microscope. A: anterior (native); B: posterior (carmine staining). bt: boring tooth; nr: nerve ring; es: esophagus; ve: ventriculus; m: mucron.

SDS-PAGE results were similar with a previous study done by Soewarlan (2016) on *Anisakis typica* larvae protein collected from skipjack tunas, wherein molecular weights 48 kDa, 38 kDa, 27 kDa, and 23 kDa were also obtained. Larvae were also suspected to carry antigenic proteins, which can be verified by western blot. Reactivity test by western blot using specific antibody obtained from immunization has identified six protein bands.

The rabbit immunized with *Anisakis* spp. larvae protein will induce B cell to produce antibodies. The parasite antigen will be recognized subcutaneously by epithelial cells, which then will activate dendritic cell acting as the antigen presenting cell (APC). This will stimulate T helper (Th) cell to differentiate into Th2 cell. Th2 cell will activate IL-4, which will activate B cell to produce immunoglobulin (IgG) as an antibody and also activate IL-5 to form eosinophils expected to kill the parasites. The eosinophils bound with IgG will attach to the parasite's surface, release toxic granules, thus eliminating them. Further, IL-4 will bind to the receptor and stimulate humoral response through B cell activation and produce IgG, IgE, and IgA.

Reactivity test of the *Anisakis* spp. larvae by Western blot showed that a primer antibody (IgG) from rabbit serum antibody (IgG) immunized with soluble larvae protein, along with the secondary antibody, conjugate IgG anti-rabbit, can recognize the larvae protein antigen holding antigenic characteristic, with molecular weights of 64 kDa, 45 kDa, 38 kDa, 30 kDa, 27 kDa and 23 kDa. Essentially, the six proteins recognized by the specific antibody were proteins with antigenic property.

Previous studies have shown that *Anisakis* spp. has antigenic proteins with different molecular sizes, depending on the parasitic species and intermediate host (Umehara *et al*., 2008; Rodero *et al*., 2002; Rodero *et al*., 2005; Soewarlan, 2016). Research by Hwang *et al*. (2003) showed the excretory-secretory (ES) product of *Anisakis* spp. larvae. Reactivity test by Western blot revealed that among antigenic bands 25 kDa to 120 kDa, the antigenic characteristic was found in 38 kDa and 49 kDa. *Anisakis* spp. third-stage larvae protein, with molecular weight of 38 kDa, was deemed to carry the antigenic character associated with hypersensitivity or allergic reactions. In contrast, Rodero *et al*. (2005) found that the antigenic proteins of *Anisakis* spp. obtained by western blot were in 40 and 60 kDa. The difference in molecular weights indicates the type of *Anisakis*. This implies the need to further characterize the antigenic proteins of the larvae (Maldonado *et al*., 2004).

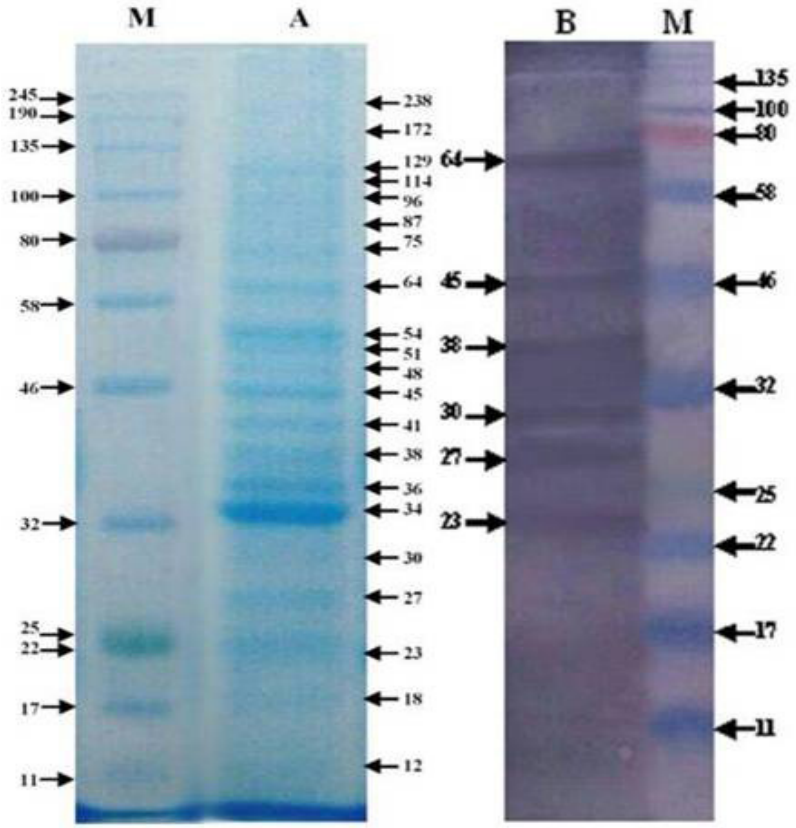

Fig. 2. Protein characterization of third-stage *Anisakis* spp. larvae isolated from mackerel tuna fish using SDS-PAGE (A), Western Blot (B), and marker (M).

This study suggests that third-stage *Anisakis* spp. larvae in mackerel tuna contains antigenic proteins, with molecular weights of 64 kDa, 45 kDa, 38 kDa, 30 kDa, 27 kDa and 23 kDa, which might play a role in hypersensitivity or allergic reactions. Further studies on these antigenic proteins are needed to determine their characteristics, such as their sensitivity and specificity, in order to develop reliable diagnostic serological kits for anisakiasis.

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RESEARCH NOTE

MORPHOLOGICAL DETECTION OF THE INTESTINAL PARASITE *Blastocystis* sp. IN FRESH AND CULTURED FECES OF PET SUGAR GLIDER (*Petaurus breviceps*) (MAMMALIA: MARSUPIALIA: PETAURIDAE) IN SURABAYA, INDONESIA

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ABSTRACT

Many deadly sugar glider diseases remain underdiagnosed. Thus, this study aimed to detect the presence of *Blastocystis* sp. in sugar gliders (*Petaurus breviceps*). Fresh stools were taken from 100 3-month to 4-year old male and female sugar gliders from enthusiasts and breeders. Samples were directly observed in wet mount, stained with iodine, methylene blue, and giemsa, and cultured on simple and RPMI 1640 media. Results showed high detection of the parasite: 87% on wet mount, 94% on iodine staining, and 100% on methylene blue, giemsa staining and cultured media. *Blastocystis* sp. in sugar glider can be described as vacuolar, granular, cyst, and amoeboid, wherein vacuolar form predominated with size 0.38–2.95 µm (average of 1.46 µm). The parasite lived for 6 days in simple culture medium and 5 days on RPMI 1640 medium. Growth peak was marked on the third day for both media. This study is the first to report *Blastocystis* sp. in sugar gliders, revealing its presence in both fresh and cultured sugar glider stools.

Key words : *Blastocystis* sp., culture medium, protozoan, staining, sugar glider

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INTRODUCTION

Recently, sugar gliders have become in demand exotic pets to animal lovers in the world, including Indonesia (Catro, 2013). However, study on their diseases is still limited, especially on the presence of *Blastocystis* sp. The main problem often faced by sugar glider lovers and breeders is the case of sudden death shortly after showing signs of diarrhea or bloating - a condition that veterinarians to this day are unable to diagnose.

Blastocystis sp. is an intestinal parasite generally found in both human and animal feces. The parasite lives in the digestive tract of humans, livestock, birds, rodents, reptiles, dogs, pigs, cats and other animals (Duda *et al.,* 1998; Yoshikawa *et al.,* 2004*;* Yoshikawa *et al.,* 2016). It causes infection with clinical symptoms, such as loss of appetite, constipation, diarrhea, urticaria, flatulence and irritable bowel syndrome (IBS) (Tan, 2008; Casero *et al*. 2015). Moreover, some researchers noted other asymptomatic cases and skin disorders (Ramirez *et al*., 2017; Khademvatan *et al*., 2018).

Parasitological surveys have often detected *Blastocystis* sp. in patient stool samples. Several methods are used for its detection: wet mount, staining, and culture methods. Common stains used are iodine lugol, iodine, giemsa, trichrome, acid-fast, and iron

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haematoxylin, a modification of Ziehl Neelsen (Leelayoova *et al*., 2002; Stensvold *et al*., 2007; Tan, 2008; Zhang *et al.,* 2012; Prasetyo, 2015). Meanwhile, there is also a number of culture media: Jones', Dulbecco's, RPMI 164, 199, formol ethyl acetate concentration, and Boeck and Drbohlav (Sakhsirisampant *et al.,* 2003; Tan, 2008; Zhang *et al.,* 2012). Culture method *in vitro* can be used to identify *Blastocystis* sp. for diagnosis in clinical and field studies. By using culture method, identification of diseases becomes more accurate, while also characterizing various forms of *Blastocystis* sp., such as vacuolar, cyst, granular and amoeboid (Dogruman *et al.,* 2010; Zhang *et al.,* 2012).

This study was conducted to detect *Blastocystis* sp. in fresh stool of sugar glider by staining and culture methods. This is the first research to detect *Blastocystis* sp. in sugar gliders, intended to assist veterinary practitioners in diagnosing possible infections.

MATERIALS AND METHODS

The study was conducted from August to October 2017. One hundred fecal samples, from 3-month to 4-year old sugar gliders, were obtained from enthusiasts and breeders in Surabaya, Indonesia. Age, sex, health status (healthy, presence of diarrhea or bloating), feed type and feeding frequency were recorded. Fecal samples were fresh stools that were just released during observation of health status. About 1 g of feces was collected per sugar glider and stored in sterile Eppendorf tubes® containing 1 ml of 2.5% potassium dichromate (Merck, Germany).

The study was conducted at the Laboratory of the Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga. Fecal samples were centrifuged at 1,500 rpm for 5 min, and the pellets of each sample were smeared on four object glasses, one for wet mount and three were stained using iodine (povidone-iodine, Mahakam Beta Farma, Indonesia), methylene blue (Merck, Germany) and 20% giemsa solution (Merck, Germany). The remaining pellets were resuspended with aquadest to a volume of 0.5 ml for culture. The suspension was divided into two, one mixed with simple culture medium and the other with RPMI 1,640 medium (Gibco® Life Technologies™, USA) to a volume of 1.5 ml. Cultures were incubated at 37°C. Development of protozoan in the culture was observed daily. According to Mohammed *et al.* (2015), the composition of simple medium are 500 ml ringer solution (Otsu-RL® Otsuka, Indonesia), 0.5 g yeast extract (Merck, Germany), 5 g peptone (Merck, Germany), 20 ml boiled rice water and 50-100 mg oxytetracyclin (Vet-oxy LA, Sanbe, Indonesia.) RPMI medium contains 10.4 g RPMI 1640 in 1 liter of double-distilled water (Zhang *et al*., 2012). Morphology of *Blastocystis* sp. was observed and measured under a light microscope (Nikon® E100, Japan) connected to a camera (Optilab® MTN001, Indonesia).

RESULTS AND DISCUSSION

Only a few *Blastocystis* sp. can be detected in fresh stools, where vacuolar form dominated (Fig. a-d); meanwhile, all forms (vacuolar, granular, cyst and amoeboid) were found in cultured samples, and vacuolar form was also the most evident (Fig. d). The diameter of *Blastocystis* sp. in sugar glider was 0.38-2.95 µm (average=1.46 µm). This is smaller in terms of size compared to *Blastocystis* sp. found in dogs, cats and humans (Stenzel and Boreham, 1996; Duda *et al.,* 1998).

Microscopic observations showed high detection of parasite on wet mount (87%), iodine staining (94%), and methylene blue and giemsa staining (100%) (Table 1). This suggests that all samples were positive for *Blastocystis* sp., and methylene blue and giemsa staining had the highest occurrence of *Blastocystis*. These results coincide with the study by Zhang *et al.* (2012), wherein staining with methylene blue and giemsa resulted to a more effective method of detection compared to wet mount smears with iodine.

Moreover, cultured stools also showed 100% detection of *Blastocystis* sp., growing both in simple and RPMI 1640 media. The development of *Blastocystis* sp. in cultured media was observed for 7 days (Table 2).

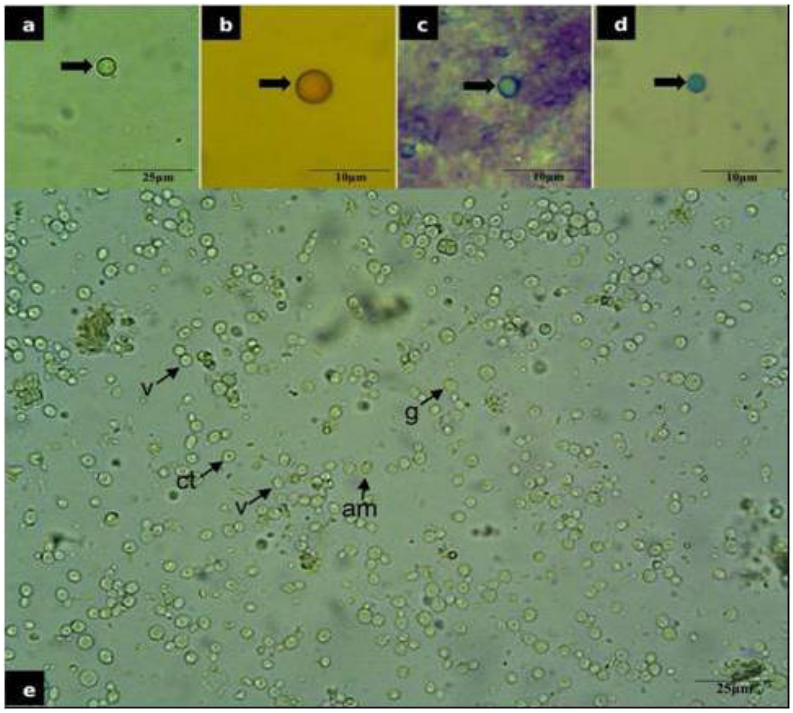

Fig. *Blastocystis* sp. (black arrows) in sugar glider stool. a: wet mount, b: iodine stain, c: giemsa stain, d: methylene blue stain, and e: cultured medium. am: amoeboid, ct: cyst, v: vacuolar, g: granular.

Growth in RPMI 1640 medium was relatively faster but so was the occurrence of death. In simple medium, growth was seen on day 2, alive until day 6; while on RPMI medium, growth was already visible on day 1, surviving only until day 5. Third day marked the peak of growth of *Blastocystis* for both media. The simple medium can then be considered a new, cheap and convenient to produce culture medium, without adding either human or horse serum (Mohammed *et al*., 2015).

Such high occurrence of *Blastocystis* sp. in sugar glider feces raises the question of whether it is actually a commensal

Table 1. *Blastocystis* sp. occurrence on fresh and cultured stools of sugar glider.

Sample	Method	Positive for *Blastocystis* sp. (%)
Fresh stool	Wet mount	87
	Iodine staining	94
	Giemsa staining	100
	Methelyne blue staining	100
Cultured stool	Simple medium	100
	RPMI 160	100

protozoan or a pathogen. A review by Parija and Padukone (2016) argues that although *Blatocystis* has been identified a century ago, its taxonomy, biology and pathogenicity are not yet fully understood. In the recent decades, however, many researchers have focused on the pathogenicity of *Blastocystis* sp. One study, for instance, has shown that stressful conditions can increase the infectivity, pathogenesis, and growth of the parasitic *B.*

Table 2. Presence of *Blastocystis* sp. from stools of sugar glider on cultured media.

Day	Culture media	
	RPMI 1640	Simple
1	Live, light	-
2	Live, light	Live, light
3	Live, medium	Live, plentiful
4	Live, light	Live, medium
5	Live, light	Live, light
6	Dead	Live, light
7	Dead	Dead

Light: growth of <10 *Blastocystis* sp. in one field of view (FOV); Medium: growth of 10-20 *Blastocystis* sp. in one FOV; Plentiful: full growth of *Blastocystis* sp. in one FOV.

Table 3. Characteristics of sugar glider samples from stools of sugar glider from Surabaya.

Parameter		*n*
Sex	Male	29
	Female	71
Age	≤ 1 yr	33
	> 1 yr	67
Food ration	Once	20
	Twice	80
Food type	Porridge	100
Drinking water type	Raw water	55
	Bottled water	23
	Water refill (filtered water)	22
Health status	Healthy	79
	Sick	21
Incidence of sickness	Present	79
	Absent	21
Incidence of death	Present	94
	Absent	6
Cage size	30×22×26 cm^3	32
	46×30×32 cm^3	66
	60×50×42 cm^3	2
Cage population	1 sugar glider	2
	2 sugar gliders	75
	>2 sugar gliders	23
Cleaning frequency of cage per month	Once	26
	Twice	56
	Four times	18

hominis (Chandramathi *et al*., 2014)

As shown in Table 3, *Blastocystis* sp. was found in both sugar gliders with or without clinical symptoms. Twenty-one sugar gliders manifested weakness, diarrhea, and bloating. These observations are similar to human studies. Some researchers have found *Blastocystis* in both symptomatic and asymptomatic patients, and clinical symptoms varied from skin disorders (itching) to intestinal

symptoms (nausea, diarrhea, flatulence, and irritable bowel syndrome (Ramirez *et al*., 2017; Khademvatan *et al*., 2018). There are 17 subtypes (ST) of *Blastocystis* in mammals and birds, 9 subtypes (ST1-9) of which can infect humans (Cian *et al*., 2017). In human cases, according to Ramirez *et al*., (2017), clinical outcome of *Blastocystis* sp. infection is not likely associated with a specific subtype of *Blastocystis* sp., but Zulfa *et al*. (2017) argues that ST3 subtype is more likely to be associated with diarrhea in children.

This research also found that the number of *Blastocystis* in sugar gliders with clinical symptoms was higher than those without clinical symptoms. Based on this, if sugar gliders show symptoms as stated, this often leads to sudden death. Sugar gliders with symptoms are assumed to have originated from colonies given raw drinking water and whose cages were only cleaned once a month. Water quality, contamination of food and drinking water, and sanitation influence the rate at which *Blastocystis* sp. infection can occur (Abdulsalam *et al*., 2012; Canete *et al*., 2012). Further research using molecular markers is needed to understand the dynamics of *Blastocystis* sp. infection and its role in health and disease of sugar gliders.

Several studies have shown that *Blastocystis* infection has the potential to be a zoonotic disease, with the discovery of the same subtype affecting both animals and humans (Osman *et al*., 2015). Transmission of *Blastocystis* infection can be oral or fecal (Yoshikawa *et al*., 2004). Considered as the infective stage, *Blastocystis* cysts in some animals can contaminate water, an easy source of transmission (Lee *et al*., 2012). Thus, *Blastocystis* subtypes in sugar gliders and their zoonotic potential entail further research.

This study demonstrates that *Blastocystis* sp. exist in fresh stool of sugar gliders, with the highest occurrence noted for methylene blue, giemsa stained and cultured media samples. Morphology of *Blastocystis* sp. was vacuolar (dominant form), granular, cyst and amoeboid, having a diameter of 0.38-2.95 μm, with an average of 1.46 μm. Day 3 marked growth peak in both media. This is the first report of *Blastocystis* sp. in sugar glider.

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