The PHILIPPINE JOURNAL OF

Veterinary Medicine

Volume 55 **Special Issue** **December 2018**

Published by the College of Veterinary Medicine
University of the Philippines Los Baños

ISSN 0031-7705

The 2nd Veterinary Medicine International Conference Surabaya, Indonesia 4-5 July 2018

The Philippine Journal of Veterinary Medicine

Volume 55 Special Issue December 2018

The Philippine Journal of Veterinary Medicine is a peer-reviewed international journal of basic and applied research in veterinary medicine and science. It is published semi-annually, for the periods January-June and July-December each year, by the College of Veterinary Medicine, University of the Philippines Los Baños. All articles are subjected to double-blind review.

Authors of articles appearing in the journal are solely responsible for opinions expressed therein.

The annual subscription price is US$100.00 (net) for foreign subscribers (inclusive of mailing cost) and Philippine PhP1,500.00 plus mailing cost for local subscribers. Prices for current single issue and back issues are available on request. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by air delivery to foreign subscribers.

All communications should be addressed to:

The Editor-in-Chief
Philippine Journal of Veterinary Medicine
College of Veterinary Medicine
University of the Philippines Los Baños
Laguna, Philippines 4031
Telefax Nos. +63-49-536-2727, +63-49-536-2730
Email: pjvm1964@gmail.com, vetmed_uplb@yahoo.com.ph

This journal is Abstracted/Indexed by: SCOPUS, Biological Abstracts, Focus on: Veterinary Science & Medicine, Zoological Records, CAB Abstracts, Index Veterinarius, Veterinary Bulletin, Parasitology Database, Helminthological Abstracts, Protozoological Abstracts, Review of Medical and Veterinary Entomology, EBSCO, ASEAN Citation Index, Prescopus Russia, *i*-journals (www.ijournals.my), *i*-focus (www.ifocus.my), *i*-future (www.ifocus.my), Philippine E-Journals (https://ejournals.ph) and UPLB Journals Online (http://journals.uplb.edu.ph/index.php/PJVM).

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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

Fifit Natalia[1], Lucia Tri Suwanti[*2,4], Endang Suprihati[2], Kusnoto[2], Setiawan Koesdarto[2] and Pudji Srianto[3]

[1]Program of Medicine Science and Veterinary Public Health; [2]Department of Parasitology; [3]Department of Reproduction; [4]Institute of Tropical Disease, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

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

Philipp. J. Vet. Med., 55(SI): 91-96, 2018

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

***FOR CORRESPONDENCE:**
(email: tswant@gmail.com)

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).

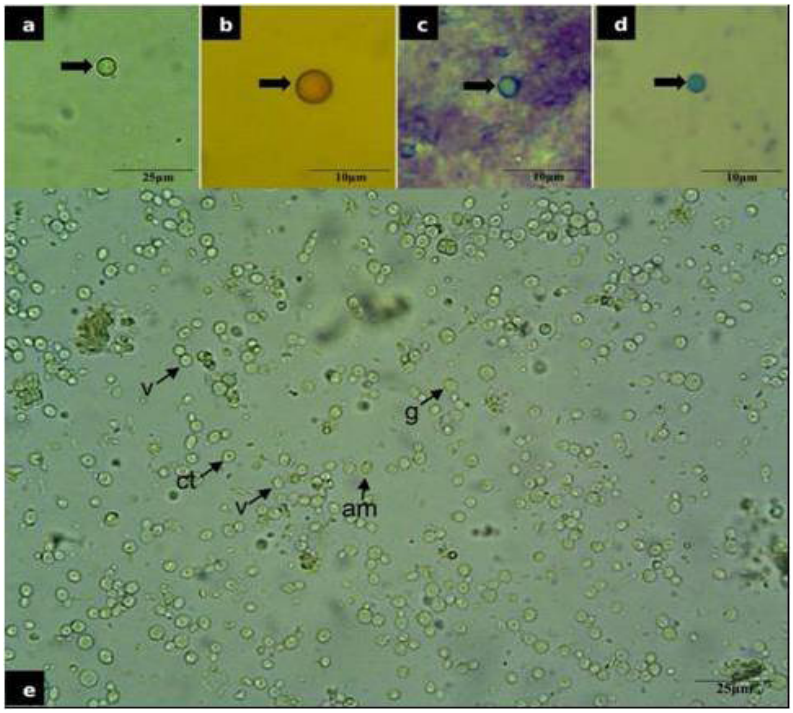

Fig. *Blastocystis* sp. (black arrows) in sugar glider stool. a: wet mount, b: iodine stain, c: giemsa stain, d: methylene blue stain, and e: cultured medium. am: amoeboid, ct: cyst, v: vacuolar, g: granular.

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.

ACKNOWLEDGMENT

The researchers would like to thank the sugar glider enthusiasts and breeders for their permission to use their pets as experimental models and for their assistance during stool collection. Huge thanks as well to the Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga and the acting Dean for providing the facilities which enabled the conduct of the study.

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2.	Identification of Active Compounds of Ethanol Extract of Citrus amblycarpa leaves by Analysis of Thin-layer Chromatography and Gas Chromatography-Mass Spectrometry as Bioinsecticide Candidates for Mosquitoes	2020
3.	Histopathological studies on *Leucocytozoon Caulleryi* infection on broiler in endemic area of Indonesia	2020
4.	Potential Extract Ethanol Citrus Amblycarpa as a Bioinsecticide Against Aedes Aegypti Larvae	2021
5.	Protein Profile of Sporozoite of Leucocytozoon sp. from Culicoides sp.	2010
6.	Deteksi Cryptosporidium canis pada Anjing di Kota Surabaya	2020
7.	Eksplorasi Protein Antigenik *Leucocytozoon caulleryi* sebagai Kit Diagnostik Leucocytozoonosis pada Ayam Broiler	2013

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Adapun penelitian tersebut tidak perlu dilakukan ***Uji Etical Clearence*** karena tidak menggunakan hewan coba.

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