Tropical Biomedicine 40(4): 392-399 (2023)
https://doi.org/10.47665/tb.40.4.003
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RESEARCH ARTICLE

Wild hosts and microscopic worlds: Investigating the morphology and surface ultrastructure of *Blastocystis* sp. in avian and non-human primate species

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ARTICLE HISTORY

Received: 21 July 2023
Revised: 28 August 2023
Accepted: 28 August 2023
Published: 31 December 2023

ABSTRACT

Blastocystis is a prevalent infectious agent found in the gastrointestinal tract of humans and animals. While the morphology of *Blastocystis* has been extensively studied, there is still a lack of comprehensive research on its ultrastructure, especially regarding surface characteristics and their correlation with pathogenic potential. Additionally, the subtyping of *Blastocystis* does not provide information on the isolate's pathogenicity. This study aimed to examine the morphology and the cell surface of *Blastocystis* in avian and non-human primates, including peafowl, pheasant, and lion-headed tamarin. By employing light microscopy and scanning electron microscopy (SEM), this study provides the first evidence of the cellular and surface features of *Blastocystis* in these animal species. Our findings revealed distinct variations in cell size, shape, and surface morphology among the different host species. Notably, the isolates from peafowl exhibited larger cell sizes compared to the isolates from the pheasant. However, interestingly, both animal species were found to exhibit the same *Blastocystis* ST6. It was also observed that the surface structure of *Blastocystis* from different hosts displayed a diverse range of patterns, including mesh-like appearances, deep indentations, and attachments to bacteria. Additionally, findings also revealed the presence of a rough surface structure in peafowl, a characteristic that has been previously linked to pathogenicity and symptomatic infection in animals, as indicated by earlier studies. The findings contribute to our understanding of the morphological features and the surface characteristic of *Blastocystis* in different host species, shedding light on the parasite's adaptations and potential implications for host health.

Keywords: *Blastocystis*; morphology; ultrastructure; scanning electron microscopy; wild animals.

INTRODUCTION

Blastocystis is a prevalent infectious agent found in the gastrointestinal tract of humans and animals (Stenzel & Boreham, 1996; Kumarasamy *et al*., 2014). Understanding the morphology and ultrastructure of *Blastocystis* is crucial for revealing its biology including its life cycle, transmission modes, and potential pathogenicity. While the morphology of *Blastocystis* has been extensively studied, there is still a lack of comprehensive research on its ultrastructure, especially regarding the surface characteristics and their correlation with pathogenic potential (Tan, 2004; Zhang *et al*., 2012). *Blastocystis* infection is zoonotic, with the same subtypes being identified in both humans and animals, emphasizing the importance of studying its transmission dynamics and host specificity (Osman *et al*., 2015). It is primarily transmitted through contaminated water and food, and poor hygiene practices in close contact with animals contribute to its higher prevalence in developing countries (Lee *et al*., 2012; El Safadi *et al*., 2016). Symptoms of *Blastocystis* are non-specific, and while most cases are asymptomatic, *Blastocystis* has been associated with irritable bowel syndrome and precancerous polyp formation, raising concerns about the potential impact on human health (Parija & Jeremiah, 2013; Ragavan *et al*., 2014; Kumarasamy *et al*., 2017). Conventional diagnostic methods based on morphological examination of *Blastocystis* in faecal samples have limitations in distinguishing each isolate from humans and animals (Zhang *et al*., 2012; Wawrzyniak *et al*., 2013). Furthermore, relying solely on subtyping does not provide a reliable prediction of the isolate's pathogenicity (Nagel *et al*., 2012). To overcome this challenge and gain deeper insights into its structure, scanning electron microscopy (SEM) has emerged as a powerful tool. SEM enables detailed visualizations of the surface ultrastructure, topography, and variation in surface coats of microorganisms (Cassidy *et al*., 1994; Widisuputri *et al*., 2021). Previous SEM studies have highlighted variations in the surface coats of *Blastocystis* isolates from different host species, suggesting potential differences in pathogenic potential (Yason & Tan, 2015; Farah Haziqah *et al*., 2018a, 2018b).

Research involving electron microscopy has examined the ultrastructure of *Blastocystis* sp. across a range of species, including humans (primarily), monkeys, pigs, chickens, ducks, ostriches, dogs, cockroaches, and cattle. These studies have unveiled insights into the surface coat and organelles of *Blastocystis* (Cassidy *et al*., 1994; Stenzel & Boreham, 1994; Duda *et al*., 1998; Lee & Stenzel, 1999;

Published by Malaysian Society of Parasitology and Tropical Medicine.

Zhang *et al*., 2012; Farah Haziqah *et al*., 2018a, 2018b; Yason & Tan, 2018; Widisuputri *et al*., 2021). The absence of studies investigating the surface ultrastructure of *Blastocystis* in peafowl, pheasant, and lion-headed tamarin leaves a gap in our understanding of this topic

The surface coat of *Blastocystis* isolates is commonly depicted as fibrillar, and it is theorized to play roles in cellular nutrition, pathogenicity, and evading the host's immune system (Cassidy *et al*., 1994; Stenzel & Boreham, 1994; Zaman *et al*., 1997, 1999; Tan, 2008). Nevertheless, various researchers have observed differences in the properties of the surface coat among different *Blastocystis* isolates.

While previous emphasis was placed on the morphological examination of *Blastocystis*, recent years, particularly after the successful classification of *Blastocystis* within the Stramenopile group through molecular and phylogenetic analysis, have seen a shift in focus. This shift has moved away from purely morphological investigations to also consider biochemical, general cellular biology, and pathological mechanisms, acknowledging that morphological findings still have their place in understanding this organism's mechanism.

Therefore, this study aims to investigate the morphology and ultrastructure of *Blastocystis* using scanning electron microscopy, focusing on peafowl, peasant, and golden-headed lion tamarin isolates. By analyzing the surface characteristics, this study aims to provide valuable insights into the morphology, ultrastructure, subtyping, and potential pathogenicity of *Blastocystis*, contributing to our understanding of the biology of this parasite and its implication for zoonotic transmission and the health of both humans and animals.

MATERIALS AND METHODS

Ethical Approval

All animals involved in this study were handled in compliance with the Institutional Animal Care and Use Committee (IACUC) (USM/IACUC/2021/(EXP) (1159) of Universiti Sains Malaysia. Upon receiving approval from the Ethics Committee of the School of Biological Sciences, Universiti Sains Malaysia, permission was also sought from the Malaysian Wildlife Department (PERHILITAN). The necessary written authorization was obtained from the appropriate authorities to conduct the study. The collection of animal samples consistently adhered to the daily husbandry and animal management protocols implemented at each institution, ensuring minimal disruption to the animals' care and well-being.

Sample Collection

Sampling was carried out between January 2021 and March 2022 in the northwest Malaysian state of Perak. A total of 35 fresh faecal samples were gathered from pheasants, peafowl, and lion-headed tamarin from several zoological gardens across the states.

To ensure freshness, the faeces were collected early in the morning before cleaning the cages. In group housing, all faeces deposited by the animals were collected from the floor and combined. For animals kept alone in enclosures, separate collection was performed. Using an applicator stick, the faeces were collected from the ground and placed in sterile containers. Each container was appropriately labelled with information including the animal's type, serial number, farm location, and the date and time of collection. Without the addition of any preservatives, the samples were promptly transported to the laboratory within a few hours of being collected.

Sample Processing and Detection of *Blastocystis* sp.

A small amount of each faecal sample, about the size of a pea, was placed into a sterile screw-top container containing 3 ml of Jones' medium, which had been supplemented with 10% heat-activated horse serum. The sample was then incubated upright at a temperature of 37°C for a period of 48 to 72 hours. To determine the presence of *Blastocystis* sp., a drop of sediment from the faecal sample was examined using light microscopy at magnifications of 100x, 400x, and 1000x. If no growth was observed, the sediment was transferred to a new medium and further incubated for an additional 48 hours at 37°C. Any positive findings from the faecal smears were preserved in methanol and stained with 10% Giemsa stain to enable detailed morphological observation using light microscopy at magnifications of 400x and 1000x. Identification of *Blastocystis* in the faecal samples was done through both morphological examination and genetic analysis targeted at the 18S rRNA gene. Isolates of *Blastocystis* from symptomatic and non-symptomatic were selected. Prior to scanning electron microscopy (SEM) screening, the supernatant of the culture medium was collected and centrifuged at 1300 rpm for 10 minutes. The liquid portion was discarded, and the solid residue from each sample was fixed in 2.5% glutaraldehyde and preserved for further processing.

Scanning Electron Microscopy

The *Blastocystis* sp. cells were rinsed three times with PBS (pH 7) through centrifugation at 3000 rpm for 5 minutes. Subsequently, each sample was fixed by the addition of 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. The isolated cells were then mounted on a polycarbonate membrane and dehydrated using a sequential ethanol series of 30%, 50%, 70%, 80%, 90%, and 100%. Each ethanol step lasted for 15 minutes, and the final step involved the addition of amyl acetate. Carbon dioxide was employed for Critical Point Drying (CPD), followed by coating the specimen with a layer of gold. The observations were conducted using a Scanning Electron Microscope (SEM) at the School of Biological Sciences, Universiti Science Malaysia, as described by Ragavan *et al*. (2014). SEM images were captured during this process (Zeiss Supra 50vp).

Statistical Analysis

One-way analysis of variance (ANOVA) was employed to calculate the mean cell sizes of *Blastocystis* isolates from a different group of animals.

Genomic DNA preparation and subtyping of *Blastocystis* isolates

The Nucleospin DNA Stool Kit (Macherey-Nagel, Germany) was used to extract DNA from culture following the manufacturer's protocol. The presence of *Blastocystis* was determined using a PCR method. The eukaryote-specific primer RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') (Clark, 1997) were utilized as forward primer and, *Blastocystis-specific* primer BhRDr (5'-GAGCTTTTTAACTGCAACAACG-3') was used targeting the small subunit (SSU) ribosomal RNA (rRNA) (*Scicluna et al*., 2006). These primers target the small subunit ribosomal RNA (rRNA) gene of *Blastocystis*, amplifying a 600 bp region that enables clear identification of subtypes in the samples *(Scicluna et al*., 2006). The PCR reaction was performed using 50 µl of Vivantis 2X Taq Master Mix, 2.5 mM MgCl, , 0.5 µl of each primer, and 2 µl of DNA. A Bio-Rad Thermo Cycler was used, with an initial denaturing step of 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, 56.3°C for 1 minute and 30 seconds, and 72°C for 1 minute. A final elongation step of 72°C for 10 minutes was included. The PCR products were visualized on a 1.5% agarose gel and subsequently purified. The classification of subtypes for each *Blastocystis* isolate was determined according to the standard terminology.

RESULTS AND DISCUSSION

The morphology and cell diameter of *Blastocystis*

Despite the lack of an established study on *Blastocystis* morphology in wild species in Malaysia, this study explores the morphological and surface ultrastructure features of *Blastocystis* in avian and non-human primate species in Perak, Malaysia. There were few morphological studies on a diverse group of animals in cattle by

Widisuputri *et al*. (2021); Highland bull, llama, and camel by Stenzel *et al*. (1993); pig by Cassidy *et al*. (1994); chicken by Stenzel *et al*. (1993), Cassidy *et al*. (1994), Farah Haziqah *et al*. (2014); ostrich by Stenzel *et al*. (1993), Chandrasekaran *et al*. (2014); monkey by Cassidy *et al*. (1994), Stenzel *et al*. (1997); dog by Duda *et al*. (1998), and cockroach by Farah Haziqah *et al*. (2017); while large-billed crow by Yong *et al*. (2008), turkey by Siti Alawiyah *et al*. (2021) have been conducted worldwide.

A total of 35 *Blastocystis*-positive fresh faecal samples were collected from three species: 25 from pheasants, 8 from peafowl, and 2 from a lion-headed tamarin (Table 1).

In pheasants, granular and vacuolar forms with round shape cells were observed. In the peafowl samples, oval, and round shapes with large central bodies, along with peculiar, granular, and distinct bold forms were observed (Figure 1a). Peafowl showed larger cells compared to pheasants, and there was significant variation in cell size within the peafowl species. Previous studies reported similar sizes in fowls, ranging from 5 to 18 µm, 9 to 28.3 µm, and 10 to 30 µm, respectively (Cassidy *et al*., 1994; Bergamo Do Bomfim & Machado Do Couto, 2013; Farah Haziqah *et al*., 2014). However, the cell size of peafowl has not been documented previously. The lion-headed tamarin found is round shaped with mostly vacuolar form (Figure 1e, 1f). This study reports the first recorded cell size of *Blastocystis* isolates in pheasants, peafowl, and lion-headed tamarins. The measured cell diameters of *Blastocystis* in pheasant, peafowl, and golden-headed lion tamarin ranged from 5.32 µm to 11.62 µm; 5.32 µm to 17.09 µm; 4.71 µm to 15.27 µm. The average cell diameter observed in pheasant was 8.52±1.78 µm, in peafowl it was 10.95±3.86 µm, and in golden-headed lion tamarin, with 8.01±2.08 µm. *Blastocystis* sp. exhibits a unique diversity of shapes and sizes, with extensive variation observed within and between isolates (Zierdt, 1991; Parija & Jeremiah, 2013). The significant differences in cell diameter observed between *Blastocystis* isolates from animals in this study may be attributed to the specific gut characteristics of the hosts, as animals differ in gut structure and diet.

In recent years, scanning electron microscopy (SEM) has been widely used to study the morphological features of cells, including *Blastocystis*, focusing on the cell's structural surface (Boreham & Stenzel, 1993; Zaman *et al*., 1999; Tan, 2008; Zhang *et al*., 2012; Ragavan *et al*., 2014). SEM has provided valuable insights into the external morphological shape of *Blastocystis* cysts and the various surface coat structures of the parasite (Zhang *et al*., 2012). The studies on the ultrastructure of wild animals are limited (Table 2). This is the first study on the surface structure of *Blastocystis* in peafowl, golden-headed lion tamarin, and pheasant. Samples from symptomatic pheasants and peafowl were used for the ultrastructure study analysis, while samples from non-symptomatic pheasants and lion-headed tamarin were employed for the same study. The surface structure of *Blastocystis* from different hosts shows variability, and this study notes variations in surface morphology among peafowl, golden-headed lion tamarin, and pheasant.

Blastocystis cells from pheasants showed numerous smaller indentations off the surface with bacteria found attached to the cell surface (Figure 2a) (Cassidy *et al*., 1994). The cells exhibit a mesh-like surface structure (Figure 2b), and the shape showed greater variation compared to Figure 2a. Both cell types left a fibrillar layer and displayed irregular shapes with frequent surface projections and deep indentations. The surface of the cells appeared to be fibrillar and attached to bacteria. This surface coat appeared compact, resulting in a rough and undulating cell surface (Figure 2b). In the peafowl, the surface structure displayed deep indentations (Figure 2c), like the raft morphology observed in *Blastocystis galli* (Belova & Kostenko, 1990). These results are in consonance with the morphology of *Blastocystis* in humans reported by Boreham and Stenzel (1993). *Blastocystis* cells from *in vitro* culture typically exhibit a spherical shape (Figure 2b). Round-shaped cells were also found by Ahmed and Karanis (2019) (Figures 2a and 2d). Elsayad *et al*. (2019) showed different outer morphologies of *Blastocystis* in humans, with some covered by a rough or smooth surface coat, although no further explanation was provided for the differences. The smooth surface structure of *Blastocystis* isolates in pheasants and lion-headed tamarins is similar to that of asymptomatic human isolates as previously described by Suresh *et al*. (1994). The rough surface cell illustrations in diarrheal peafowl in this study support the results reported by Boreham and Stenzel (1993) who found rough in *Blastocystis* isolates from patients with diarrhoea. These studies found that the percentage of *Blastocystis* organisms with rough surfaces was significantly higher in peafowl compared to other animal hosts. This rough surface structure may be related to the pathogenicity of the organism and the associated symptoms. It is suggested that *Blastocystis* with a rough surface is more pathogenic than those with a smooth surface, and the surface structure features of *Blastocystis* sp. correlated with symptomatic appearance (Tan, 2008; Ragavan *et al*., 2014; Ahmed & Karanis, 2019). Rougher surfaces' excessive indentation was observed in the isolates derived from patients with irritable bowel syndrome (Ragavan *et al*., 2014) and colorectal carcinoma (Ahmed & Karanis, 2019).

The morphology of the fibrillar structure in the surface structure (Figure 2d) is more similar to that reported by Cassidy *et al*. (1994) and Stenzel and Boreham (1994). This suggests that the fibrillar structure may have a function in protecting against osmotic shock which is a condition in which cells lose water and swell.

In other protozoa, it has been suggested that the surface coat acts as protection against mechanical and chemical challenges in the environment (Nagel, 2012). Bacteria were frequently observed in close association with the surface coat, especially in the peafowl samples. The surface coat contains a variety of carbohydrates, and it is postulated to play a role in trapping and degrading bacteria for nutrition (Tan, 2008). The surface coat may facilitate non-specific bacterial attachment preceding phagocytosis (Dunn *et al*., 1989), exert a toxic effect on bacteria (Silard & Burghelea, 1985), or control the permeability of ions and molecules. Zaman *et al*. (1997) suggested that the surface coat is involved in energy capture, allowing *B. hominis* to obtain nutrition from bacteria. Further studies are needed to determine the exact role and composition of the surface coat of *Blastocystis*. Although some functions of the surface coat are not yet fully understood, it is believed to serve as a mechanism for trapping bacteria for nutritional purposes and attachment to the intestinal epithelial lining (Zaman *et al*.,

Table 1. Number of samples collected from symptomatic and non-symptomatic wild animals from several zoological gardens

Host	Genus/species	Symptomatic	Non-symptomatic	Subtype
Pheasant	*Phasianus colchicus*	5	20	ST6
Peafowl	*Pavo cristatus*	5	3	ST6
Lion-headed tamarin	*Leontopithecus chrysomelas*	0	2	Unknown
Total		**10**	**25**	

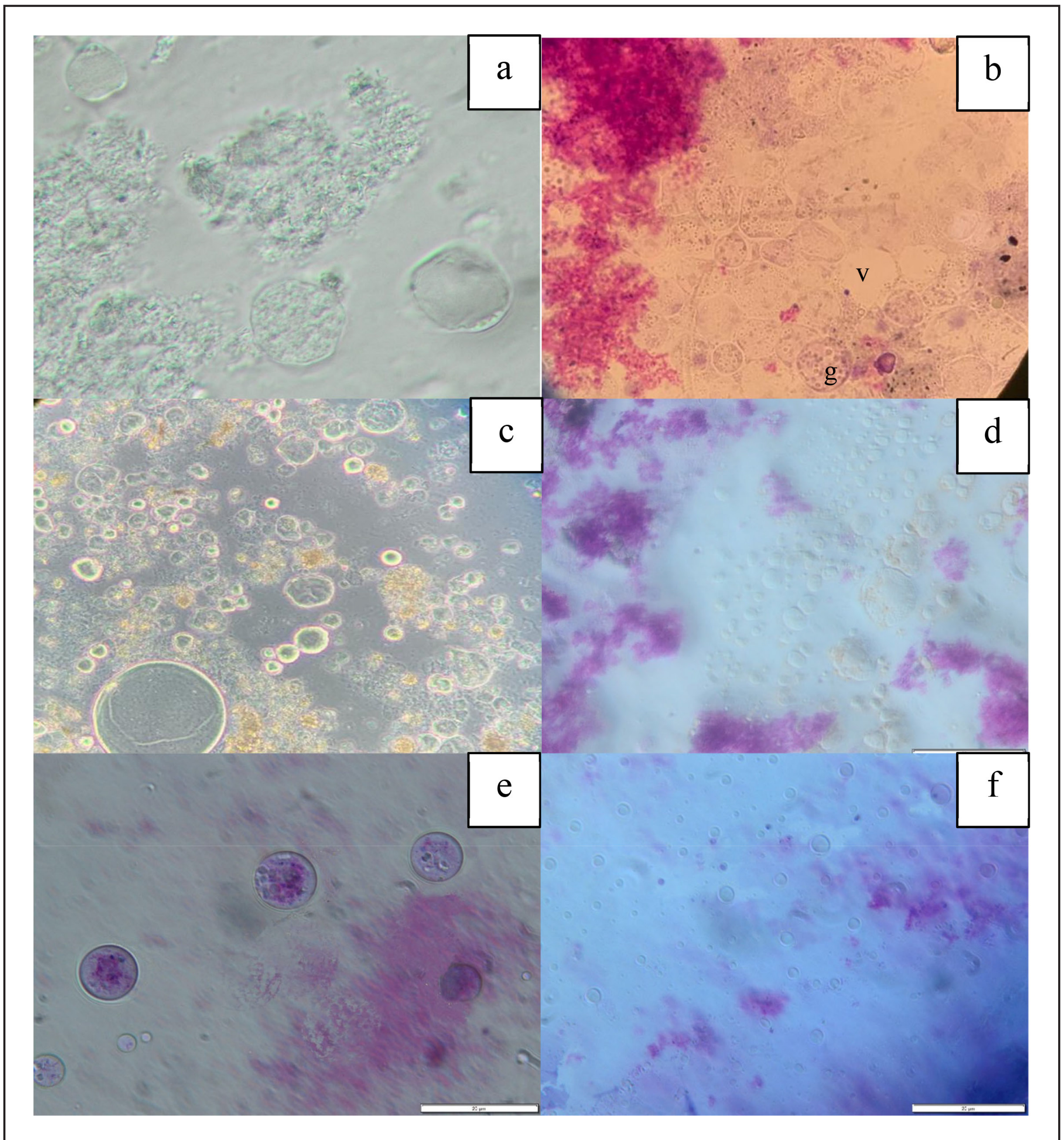

Figure 1. Morphology of *Blastocystis* isolated from positive samples based on the group of animals

a. Vacuolar and granular forms from peafowl/*Pavo cristatus* isolate – Aves (unstained)
b. Vacuolar (v), granular (g), and from Amoeboid (a) from peafowl/*Pavo cristatus* isolate – Aves (stained)
c. Vacuolar and granular forms from pheasant/ *Phasianus colchicus* isolate (unstained)
d. Vacuolar and granular forms from pheasant/ *Phasianus colchicus* isolate (stained)
e. Granular forms from golden-headed lion tamarin/*Leontopithecus chrysomelas* isolate (stained)
f. Vacuolar form from golden-headed lion tamarin/*Leontopithecus chrysomelas* isolate (stained)

Table 2. Surface ultrastructure of *Blastocystis* isolated from various sources between 2014-2023

Host (Scientific name)	Source of isolates (Faecal/ culture)	Morphological forms/shape	Cell Diameter	Description	Subtype	References
Cattle / *Bos taurus*	*In-vitro* cultivation	Vacuolar	–	- Rough surface structure - associated with diarrhoea - Smooth surface – non-diarrhea	–	Widisuputri *et al*. (2021)
Chicken/ *Gallus gallus*	*In-vitro* cultivation	Vacuolar, Granular Spherical	10-100 µm	- Free-range chicken-smooth surface structure - Barn-reared chicken - coarse with folds surface structure	ST1, ST6, ST8, ST7	Farah Haziqah *et al*. (2018b)
Ostrich/ *Struthio*	*In-vitro* cultivation	Cyst-rounded or slightly irregular in shape	3.0-7.0 µm	- Thick compact surface coat with attached bacteria	ST6	Chandrasekaran *et al*. (2014)
Turkey/ *Meleagris*	*In-vitro* cultivation	Vacuolar, Granular Spherical	5-30 µm	- Rough surface coat - Undulating cell surface - Gouges or deep furrows on the surface	ST7	Siti Alawiyah *et al*. (2021)
Cockroach /*Blattodea*	*In-vitro* cultivation	Vacuolar Granular Spherical	7-20 µm	- Smooth - Coarse - Folded surface	ST3	Farah Haziqah *et al*. (2017)
Human/ *Homo sapiens*	*In-vitro* cultivation	Vacuolar		- Uniform surface - Uneven surface - Mesh-like surface	ST1, ST4, ST7	Yason and Tan (2018)
Pheasant/ *Phasianus colchicus*	*In-vitro* cultivation	Vacuolar, Granular Spherical Ovoid	5.32-17.09 µm	- Without the fibrillar layer. - Smooth surface with small indentions on the surface -Bacteria attached	ST6	Present study
Peafowl/ *Pavo cristatus*	*In-vitro* cultivation	Vacuolar, Granular Aamoeboid Spherical Ovoid irregular	5.32-11.62 µm	- Compact with rough and undulating cell surface - Mesh-like appearance - Round and irregular - Showed projections and deep indentations	ST6	Present study
Golden-headed lion tamarin/ *Leontopithecus chrysomelas*	*In-vitro* cultivation	Vacuolar, Granular, Spherical Avoid	4.71-15.27 µm	- Smooth surface structure - Attached with a fibrillar layer - Bacteria attached	Unknown	Present study

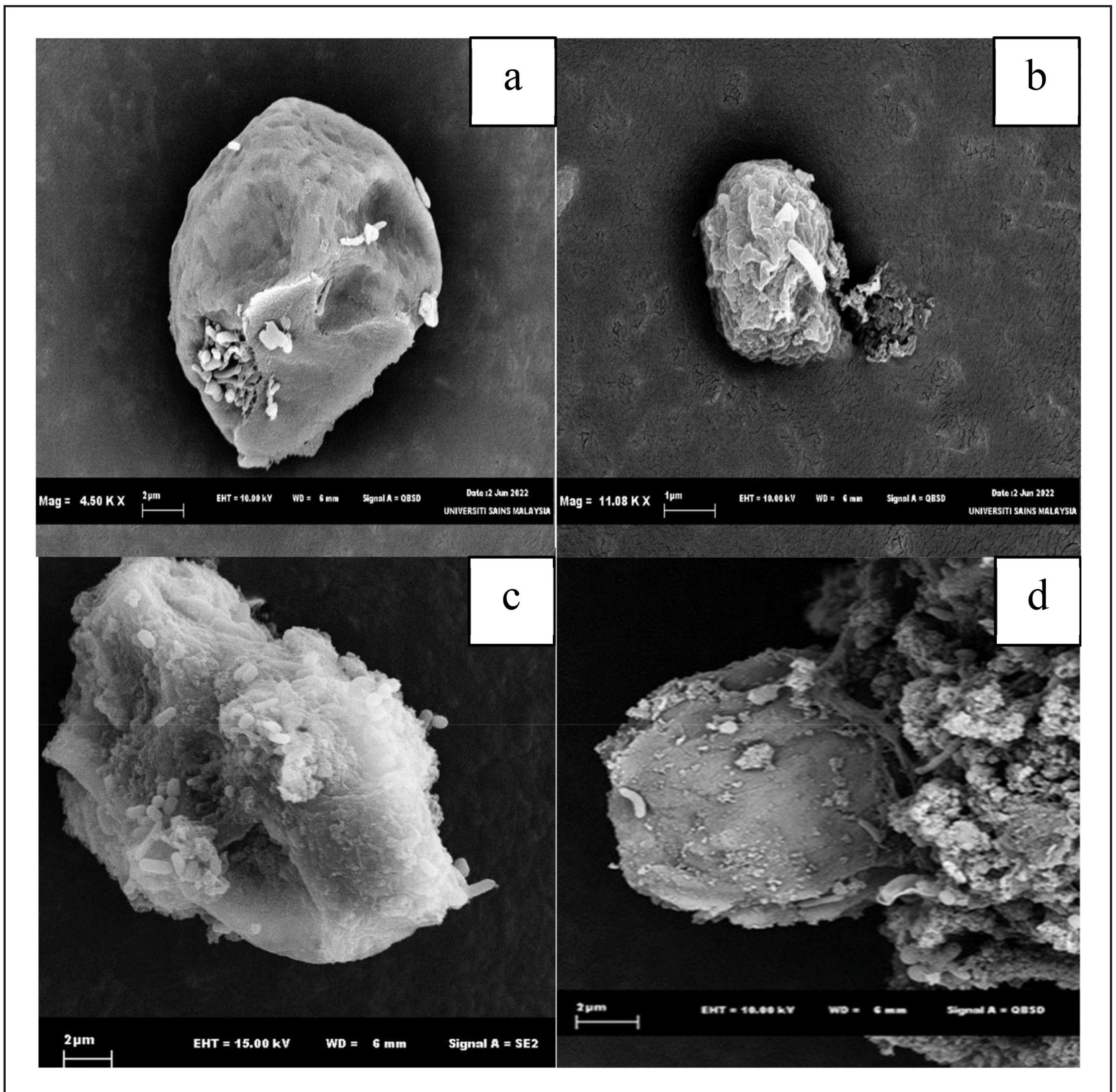

Figure 2. Scanning electron microscopy (SEM) of *Blastocystis* in wild animals

a) The cell without the fibrillar layer. Smooth surface with a small indention on the surface in pheasant.
b) Granular form of the cell. Extremely folded of a rough surface in peafowl.
c) Irregular shape with projections and deep indentations. The surface coat of the *Blastocystis* appeared to be attached to bacteria. The surface seems with a deep indention in peafowl.
d) Round shape with smooth surface, attached with fibrillar layer and bacteria in golden-headed lion tamarin.

1999). The role of bacteria in the surface structure of *Blastocystis* is not well understood. However, in the year 1989, Dunn and his colleagues made a significant observation that specific bacteria cells attached to *Blastocystis* displayed noticeable modifications within their cytoplasm, resulting in a decrease in electron density within these bacterial cells. This occurrence prompted suspicions regarding a potential adverse influence originating from *Blastocystis*' surface coat on the associated bacteria. It is noteworthy that the possibility of phagocytosis was dismissed due to the lack of any supportive evidence indicating the presence of bacteria within *Blastocystis* cells. This conclusion not only finds resonance in the research conducted by Cassidy *et al*. (1994) but also persists to the present day. According to Yason & Tan (2018), the surface coat of *Blastocystis* is associated with potentially pathogenic *Blastocystis* subtypes. There is a hypothesis that the surface coat protects the organism from the host's innate immune response and contributes to greater adhesion during colonization (Yason & Tan, 2018).

Subtyping of *Blastocystis*

The *Blastocystis* subtypes identified both in all the pheasants and peafowl samples were confirmed to be ST6 through BLAST calls in the NCBI database. However, the specific subtype of *Blastocystis* found in lion-headed tamarins remains unknown.

CONCLUSION

This research has shed light on the cell surface characteristics of *Blastocystis* in peafowl, pheasant, and lion-headed tamarin, making it the first study of its kind worldwide. The findings revealed variations in cell size and shape within and between species, indicating the influence of the host's gut characteristics on *Blastocystis* morphology. Scanning electron microscopy (SEM) analysis provided insights into the surface structures of *Blastocystis* from different hosts, showing variations in surface morphology. The presence of bacteria associated with the surface coat and the role of the coat in trapping and degrading bacteria for nutrition were observed. The rough surface structure of *Blastocystis* isolates in certain hosts may be linked to pathogenicity and symptomatic appearance. Understanding the morphological and surface ultrastructure features of *Blastocystis* in different host species contributes to our knowledge of this parasite and its potential impact on host health. Further research is needed to explore the exact role and composition of the surface coat and its association with pathogenicity in different *Blastocystis* subtypes.

ACKNOWLEDGEMENT

We would like to express our gratitude to the zoological gardens in Perak for granting permission for sample collection. We extend my appreciation to the Department of Wildlife and National Parks, Peninsular Malaysia for their support. Additionally, we would like to acknowledge the Electron Microscopy Unit for their invaluable assistance and guidance in examining the cells.

Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical Approval

Ethical approval was obtained for this study from the Universiti Sains Malaysia Institutional Animal Care and Use Committee (IACUC) (USM/IACUC/2021/(EXP) (1159). The study was conducted in accordance with the guidelines provided by the Malaysian Wildlife Department.

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