PREVALENCE, MORPHOLOGICAL CHARACTERIZATION, AND SUBTYPE DISTRIBUTION OF *Blastocystis* sp. FROM SELECTED CAPTIVE WILD ANIMALS IN PERAK, MALAYSIA

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by

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LIST OF SYMBOLS

*	Asterisk
bp	Base pair
χ2	Chi-squared test
º	Degree
DNA	Deoxyribonucleic acid
=	Equal to
g	Gram
km	Kilometer
µl	Microliter
µm	Micrometer
mA	Milli Amperes
mg	Milligrams
M	Molar
nm	Nanometer
%	Percentage
±	Plus or minus

*	Asterisk
bp	Base pair
χ2	Chi-squared test
º	Degree
DNA	Deoxyribonucleic acid
=	Equal to
g	Gram

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BLAST	Basic Local Search Alignment Tool
BIC	Bayesian Information Criterion
BMPT	Bukit Merah Petting Zoo
C	Celsius
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
GIS	Geographic Information System
GLPT	Gunung Lang Petting Zoo
IL	Illinois
Inc.	Incorporated
IACUC	Institutional Animal Care and Use Committee
IBS	Irritable Bowel Syndrome
$MgCl^2$	Magnesium Chloride
ML	Maximum Likelihood
MLST	Multilocus Sequence Typing
NCBI	National Centre for Biotechnology Information
NJ	Neighbour Joining
NGS	Next Generation Sequencing
NHP	Non-Human Primate
OD	Odd Ratio
OUI	Orang Utan Island

PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
P	Probability
QUEST	Quest International University
RAPD	Random Amplified Polymorphic DNA
qPCR	Real-time Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution per minute
rpm	Revolutions per minute
RNA	Ribonucleic acid
SEM	Scanning electron microscope
SEM	Scanning electron microscopy
sec	Second
STS	Sequence Tagged Site
SSU rRNA	Small sub-unit ribosomal ribonucleic acid
sp.	Species
SPSS	Statistical Package for Social Science
ST	Subtype
SWCC	Sungkai Wildlife Conservation Centre
T92	Tamura 3-Parameter

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PREVALENS, PENCIRIAN MORFOLOGI DAN TABURAN SUBJENIS BAGI *BLASTOCYSTIS* SP. DARIPADA HAIWAN LIAR KURUNGAN TERPILIH DI PERAK, MALAYSIA

ABSTRAK

Blastocystis, suatu eukariot simbion yang merupakan patogen berpotensi yang menjangkiti manusia dan haiwan secara global. Malaysia, dengan kepelbagaian spesies haiwan liar di zoo dan taman haiwan liar mewujudkan tetapan tersendiri bagi kajian *Blastocystis*. Haiwan-haiwan ini yang mempunyai hubungan rapat bersama manusia, memudahkan potensi penularan parasit antara haiwan dan manusia. Ini menunjukkan sebab yang munasabah untuk mengkaji *Blastocystis*. Meskipun terbukti risiko zoonotik, kajian yang minima mengenai prevalens dan subjenis *Blastocystis* dalam populasi hidupan liar di Malaysia. Mengenali jurang pengetahuan ini, kajian ini adalah bertujuan untuk meneliti prevalens, pencirian morfologi, dan taburan subjenis *Blastocystis* dalam populasi haiwan liar dalam kurungan di pelbagai lokasi di Perak, Malaysia. Sejumlah 352 sampel tinja haiwan liar dalam kurungan dari pelbagai kumpulan taksonomi menjalani pemeriksaan dengan menggunakan pengkulturan in-vitro dan kaedah kod bar DNA. Keutamaan pemilihan spesies adalah terdiri daripada spesies yang terancam yang menyumbang kepada data awal mengenai prevalen jangkitan *Blastocystis* dalam kalangan pelbagai haiwan liar kurungan. Keseluruhan prevalens jangkitan *Blastocystis* di kalangan haiwan yang dikaji ialah 28.8%. Unggas, mempunyai prevalens tertinggi dengan 57.7%, menekankan kepentingannya sebagai takungan berpotensi. Karnivor mempunyai kadar prevalens yang kedua tertinggi iaitu 37.1% diikuti dengan 26.7% dalam tikus, primat bukan manusia (16.7%), manakala reptilia, Marsupial, Artiodactyla, dan Perissodactyla masing-masing dengan kadar

prevalens yang berbeza-beza (10.0 - 35.3%). Walau bagaimanapun, Proboscidea menunjukkan kadar jangkitan sifar, menunjukkan sama ada kerentanan rendah atau saiz sampel yang sedikit untuk mengesan kewujudan *Blastocystis* dalam gajah. Faktor-faktor seperti kekerapan sentuhan dalam kalangan haiwan, tempat terkurung, dan kaedah minuman dikenal pasti sebagai pengaruh penting kepada dinamik penularan. Haiwan dengan keadaan najis cirit-birit menunjukkan risiko jangkitan *Blastocystis* yang tinggi mencadangkan potensi hubungan potensi antara gangguan gastrousus dan kerentanan kepada parasit (p=0.006). Tambahan pula, kajian ini mendapati kaitan yang signifikan antara meminum air sungai dan jangkitan *Blastocystis* dalam spesies burung yang menekankan potensi peranan penularan bawaan air dalam persekitaran zoo (p=0.040). Sementara itu, pemerhatian melalui mikroskop cahaya mendedahkan pelbagai bentuk vakuol dan granul bagi *Blastocystis* dalam haiwan liar dengan pencilan daripada spesies Avian kelihatan saiz lebih besar (5.32 - 17.09 μm) berbanding kumpulan haiwan lain. Pengimbas mikroskop elektron memaparkan struktur permukaan yang pelbagai termasuk rupa seperti jejaring, lekukan dalam dan lekatan pada bakteria. Terutamanya, pencilan tertentu yang mempamerkan struktur permukaan kasar yang dikaitkan dengan patogenik dan jangkitan simptomatik, termasuk cirit-birit pada haiwan perumah. Analisis subjenis mendedahkan 13 subjenis *Blastocystis* (ST1-ST9, ST14, ST27, ST41 dan NMA) di mana spesies Avian menunjukkan prevalens ST27 yang ketara mencadangkan perkaitan unik dalam kumpulan ini manakala ST41 dilaporkan dalam burung budgerigar, menambahkan dapatan sebelum ini dengan kewujudan ST41 yang dilaporkan pada manusia di Colombia. Penemuan *Blastocystis* dalam perumah baharu, termasuk singa Afrika (ST3), harimau (ST4), memerang berkuku kecil (ST7), walabi (ST1), dan tapir (ST8), merupakan suatu sumbangan penting. Tambahan pula, analisis filogenetik menyokong

pertalian evolusi antara jujukan *Blastocystis* yang memperluaskan pemahaman tentang kepelbagaian genetik parasit ini. Penemuan ini menyumbang kepada pemahaman terkini tentang kepelbagaian genetik *Blastocystis*, julat perumah, takungan haiwan, dan potensi zoonosis dalam kurungan haiwan liar di Malaysia. Kajian meluas diperlukan untuk mengukuhkan pemahaman kita tentang epidemiologi *Blastocystis* dan impaknya terhadap kesihatan haiwan dan manusia.

PREVALENCE, MORPHOLOGICAL CHARACTERIZATION, AND SUBTYPE DISTRIBUTION OF *BLASTOCYSTIS* SP. FROM SELECTED CAPTIVE WILD ANIMALS IN PERAK, MALAYSIA

ABSTRACT

Blastocystis, a eukaryotic gastrointestinal symbiont is a potential pathogen affecting both animals and humans globally. Malaysia, with its diverse captive wild animal species in zoos and wildlife parks offers a distinctive setting for studying *Blastocystis*. These animals that have close human contact, facilitating potential parasite transmission between animals and humans. Despite the evident of zoonotic risk, less studies were done on *Blastocystis* prevalence and subtype distribution in Malaysian wildlife. Recognizing this knowledge gap, this study aims to examine the prevalence, morphological characteristics, and subtype distribution of *Blastocystis* in captive animals at various locations in Perak, Malaysia. A total of 352 animals faecal samples from diverse taxonomic groups underwent examination using *in vitro* cultivation and DNA barcoding methods. The selected species predominantly comprised endangered species contributing initial recorded data on *Blastocystis* infection prevalence in a diverse array of captive wild animals. The overall *Blastocystis* infection prevalence among studied animals was 28.8%. Aves had a high prevalence of 57.7%, emphasizing their significance as potential reservoirs. Carnivora had the second highest prevalence rate of 37.1% followed by 26.7% in rodentia, non-human primates (16.7%), whereas reptiles, Marsupials, Artiodactyla, and Perissodactyla each with varying prevalence rates (10.0 - 35.3%). However, Proboscidea showed zero infection rates, indicating either low susceptibility or insufficient sample size to detect occurrence of *Blastocystis* in elephants. Factors such

as contact frequency among animals, confined environments, and the method of drinking water were identified as the significant influencers of transmission dynamics. Animals with diarrheal faecal condition exhibited a higher risk of *Blastocystis* infection suggesting potential links between gastrointestinal disturbances and susceptibility to the parasite (p=0.006). Furthermore, the study found a significant association between drinking river water and *Blastocystis* infection in avian species, emphasizing the potential role of waterborne transmission in the zoo environment (p=0.040). Meanwhile, observations through light microscopy revealed numerous vacuolar and granular forms of *Blastocystis* in wild animals with isolates from avian species appearing larger in size (5.32-17.09 μm) than those from other animal groups. Scanning electron microscopy displayed diverse surface structures including mesh-like appearances, deep indentations, and attachments to bacteria. Notably, certain isolates exhibited a rough surface structure associated with pathogenicity and symptomatic infection, including diarrhea in host animals. The subtype analysis revealed 13 *Blastocystis* subtypes (ST1-ST9, ST14, ST27, ST41 and NMA) in which Avian species displayed a notable prevalence of ST27 suggesting a unique association within this group whereas ST41 was reported in budgerigars, expanding on the previously reported on the occurrence of ST41 found in humans in Colombia. The discovery of *Blastocystis* in new hosts, including African lions (ST3), tigers (ST4), small-clawed otters (ST7), wallabies (ST1), and tapirs (ST8), constitutes significant contributions. Moreover, the phylogenetic analysis supported evolutionary connections among *Blastocystis* sequences expanding the understanding of the genetic diversity of this parasite. These findings contribute to the current understanding of *Blastocystis* genetic diversity, host range, animal reservoirs, and zoonotic potential in captive wild animals in Malaysia. Further investigations are warranted to deepen our

understanding of *Blastocystis* epidemiology and its impact on animal and human health.

CHAPTER 1

INTRODUCTION

1.1 Research background

Blastocystis, a single-cell parasite belonging to Stramenopiles group of eukaryotes is a common inhabitant of the gastrointestinal tract of both animals and humans. The prevalence rates vary depending on the population studied (Parija & Jeremiah, 2013). It was discovered over a century ago and *Blastocystis* has been the subject of intense research due to its potential to cause serious public health issues to the host. Understanding this parasite is crucial, as it poses ongoing human and animal health risks, impacting millions globally. The persistent research reflects the urgency in uncovering its effects, controlling its spread, and protecting public health (Petrášová *et al*., 2011). As an anaerobic organism, *Blastocystis* can survive without oxygen which may contribute to the ability to thrive in the intestinal environment (Parija & Jeremiah, 2013; Stensvold & Clark, 2016).

Blastocystis is known for its unique morphology which has been described as vacuolar, granular, amoeboid, cyst, avacuolar, and multivacuolar (Tan, 2008; Parija & Jeremiah, 2013; Ahmed & Karanis, 2019). The vacuolar and granular forms are the most observed morphologies. However, the amoeboid form is less common but considered to be more pathogenic (Moosavi *et al*., 2012). Meanwhile, the cyst form is typically more infectious than the other form and can be transmitted through faecal-oral transmission (Stensvold, 2013b; Ahmed & Karanis, 2019). Moreover, the avacuolar and multivacuolar forms are less common and normally overlooked than the other forms. *Blastocystis* may have different pathogenicity and virulence characteristics (Ahmed & Karanis, 2019). The ability of this protozoan to change

between different morphologies may contribute to its pathogenesis and the ability to survive in different environments (Ajjampur & Tan, 2016). Therefore, the morphological features of *Blastocystis* can be highly variable making it difficult to differentiate between different subtypes or identify as new subtypes (Stensvold *et al*., 2007).

Hence, the genetic polymorphism has emerged as more reliable and accurate method for *Blastocystis* classification and subtyping (Stensvold, 2013b; Yoshikawa & Iwamasa, 2016; Maloney & Santin, 2021). A genetic marker based on differences in the small subunit ribosomal RNA (SSU rRNA) was used to differentiate between different subtypes and provide insight into the evolutionary relationship between *Blastocystis* isolates (Stensvold, 2013b). According to Yoshikawa & Iwamasa (2016), *Blastocystis* organisms exhibit significant genetic variability which refers to the differences in DNA sequence and gene expression among different subtypes (Puthia *et al*., 2008; Wang *et al*., 2013; Maloney & Santin, 2021). However, genetically similar isolates can also be identified from different host species. As a result, assigning species names to *Blastocystis* isolates based on their host origin is not practical and can be misleading. Therefore, to address this issue, Stensvold (2013b) proposed a standardized naming system based on genetic variation in *Blastocystis*. The system uses a combination of letters and numbers to designate the different subtypes with the letter indicates the subtype (ST) and the number (*n*) indicates the genetic subtype within that subtype. To date, the SSU rRNA gene sequence used in taxonomic analysis has revealed that the genus *Blastocystis* has 41 different subtypes (STs) (Valença Barbosa *et al*., 2017). To date, ST1 to ST9 and ST12 have been detected and documented in humans with variable levels of prevalence in which ST1 to ST4 accounted with 95% prevalence in humans as the most common *Blastocystis* subtypes

(Valença Barbosa *et al.*, 2017). As in animals, rodents, primates, and hoofed animal have all been found to harbor *Blastocystis* ST1 to ST4 (Li *et al*., 2019). Moreover, other subtypes such as ST5 to ST9 and ST12 were more frequently found in animals than in humans as these animals are the reservoirs for this protozoan (Gabrielli *et al*., 2021).

The prevalence of *Blastocystis* infection varies greatly across different populations. The infection rate ranged between 30% to 76% in many impoverished nations but only ranged from 0.5% to 30% in European nations, even though *Blastocystis* infections occur everywhere (Clark *et al*., 2013; Wawrzyniak *et al*., 2013). Without a doubt, the high prevalence of infections observed in underdeveloped countries has been linked to inadequate sanitation facilities, the high prevalence of filthy living circumstances, and habits that encourage the consumption of contaminated food and water (Parija & Jeremiah, 2013). Also, it has been demonstrated that visiting tropical countries significantly raises the risk of getting *Blastocystis* (Javanmard *et al*., 2019). Apart from that, contact with animals has been identified as a possible factor that speeds up the spread of *Blastocystis* infection (Fahim *et al*., 2021).

It is difficult to make a direct comparison of the infection rates of *Blastocystis* in animals and humans due to differences in the population studies diagnostic methods and other factors (Poirier *et al*., 2012; Cian *et al*., 2017). However, it is generally believed that *Blastocystis* infection is more common in animals than in humans, particularly in certain animal populations (Betts *et al*., 2020). Additionally, infection in wild animals has been extensively studied worldwide with a wide range of wild and captive species found to be affected by this protozoan. *Blastocystis* infection in non-

human primates (NHPs) has been reported by Li *et al*. (2020); Parkar *et al*. (2010), in mammals such as Artiodactyla by Betts *et al*. (2020), Chen *et al*. (2021) and Zhao *et al*. (2017), in Proboscidea by Li *et al*. (2019) and Roberts *et al*. (2013), and in Perissodactyla by Zhao *et al*. (2017) and Cian *et al*. (2017). Besides, it has also been found in birds such as ostriches (Chandrasekaran *et al*., 2014; Zhao *et al*., 2017), pheasants (Maloney & Santin, 2021), and peafowl (Deng *et al*., 2019; Maloney & Santin, 2021). Moreover, *Blastocystis* has also been detected in reptiles (Cian *et al*., 2017; AbuOdeh *et al*., 2019), Marsupials (Zhao *et al*., 2017), and rodents (Valença-Barbosa *et al*., 2019; Li *et al*., 2020b; Chen *et al*., 2021).

The transmission of *Blastocystis* occurs through faecal-oral transmission (Ramírez *et al*., 2014). This protozoan is transmitted by the cysts form in the faeces of infected hosts via contaminated food, water, or surfaces. Direct contact with infected individuals or animals can also result in the transmission of this parasite (Cian *et al*., 2017). There is evident showing that overlapping of sequence subtype (STs) and identical SSU rRNA between humans and animals reveals the possibility of cross-transmission between these hosts (Yoshikawa *et al*., 2009; Nagel *et al*., 2012; Alfellani *et al.,* 2013). Farmers, veterinarians, animal handlers, and researchers who work with animals are at higher risk of contracting *Blastocystis* infections as they may encounter animal faeces or be exposed to contaminated food and water sources (Rauff-Adedotun *et al*., 2022).

There are several studies on *Blastocystis* in Malaysia in which most of these studies concentrated on the genetic diversity, morphology, and classification of *Blastocystis* in humans (Vennila *et al*., 1999; Tan & Suresh, 2006b; Chandramathi *et al*., 2010; Anuar *et al*., 2013; Kumarasamy *et al*., 2014; Ragavan *et al*., 2015;

Noradilah *et al*., 2017; Kodio *et al*., 2019; Ho *et al*., 2022). Meanwhile, freshwater fish, chickens, dairy goats, and goat meat were all recently studied for *Blastocystis* in Malaysia (Rauff-Adedotun *et al*., 2022). Studies on *Blastocystis* in livestock animals have been extensively studied as compared to the wild or captive wild animals in Malaysia with only a few groups of captive wild animals were studied, namely deer (Hemalatha *et al*., 2014; Mohd Zain *et al*., 2017; Mohammad *et al*., 2018a), gaur (Hemalatha *et al*., 2014), ostrich (Chandrasekaran *et al*., 2014), giant bill crow (Yong *et al*., 2008), non-human primates (Hemalatha *et al*., 2014; Adrus *et al*., 2019), mouse deer, and the water monitor lizard (Hemalatha *et al*., 2014; Adrus *et al*., 2019).

1.2 Justification of study

Zoological gardens in Malaysia play significant roles in wildlife conservation, education, and research by providing shelter for diverse animal species and contributing to agriculture and local well-being. Gastrointestinal parasites threaten wild animals worldwide, but Southeast Asia has been a less studied region (Yong *et al*., 2008; Mohd Zain *et al*., 2017; Mohammad *et al*., 2018a; Adrus *et al*., 2019). Despite being discovered over 100 years ago, the pathogenic potential of *Blastocystis* remains uncertain, hindering elimination strategies (Abe *et al*., 2002; Andersen & Stensvold, 2016). *Blastocystis* exhibits high prevalence in various animal populations globally (Abe *et al*., 2002; Rauff-Adedotun *et al*., 2020; Sanggari *et al*., 2022). Understanding *Blastocystis* prevalence and subtype distribution allows the identification of vulnerable wildlife populations for targeted conservation measures (Abe *et al*., 2002; Andersen & Stensvold, 2016). This knowledge is crucial for maintaining animal population health and sustainability where *Blastocystis* is prevalent. Besides, infectious diseases from wild animals threaten human health,

putting animal handlers and zoological garden visitors at higher infection risk (Cian *et al*., 2017; Hublin *et al*., 2021). *Blastocystis* transmission occurs faecal-orally, enabling animal-human transmission (Hublin *et al*., 2021). Investigating *Blastocystis* epidemiology in animals, especially wildlife, provides insights into zoonotic subtypes and transmission potential, wildlife health impacts, and interventions to reduce wildlife-human transmission risk. This holistic approach safeguards both wildlife and human health. In addition, examining *Blastocystis* morphology reveals attachment, invasion, and colonization mechanisms, furthering understanding of pathogenic potential and host-parasite relationships (Tan, 2004). Though previous ultrastructural studies focused on isolates from certain hosts (Teow *et al*., 1991; Cassidy *et al*., 1994; Stenzel & Boreham, 1996; Singh *et al*., 1996; Yoshikawa *et al*., 2004; Chandrasekaran *et al*., 2014), comprehensive *Blastocystis* studies across many wild hosts are still lacking.

1.3 Research objectives

This study embarks on the following objectives:

1. To determine the prevalence of *Blastocystis* sp. infection in the following captive wild animals from several zoological gardens in Perak, Malaysia.
    a) Aves
    b) non-human primates (NHPs)
    c) Artiodactyla
    d) Reptilia
    e) Carnivora
    f) Rodentia
    g) Perissodactyla
    h) Proboscidea
    i) Marsupialia

2. To examine the morphological characteristics of *Blastocystis* sp. of several selected *Blastocystis* positive isolates from various captive wild animal groups.
    a) General morphology based on the staining characteristics using Giemsa stain.
    b) Ultrastructural descriptions using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

3. To determine the subtype characteristics of *Blastocystis* sp. from captive wild animals from several zoological gardens in Perak, Malaysia using the DNA barcoding method.
    a) Subtype diversity and frequency of *Blastocystis* sp.
    b) Subtype distribution of *Blastocystis* sp.

4. To investigate the evolutionary relationships of *Blastocystis* positive isolates from various captive wild animal groups.

CHAPTER 2

LITERATURE REVIEW

2.1 Classification of *Blastocystis* sp.

2.1.1 History

Blastocystis was first describe by Alexieff in 1911 as *Blastocystis enterocola* (Zierdt, 1991). Later Brumpt (1912), renamed this single-celled organism as *Blastocystis hominis* and classified it under the yeast family. Over the last 100 years, *Blastocystis* has been mistakenly classified as various organisms not only as yeasts, flagellate cysts, amoebas, and sporozoans. Later, in 1976, Zierdt identified this organism as protozoan due to its protozoan feature (Zierdt & Tan, 1976) (Table 2.1). The accurate taxonomy of *Blastocystis* was then accomplished by Silberman *et al*. (1996) which was based on molecular analysis of the small subunit ribosomal RNA (SSU rRNA). The organisms were not monophyletic to yeast, sarcodines, sporozoans, or fungi. This led to a later reclassification of it within the Stramenopiles. The accuracy of the classification has been verified through molecular analysis of other *Blastocystis* genes (Arisue *et al*., 2002). Moreover, a considerable degree of genetic diversity with a maximum variation of 7% in amino acid sequences has been observed within the 18S DNA coding rDNA subtypes (Noël *et al*., 2005).

Table 2.1 Reclassification of *Blastocystis* from fungi to protozoa (Zierdt, 1991).

Characteristic	**Yeast**	*Blastocystis hominis*
Strict anaerobe	No	Yes
Grow on bacteriological or fungal media	No	Yes
Grow on solid media	Yes	No
Growth depends on the presence of bacteria	No	Yes
Ingest bacteria and other particulate matter	No	Yes
2-3 days lifespan at 22° C	No	Yes
Die overnight at 4°C	No	Yes
Cell growth stop at below 33 °C, and die at 30°C	No	Yes
Optimal growth at 37°C	No	Yes
Natural pH	No	Yes
pH 55.5	No	Yes
Resistant to 400µg of amphotericin per ml	No	Yes
Susceptible to anti-protozoal drugs used for intestinal infections	No	Yes
Cell wall	No	No
Reproduction mode-endodyogeny, schizogony, binary fission, and plasmotomy	No	Yes
Bacterial endosymbiont	No	Yes
Budding	Yes	No
Feeding pseudopodium	No	Yes
Locomotion pseudopodium	No	Yes
Limiting membrane with macropinocytotic vesicles	No	Yes
Membrane bound compartment	No	Yes
Vacuole	No	Yes
Mitochondria-cause the saccate cristae entered a resting state	No	Yes

Scientific classification of *Blastocystis* sp.

Kingdom: Stramenopiles/Chromista

Infrakingdom: Heterokonta

Subkingdom: Chromobiota

Infrakingdom: Heterokonta

Subphylum: Opalinata

Class: Blastocystea

Order: Blastocystida

Family: Blastocystidae

Genus: *Blastocystis*

2.1.2 Specification

Previously, the isolates of *Blastocystis* that have been found in humans are known as *B. hominis*. However, *Blastocystis* isolated from animals is thought to be a different species of *Blastocystis*. Special names have been given to *Blastocystis* isolated from some animals including rats and reptiles. The karyotypic pattern of *Blastocystis* in rats is obviously different from that of the isolates of *B. hominis* from humans. Due to its unique karyotypic pattern, *Blastocystis* was subsequently proposed as *Blastocystis ratti* by Chen *et al*. (1997). Additionally, several studies have demonstrated that some reptilian isolates differ from hu

man isolates and homeothermic animals including sea snakes, reticulated python, rhino iguanas, and red-footed tortoises in terms of growth features, karyotypic patterns, and chromosomal patterns. The proposed species names for *Blastocystis* in these animals are *B. geocheloni* sp. for the red-footed tortoise, *B. cycluri* sp. for the rhino iguana, and *B. pythoni* sp. for the reticulated python (Singh *et al*., 1996).

Moreover, several studies on the morphological and host variations among *Blastocystis* origins have been made. These consist of *B. galli* from chickens (Lee & Stenzel, 1999), *B. aseri* from geese and *B. anatis* from ducks (Belova, 1991; Belova, 1992). As *Blastocystis* has a variable morphology, the morphological characteristics are insufficient for species classification. However, Tanizaki *et al*. (2005) disagree to these standards of specifying *Blastocystis* isolates from birds particularly quails, geese, and chickens. Due to the significant genetic diversity found in the host as determined by investigations of the small-subunit ribosomal RNA gene (SSU-rRNA), it was discovered that the host-specific name was unclear. According to Stensvold *et al.* (2007b), the classification of mammalian and avian host isolates should be based on subtypes. Since not all the mammalian and avian clades can be classified as *Blastocystis hominis* due to genetic differences among them, *Blastocystis* sp. was used instead of *Blastocystis hominis*. As a result, assigning species names to *Blastocystis* isolates based on their host origin is not practical and can be misleading. Therefore, to address this issue, Stensvold (2013b) proposed a standardized naming system based on genetic variation in *Blastocystis*. The system uses a combination of letters and numbers to designate the different subtypes with the letter indicates the subtype (ST) and the number (*n*) indicates the genetic subtype within that subtype.

2.2 Biology of *Blastocystis* sp.

2.2.1 Morphology

Blastocystis is a unicellular parasite with a diameter ranging from 5 to 30 µm that can exist in different morphological form. The morphology of *Blastocystis* has been described in several studies based on light and electron microscopy (Boreham & Stenzel, 1993; Stenzel & Boreham, 1996). Boreham & Stenzel, (1993) described *Blastocystis* as polymorphic organism that exist in different morphological forms, including vacuolar, granular, ameboid, avacuolar, multivacuolar, and cyst forms. It was also documented that the vacuolar, granular, ameboid and cyst forms of *Blastocystis* are the most observed forms as compared to the avacuolar and multivacuolar forms.

Earlier studies described *Blastocystis* as mostly spherical cells with a large central body, a thin peripheral rim of cytoplasm, multiple nuclei, cytochrome free mitochondria, and a thick mucilaginous coat surrounding the organism, and each form shows distinct morphological features (Parija & Jeremiah, 2013). In fact, the appearance of *Blastocystis* depends on the environmental conditions, and ultrastructural studies demonstarted that a continuum of morphological form of *Blastocystis* exist (Stenzel & Boreham, 1996). Further studies have shown that physical factors, such as osmotic changes, the presence of certain drugs, and metabolic status can affect the morphology of *Blastocystis in vivo* and *in vitro* (Boreham & Stenzel, 1993; Stenzel & Boreham, 1996).

Meanwhile, several studies have shown that granular and vacuolar forms lose staining strength after a longer period before microscopic identification. A fresh culture used for characteristic identification is consistently stained as compared to

organisms dyed after a delay. Vdovenko & Williams (2000) also revealed degenerative alterations and fixation artifacts in the vacuolar and granular forms. Physical factors such as metabolic state, the presence of certain medications, and osmotic changes able to affect the morphology of *Blastocystis* cause modification to its shape (Boreham & Stenzel, 1993).

2.2.1(a) Cystic form

Blastocystis exists in different morphological forms in which the cyst form being the infective stage and having a spherical or ellipsoidal shape with a diameter of 5-15 µm. It contains compressed cytoplasm with mitochondria and a small vacuole, typically has two to four nuclei, and is surrounded by a multilayered wall that provides protection against environmental stresses (Parija & Jeremiah, 2013) (Figure 2.1). The cyst form of *Blastocystis* has been frequently reported in human faecal samples and is known to be resistant to the acidic pH of the stomach (Stenzel & Boreham, 1991).

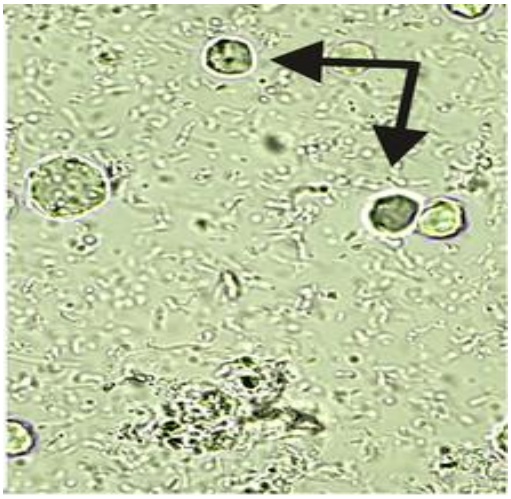

Figure 2.1 Cyst forms of *Blastocystis* indicated in black arrow (Boutahar *et al*., 2023)

Additionally, reports suggest that the cyst form also found in animal faecal samples (Moe *et al*., 1999) and water samples (Boreham & Stenzel, 1993) indicating a potential source of infection. The presence of the cyst form of *Blastocystis* has also been associated with chronic infections and its presence can indicate prolonged colonization (Yoshikawa *et al*., 2004b). The form described in Yoshikawa *et al*. (2004b) can last for two months at a temperature of 4°C and is durable, but it can be damaged by drying, high temperatures, and freezing. The survival rate of *Blastocystis* cysts in different condition is shown in Table 2.2.

Table 2.2 Survival rate of *Blastocystis* cysts in different condition.

Condition	**Survival Time**			
	Moe *et al*. (1996)	Tan (2008)	Parija & Jeremiah (2013)	Adao & Rivera (2018)
40-50°C	12 hours	NA	NA	NA
25°C water	NA	19 days	19 days	19 days
4°C water	14-28 days	NA	NA	NA
Room Temperature	24 hours	1 months	1 months	1 months
4°C air	3 days	2 months	2 months	2 months
37°C air	<2 weeks	NA	NA	NA

NA- Not Applicable

2.2.1(b) Vacuolar form

The vacuolar form of *Blastocystis* is a commonly observed morphological form in various hosts, including humans, mammals, reptiles, birds, and insects (Duda *et al*., 1998; Lee & Stenzel, 1999; Stensvold *et al*., 2007; Wawrzyniak *et al*., 2015). The vacuolar form of *Blastocystis* is characterized by a large central body or central vacuole, surrounded by a thin peripheral rim of cytoplasm, multiple nuclei with a cap of condensed chromatin, cytochrome-free mitochondria, and a thick mucilaginous coat

surrounding the organism (Boreham & Stenzel, 1993; Tan, 2008) (Figure 2.2). The size of the vacuolar form ranges from 2 µm to more than 200 µm in diameter, with the average diameter usually between 4 and 15 µm (Stenzel & Boreham, 1996). This form has been suggested to play a role in the pathogenesis of *Blastocystis* infections. Ajjampur and Tan (2016) reported that the vacuolar form is more prevalent in symptomatic patients than in asymptomatic ones. Furthermore, the vacuolar form may be involved in the formation of biofilms, leading to persistent infections as described by Stensvold & Clark (2016). Besides, study show that the vacuolar form of *Blastocystis* exhibits genetic heterogeneity, with multiple subtypes identified (Arisue *et al*., 2002). Meanwhile, Yoshikawa *et al*. (1995) reported the presence of multiple genotypes in the vacuolar form of *Blastocystis* indicating the existence of diverse strains within this morphological form.

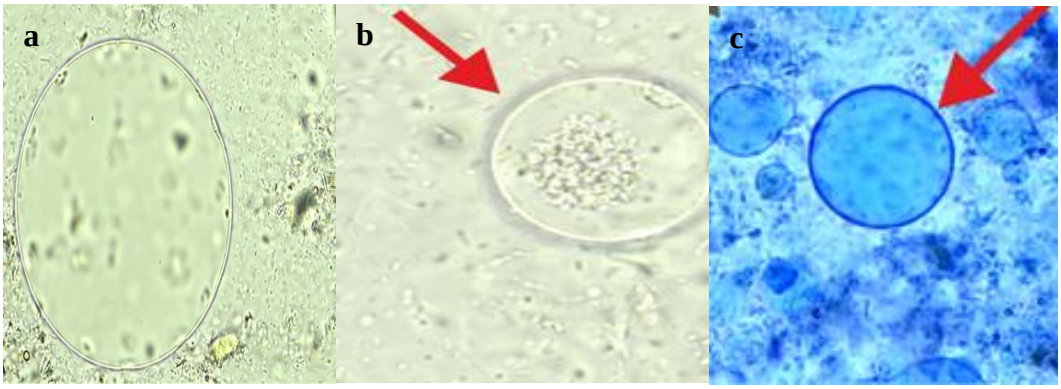

Figure 2.2 Microscopic visualization of vacuolar forms under 400x magnification, showcasing a range of sizes (a-b) without stain and (c) stained with methylene blue (Boutahar *et al.*, 2023).

2.2.1 (c) Granular form

The granular form of *Blastocystis* contains numerous granules within the central vacuole or the thin band of peripheral cytoplasm of the organism (Zierdt, 1973; Zierdt & Williams, 1974) (Figure 2.3). The granules found in the granular form have been classified into three types, namely metabolic, reproductive, and lipid granules (Dunn *et al*., 1989). The granular form has been reported to range in size from 15 to 80 µm, with the largest being 80 µm in diameter (Zierdt, 1973). The granules found in the granular form have been reported to be myelin-like inclusions, small vesicles, crystalline granules, and lipid droplets (Dunn *et al*., 1989).

In terms of morphology, the granular form is similar to the vacuolar form with a thin peripheral band of cytoplasm surrounding a large central vacuole with granules, which are also sometimes observed in its cytoplasm (Rojas-Velázquez *et al*., 2022). The granular form occurred from the vacuolar form in certain culture conditions such as the increase of serum concentration in the culture medium, axenization of the culture, or the addition of certain antibiotics (Tan & Zierdt, 1973; Dunn *et al*., 1989; Stenzel & Boreham, 1996). Reproductive granules have been suggested to play a role in schizogony-like division to produce viable progenies of *Blastocystis* sp. (Zierdt *et al*., 1967; Zierdt, 1991). However, this suggestion has been disputed by other researchers due to a lack of evidence, and the only plausible mode of reproduction continues to be binary fission (Suresh *et al*., 1994).

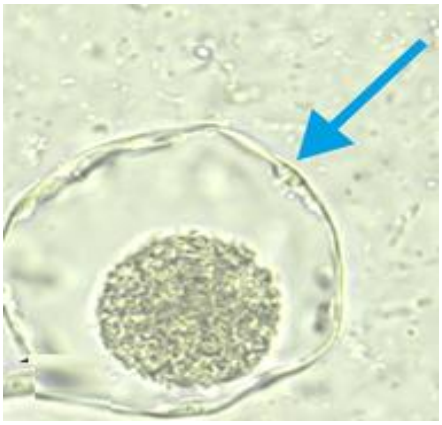

Figure 2.3 Granular form of *Blastocystis* under 400x magnification in light microscopy (Boutahar *et al*., 2023).

2.2.1(d) Amoeboid form

The amoeboid form of *Blastocystis* was described by Zierdt (1973). This form was characterized by the presence of pseudopodia that allow the organism to move and capture food. This form is considered the most motile and invasive of *Blastocystis*, with irregular shape and a size range from 10-15 μm in diameter (Tan & Zierdt, 1973) (Figure 2.4). Several studies have suggested that the amoeboid form of *Blastocystis* is associated with pathogenicity in which Singh *et al*. (1995) reported that the amoeboid form was more frequently associated with diarrhea, whereas Ragavan *et al*. (2015) observed that the amoeboid form was higher in prevalence in irritable bowel syndrome (IBS) patients. Besides, Tan and Zierdt (1973) reported that this form of *Blastocystis* may also invade and replicate within the colonic epithelial cells of mice and causing damage to the intestinal mucosa of chickens resulting in increased permeability and decreased absorptive capacity as reported by Zhang *et al*. (2012).

Apart from that, the resistance of the amoeboid form of *Blastocystis* to antibiotics as reported by Boreham & Stenzel (1993) may contribute to treatment failure in the infected patients. However, Yamada & Yoshikawa (2012) suggested that

the presence of the amoeboid form could be a useful diagnostic marker for *Blastocystis* infection as it was found to be more prevalent in symptomatic patients than in asymptomatic carriers. Overall, the amoeboid form of *Blastocystis* is an important aspect to understanding better its pathogenicity and potential diagnostic implications. The amoeboid form of *Blastocystis* plays a crucial role in advancing our understanding of how the organism causes disease (pathogenicity) and how it might be used as a diagnostic marker to identify symptomatic infections.

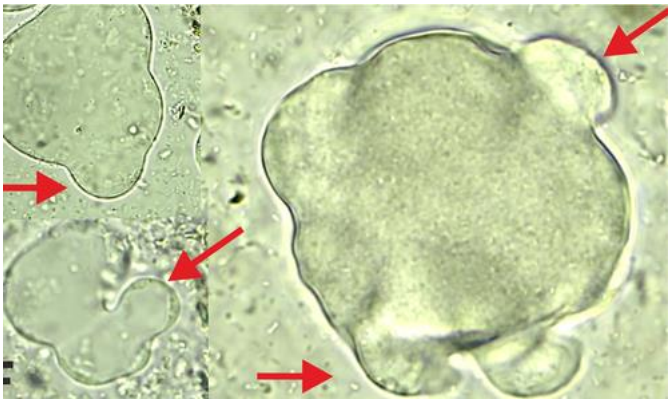

Figure 2.4 Amoeboid form *Blastocystis* showing one or multiple pseudopodia (marked with red arrow) (Boutahar *et al*., 2023).

2.2.1(e) Avacuolar and multi-vacuolar form

The avacuolar and multi-vacuolar forms are the vegetative forms of *Blastocystis* that have been identified with the size ranges from 5 to 8 μm in diameter. The multi-vacuolar form contains several small vacuoles in the cytoplasm whereas the avacuolar form is devoid of vacuoles. They can have one or two nuclei. The vegetative forms of *Blastocystis* are reportedly the most neglected due to their variable appearance and poorly understood morphology (Parija & Jeremiah, 2013; Stensvold, 2013c).

2.2.2 Life cycle

The life cycle of *Blastocystis* has been proposed by several researchers (Zierdt, 1988, 1991; Boreham & Stenzel, 1993; Stenzel & Boreham, 1996; Moe *et al*., 1997; Yoshikawa *et al*., 2004). *Blastocystis* can be spread orally via faeces in its cystic form. After the host ingests a *Blastocystis* cyst, the parasite encysts and undergoes binary fusion to form a vacuolar structure, eventually taking on an amoeboid or granular appearance (Figure 2.5). Except for cysts, there are vacuolar, granular, and amoeboid forms that have bilaminar membranes (Lepczyńska *et al*., 2017). As the cell develops into a cyst and exits the body through the faeces, the fibrillar layer of the cell is shed during the encystation process (Parija & Jeremiah, 2013). The faecal-oral pathway is believed to be the primary mode of transmission for *Blastocystis* in which the cyst forms able to persist longer in unclean water (Dumètre *et al*., 2012). *Blastocystis* cysts are shielded by a thick cell wall, allowing them to survive for extended periods in the environment (Tan, 2004). *Blastocystis* can be vulnerable to common disinfectants and high temperatures, but the spread of *Blastocystis* may be aided by close contact.

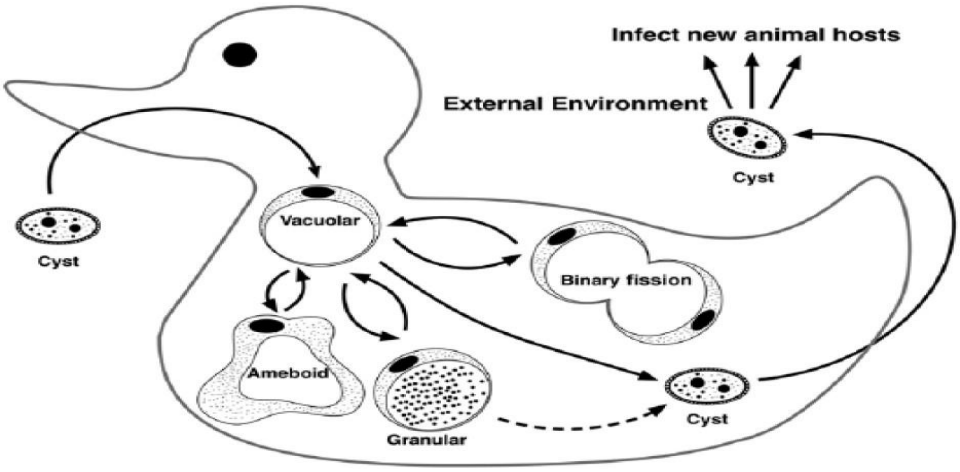

Figure 2.5 General life cycle of *Blastocystis* sp. (Parija & Jeremiah, 2013).

2.2.3 Transmission

A zoonotic transmission is a significant pathway that *Blastocystis* can be transmitted to humans. The transmission pathways of *Blastocystis* outlined in Tan (2008) highlight its spread through various modes, including human-to-human transmission via the fecal-oral route, zoonotic transmission from animals like livestock or pets, and environmental exposure through contaminated water, food, or soil (Figure 2.6). The parasite's resilient cysts facilitate indirect transmission through contact with contaminated surfaces or objects. Additionally, *Blastocystis* can circulate among animals, contributing to an environmental reservoir that poses a risk to humans. (Figure 2.6). A study by Lee *et al*. (2012) found that infected people in Italy who have frequent interaction with animals are more likely to become infected with *Blastocystis*. Besides, contaminated water is another potential source of *Blastocystis* infection. Lee *et al*. (2012) demonstrated that *Blastocystis* can survive in water for extended periods. Therefore, it is suggested that water with contaminated *Blastocystis* cysts is another potential source of infection. In Malaysia, Ithoi *et al*. (2011) found that river water contained *Blastocystis*, and both water and faeces of inhabitants in the same location contained the same *Blastocystis* subtypes. Besides, contaminated food has also been identified as a potential source of *Blastocystis* infection. Kheirandish *et al*., 2014 found that *Blastocystis* was present in the stool samples of a significant percentage (1.4%) of food handlers in Iran suggesting that food contamination may be a source of infection.

Additionally, Parkar *et al*. (2007) found that person-to-person contact and sharing of items were also another potential sources of *Blastocystis* infection among the institutionalized mentally disabled patients. Poor sanitation and hygiene practices have also been suggested to contribute to the spread of *Blastocystis*. Salim *et al*. (1999)

found that *Blastocystis* infection in a 17% of rural Malaysian households and it was suggested that poor sanitation and hygiene practices were contributing to the spread of this protozoan parasite. Meanwhile, animal-to-human transmission was also found to be a potential source of infection in animal shelters in Nepal in which Lee *et al*. (2012) reported that the prevalence of *Blastocystis* sp. in villagers also found in the animals they raised and the rivers they frequently use.

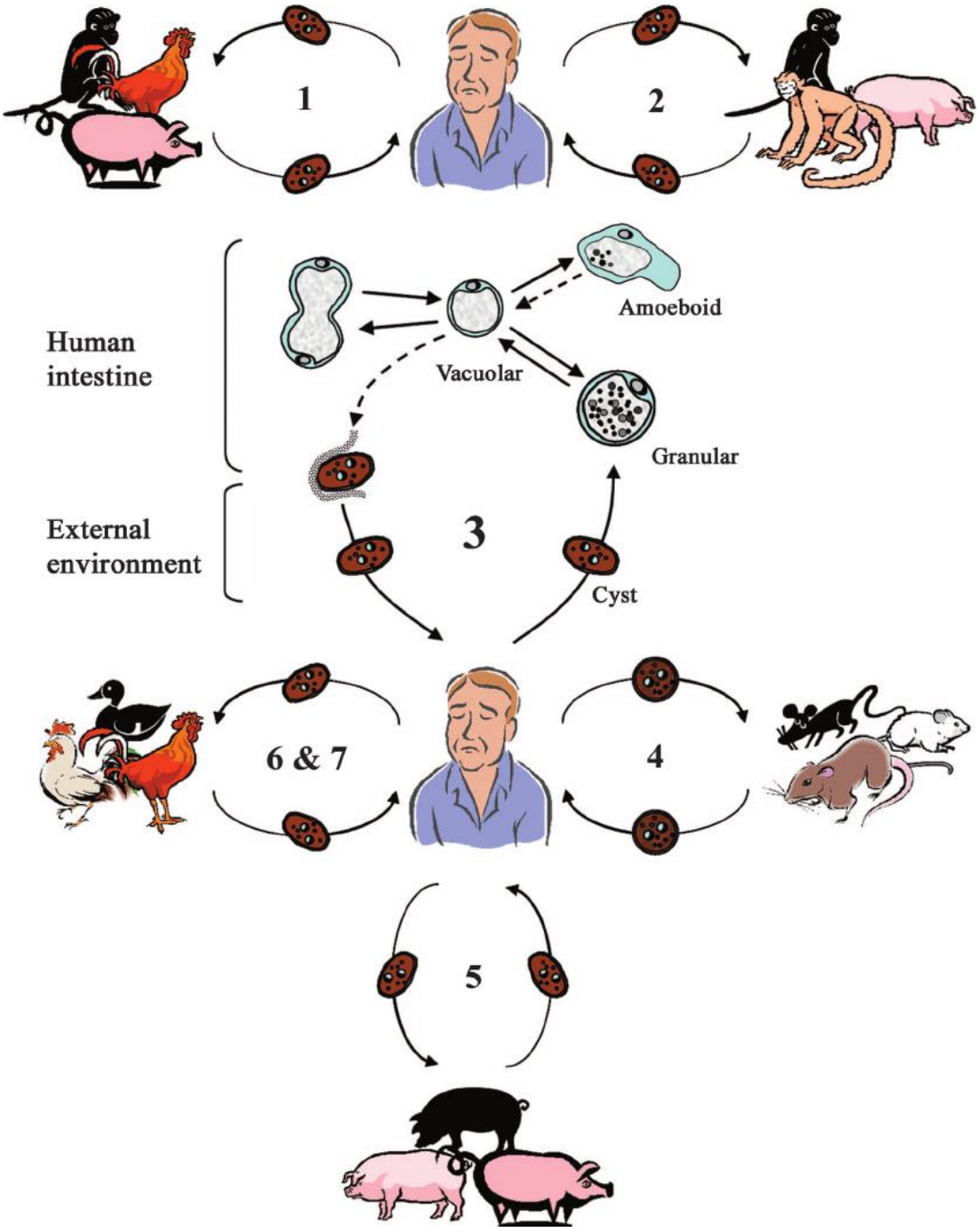

Figure 2.6 Different pathways of *Blastocystis* subtypes transmission (Tan, 2008).