MORPHOLOGICAL CHARACTERISATION, MOLECULAR SUBTYPING AND PHYLOGENY OF *Blastocystis* sp. ISOLATED FROM TURKEY (*Meleagris gallopavo*) IN PENANG, MALAYSIA

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
º	Degree
µl	Microliter
µm	Micrometer
bp	Base pair
DNA	Deoxyribonucleic acid
g	Gram
PCR	Polymerase chain reaction
pH	Power of hydrogen
RNA	Ribonucleic acid
rpm	Revolutions per minute
sec	Second
SEM	Scanning electron microscope
sp	Species
SPSS	Statistical Package for Social Science
SSU-rRNA	Small sub-unit ribosomal ribonucleic acid
ST	Subtype
Taq	*Thermus aquaticus*
TEM	Transmission electron microscopy

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PENCIRIAN MORFOLOGI, SUBJENIS MOLEKUL DAN FILOGENI BAGI *Blastocystis* sp. DIPENCILKAN DARIPADA POPULASI AYAM PIRU *(Meleagris gallopavo)* DI PULAU PINANG, MALAYSIA

ABSTRAK

Kebanyakan ladang ternakan unggas di Malaysia gemar memelihara ayam sama ada untuk telur atau / dan daging daripada ayam piru. Ini disebabkan oleh beberapa cabaran seperti bebanan parasit dan tekanan haba dalam menternak ayam piru. *Blastocystis* adalah salah satu parasit protozoa yang paling umum menjangkiti unggas. Oleh kerana tiada kajian dijalankan keatas jangkitan *Blastocystis* pada ayam piru di Malaysia, kajian ini bertujuan untuk menentukan status terkini, ciri-ciri morfologi dan subjenis *Blastocystis* daripada ayam piru yang diternak sama ada di reban tertutup atau sistem ternak bebas di Pulau Pinang, Malaysia. Didapati bahawa prevalens jangkitan *Blastocystis* sp. pada ayam piru adalah sederhana tinggi dengan 41.6% (25/60) di reban tertutup dan 45.0% (45/100) dalam sistem ternak bebas dimana jangkitan lebih tinggi pada ayam piru betina dengan tiada tanda dan gejala gastrousus. Bentuk vakuol adalah bentuk yang paling lazim ditemui dalam kultur *in vitro* berukuran antara 5 hingga 20 μm diameter dengan lapisan permukaan kasar dan permukaan sel beralun dilihat di bawah mikroskop elektron pengimbasan. Manakala, ultrastruktur sel daripada pemencilan ayam piru adalah berbeza-beza dengan vakuol legap elektron yang separa penuh kepada elektron padat dalam vakuol yang terisi penuh. Menariknya, analisis jujukan 30 pencilan *Blastocystis* positif daripada ayam piru mendedahkan satu subjenis dan tiga alel iaitu, ST7 alel 99 (73.4%, *n*=22), ST7 alel 100 (23.3%, *n*=7) dan ST7 alel 101 (3.3%, *n*=1). Ini adalah kajian pertama yang menilai prevalens, morfologi dan subjenis *Blastocystis* sp.

diasingkan daripada ayam piru ternak bebas dan reban tertutup di Malaysia. Penemuan kajian ini juga telah menambahkan pemahaman tentang jangkitan *Blastocystis* bagi menangani jangkitan parasit dalam pengeluaran ayam piru serta. Disamping itu, kesedaran terhadap penyebaran zonotik hendaklah dipertimbangkan khususnya kepada penternak ayam piru atau pekerja sembelihan yang mempunyai risiko tinggi terhadap jangkitan oleh kerana mereka berhubung rapat dengan unggas tersebut dan lebih cenderung kepada jangkitan *Blastocystis.*

MORPHOLOGY DESCRIPTION, MOLECULAR SUBTYPING AND PHYLOGENY OF *Blastocystis* sp. **ISOLATED FROM TURKEY** (*Meleagris gallopavo*) **POPULATIONS IN PENANG, MALAYSIA**

ABSTRACT

Most poultry farms in Malaysia preferred rearing chickens either for eggs or/and meat than turkeys. This is due to several challenges such as parasitic load and heat stress in rearing turkey. *Blastocystis* is one of the most common protozoan parasites infecting poultry. As no study was conducted on study of *Blastocystis* infection in turkey in Malaysia, this study aims to determine the current status, the morphological characteristics and subtyping of *Blastocystis* from turkey reared either in closed house or free-range system in Penang, Malaysia. It was found that the prevalence of *Blastocystis* sp. infection in turkeys were moderately high with 41.6% (25/60) in the closed house and 45.0% (45/100) in free-range system as infection was higher in the female turkeys with no gastrointestinal signs and symptoms. Vacuolar form was the most common form found in the *in vitro* culture ranged between 5 to 20 μm in diameter with a rough surface coat and undulating cell surface viewed under the scanning electron microscope. Meanwhile, the ultrastructure of the cells from turkey isolates were varies with partially expanded electron-opaque vacuoles to electron-dense in fully distended vacuoles. Interestingly, the sequence analysis of 30 positive *Blastocystis* isolates from turkeys revealed one ST and three alleles namely, ST7 allele 99 (73.4%, *n*=22), ST7 allele 100 (23.3%, *n*=7) and ST7 allele 101 (3.3%, *n*=1). This was the first study to evaluate the prevalence, morphological and ST of *Blastocystis* sp. isolated from free-range and close house turkeys in Malaysia. The findings of this study also added to our understanding on *Blastocystis* infection so as

and to facilitate parasitic infection in turkey production. Besides, as well as being able to give awareness on zoonotic transmission should be taken into consideration to especially to the turkey farmers or the slaughter workers who farmers might have high risk of infection as they are in constant contact with the birds and more susceptible to *Blastocystis* sp. infection.

CHAPTER 1
INTRODUCTION

1.1 Research background

Wild turkeys (*Meleagris gallopavo*) are huge, sexually dimorphic fowls with long feet, wide and curved tails, elongated necks and small heads which associates with the other members in the order Galliformes, family Meleagridae and genus *Meleagris* (Miller, 2018). They are very adjustable in various conditions, capable to live in warm environments as well as to some countries that are frequently blanketed with snow. The adult males, or known as tom or gobblers, are weigh from 10 to 15 kg throughout their range depends with the type of breeds. The adult females, or known as hens, are commonly do not surpass 10 kg, with the typical weight is from 6 to 9 kg (Cathey *et al.,* 2007). In Malaysia, turkeys are reared for many purposes such as poultry meat as well as a hobby. Turkeys are considered expensive and have a high demand especially during festive seasons such as Christmas Eve and Deepavali.

The turkey's usual behaviours are to forage food on soil, therefore, there are numerous types of organisms as well as intermediate hosts that can cause the endoparasites infection in turkeys as they are omnivorous, and they have a wide-ranging diet. Mohammad Zarith *et al.* (2017) stated that studies on the dispersion of parasitic infection in turkeys particularly in Malaysia is still scarce which probably due to Malaysian preference to eat more chicken than turkey, making study on turkey diseases economically insignificant.

Generally, turkeys are having some issues to several parasitic diseases caused by protozoan parasites. Protozoa are single-celled organisms that can be commensals or parasitic in nature. There are certain species of parasitic protozoan which include in the medical importance worldwide. In turkey population, the most common species of parasitic protozoan encountered were *Eimeria* spp. which cause coccidiosis (Sharman *et al.,* 2010; Olanrewaju and Agbor, 2014) and *Histomonas meleagridis,* the source of blackhead disease (histosomiasis) (Liu *et al.,* 2011). Other protozoan which may also infect turkeys include *Hexamita meleagridis* (hexamitiasis), *Trichomonas gallinae* (trichomoniasis) and *Cochlosoma anatis* (cochlosomiasis) (Hauck and Hafez, 2013). Apart from that, a neglected zoonotic protozoan known as *Blastocystis* sp. was also been found in turkeys (Lee, 1970; Yamada *et al.,* 1987; Belova and Kostenko, 1990; Belova, 1992a; Mokhtar and Youssef, 2018).

Blastocystis sp. is a common, non-flagellated, anaerobic stramenopiles (Gentekaki *et al.,* 2017) that inhabits the gastrointestinal tracts in many humans and various animals particularly poultry (Mokhtar and Youssef, 2018). *Blastocystis* occurs in four different morphological form namely; vacuolar, granular, amoeboid and cyst form (Tan, 2008). Binary fission is the most common reproduction mode (Adao and Rivera, 2018) in which cyst is the infective form that accountable in the transmission. The main transmission mode of this protozoan is through the faecal-oral pathway via drinking untreated water and/or poor sanitary conditions.

The occurrence of this organism has been perceived in a wide diversity of species worldwide. It has a great genetic diversity thus the genotypes were assigned using the subtyping nomenclature (ST) (Rauff-Adedotun *et al.,* 2020). Nomenclature *Blastocystis* sp. STs, ST1-ST9 was first presented in 2007 (Rauff-Adedotun *et al.,*

2020), after many of ST were proposed recently. Starting from the year 2013, new ST was recognized which was ST1-ST17 between some hosts (Alfellani *et al.,* 2013; Stensvold and Clark, 2020). Presently, a total of 29 ST have been suggested (Rauff-Adedotun *et al.,* 2020). However, four ST out of 29 ST that have been proposed namely, ST18, ST19, ST20 and ST22 was recently under question due to the probability that they were generated from memento consequently their quixotic emergence (Stensvold and Clark, 2020). The enduring 25 ST which include ST1-ST17, ST21, ST23-ST29 have encountered the existing suggested standards for distinctive ST nominations (Maloney and Santin, 2021). Additionally, ST1-ST9 and ST12 have been recovered in humans, with fluctuating stages of existence (Greige *et al.,* 2019) later the possibility of zoonotic transmission will occur (Clark *et al.,* 2013; Mohammad *et al.,* 2018; Stensvold *et al.,* 2020).

The most recent study on *Blastocystis* in poultry by Greige *et al.* (2018) reported that the avian samples specifically from chickens in Lebanon were subtyped and fitted to any ST6 or ST7, with a great majority belongs to ST6. Surprisingly, this ST also been detected among the chicken handlers which affirmed that there was zoonotic transmission of this ST as those individuals were frequently in a direct contact with the chickens. Meanwhile, Mokhtar and Youssef (2018) reported the occurrence of ST1, the zoonotic ST with a prevalence of 7.8% in poultry species among the chicken, ducks, geese and turkeys isolates in Egypt. It was also been found in humans having similar ST with the animals that they handle. Besides, the study also reported the occurrence of ST7 and ST6 in both turkeys and chickens in which both ST were represented as avian-adapted STs.

Most of the previous studies on *Blastocystis* in poultry were concentrated on *Blastocystis* in domestic chickens (Stensvold *et al.,* 2009; Alfellani *et al.,* 2013; Ramirez *et al.,* 2014; Mokhtar and Youssef, 2018; Greige *et al.,* 2018; Wang *et al.,* 2018; Deng *et al.,* 2019; Kaczmarek *et al,* 2019; Rauff-Adedotun *et al.,* 2020; Kaczmarek *et al,* 2021; Maloney *et al.,* 2021), quails (Monte *et al.,* 2018; Maloney *et al.,* 2021; Onder *et al.,* 2021), ducks (Maloney *et al.,* 2020; Rauff-Adedotun *et al.,* 2020; Fahim *et al.,* 2021; Maloney *et al.,* 2021; Muadica *et al.,* 2021) and ostriches (Chandrasekaran *et al.,* 2014; Maloney *et al.,* 2020; Rauff-Adedotun *et al.,* 2020; Deng *et al.,* 2021; Rudzinska *et al.,* 2021; Zhang *et al.,* 2021). As there are very limited study in turkey population worldwide (Lee, 1970; Belova, 1992a; Noel *et al.,* 2003; Sreekumar *et al.,* 2013; Mokhtar and Youssef, 2018; Maloney *et al.,* 2020) and none was conducted in Malaysia. Therefore, this study will help to provide a baseline study on this neglected zoonotic protozoan parasite infection in turkey population mainly in the northern region of Peninsular Malaysia as because there is no awareness on the zoonotic transmission of *Blastocystis* infection among the turkey farmers.

1.2 Objectives of study

This study embarks on the following objectives:

1. To determine the prevalence of *Blastocystis* sp. in turkey *(Meleagris gallapavo)* consisting of free-range and closed house reared populations in Penang, Malaysia.

2. To establish phenotypic characteristics of *Blastocystis* sp. isolated from turkey based on staining and ultrastructure characteristics using electron microscopy.

3. To determine the ST characterizations and phylogeny of *Blastocystis* sp. isolated from turkey through the application of DNA barcoding methods.

CHAPTER 2
LITERATURE REVIEW

2.1 Turkey *(Meleagris gallopavo)*

Poultry are domesticated birds belongs to the members of the order Galliformes and Anseriformes which contain of family Anatidae or generally known as water fowl or domestic geese and ducks (Eaton, 1992). Turkeys are native to Latin America (Silberman *et al.,* 1996) and the largest birds in the farming system. Their body weight ranges between 7 to 8 kg in males and 4 to 5 kg in hens. Besides, they have good meat conformation, produce about 90 eggs per year and have medium to good hatchability. They are more susceptible to diseases compare to chickens or ducks (Jahan *et al.,* 2018).

According to Pearson and Sharples (1995), birds are animal protein sources formed inside the dissolute likely period. Consequently, the request for poultry meat is accomplishment more from year to year all over the world as the human residents rises. In Malaysia, turkey meat is less consumed as compared to the western countries such as Europe and United States (Mohammad Zarith *et al.,* 2017).

The American Livestock Breeds Conservancy (2007), documented eight variations of heritage turkeys includes Beltsville small white turkey (Figure 2.2), Black turkey (Figure 2.3), Blue state turkey (Figure 2.4), Bourbon reds turkey (Figure 2.5), Narragansett turkey (Figure 2.6), Standard bronze turkey (Figure 2.7), Royal palm turkey (Figure 2.8) and White Holland turkey (Figure 2.9). In Malaysia, they were regionally known as ‘ayam piru’ (Mohammad Zarith *et al.,* 2017). Usually, turkey meat sold at RM40 to RM45 per kg on average in around poultry

farm. Even though turkey meat is much costly than broiler and scavenging chickens, Udoh *et al.,* (2014) stated that turkey meat comprises lesser calories and fat, and higher in protein, than other meats. Nutritious value as shown in Figure 2.1 for sampled turkey meat has the peak crude fat, fibre and protein content constructed on the type of diet (Ogunmola *et al.,* 2013). This designates that turkey meat could deliver more energy and other useful minerals than other meat sources (Mohammad Zarith *et al.,* 2017). Recently in Malaysia there have been an increase demand for turkey meat especially during Christmas Eve and Deepavali.

Nutrition Facts **Serving Size: 1 unit (Yield from 1 lb ready-to-cook turkey) (111g)**	
Amount Per Serving	
Calories 123	**Calories from Fat** 6
	% Daily Value *
Total Fat 0.72 g	**1%**
Saturated Fat 0.233 g	**1%**
Trans Fat 0.014 g	
Cholesterol 69 mg	**23%**
Sodium 54 mg	**2%**
Potassium 325 mg	**9%**
Total Carbohydrate 0.00 g	**0%**
Dietary Fiber 0.0 g	**0%**
Sugars	
Protein 27.31 g	**55%**
Vitamin A 0 IU	**0%**
Vitamin C 0.0 mg	**0%**
Calcium 11 mg	**1%**
Iron 1.30 mg	**7%**
*Based on a 2000 calorie diet	

Figure 2.1: Nutrition facts for turkey meat (The Poultry Guide, 2013).

Figure 2.2: Beltsville small white turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.3: Black turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.4: Blue state turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.5: Bourban reds turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.6: Narragansett turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.7: Standard bronze turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.8: Royal palm turkey (American Livestock Breeds Conservancy, 2007).

Figure 2.9: White Holland turkey (American Livestock Breeds Conservancy, 2007).

2.2 Gastrointestinal protozoan parasites in turkey

Gastrointestinal protozoan parasites are single-celled eukaryotes found in humans and animals in which the infection range from asymptomatic to life threatening, depending on the species or strain of the parasite and the resistance of the host (Yaeger, 1996). Infections may be inapparent or mild in normal or healthy hosts, but they can be life-threatening in immunosuppressed hosts (Janoff and Smith, 2001; McDougald *et al.,* 2019). In animals, they are responsible for significant losses of production and several gastrointestinal protozoan parasites are of zoonotic importance which cause severe morbidity and mortality, thus affect the economy of livestock.

Blackhead disease, also called histomoniasis is caused by the protozoan parasite called *Histomonas meleagridis*. It has a significant economic impact on turkey as well as chicken production. It is carried by the relatively harmless caecal worm, *Heterakis gallinarum* infected with this protozoan (Reid, 1967). The usual signs and symptoms of this disease include bright yellow diarrhoea, dullness and a very special cases, turkey will develop a black coloured head. To date, there is currently no treatment for this disease, hence successful control of caecal worms is an important step for the control of Blackhead disease (McDougald, 2005; Lister and Houghton-Wallace, 2012).

Besides, coccidiosis is an important disease of turkey caused by protozoan parasites belonging to the genus *Eimeria* (Rathinam, 2014). It was first observed in the caeca of a turkey by Smith (1895) while studying the disease histomoniasis or blackhead. This protozoan parasite develops in the epithelial cells of the alimentary canal and transmitted mainly via faecal contamination. It distresses frequently young

birds, and the signs in turkeys are not pathognomonic which include loss of appetite, listlessness, huddling, constant cheeping, drooping wings, and ruffled feathers (Reid, 1972). There are different coccidial species which infects turkeys namely, *E. meleagridis, E. adenoeides,* and *E. gallopavonis* develop in the lower intestine and/or caeca, while *E. meleagrimitis*, *E. dispersa*, *E. innocua*, and *E. subrotunda* develop in the upper and mid-intestine (Rathinam, 2014).

Trichomonas gallinae is also an economically important pathogen since it affects wild and livestock birds including turkeys (Mirzaei *et al.,* 2014; Albeshr and Alrefaei, 2019). It was first identified in Europe by Rivolta (1878). Transmission of this protozoan parasite is most likely to be via birds feeding one another with regurgitated food or through food or water sources contaminated from an infected bird (Saif *et al.,* 2003; Lawson *et al.,* 2018). Different strains may differ in their virulence from apathogenic to very virulent. *Trichomonas gallinae* is extremely common in pigeons (Mayahi *et al.,* 2007; Borji *et al.,* 2011; Albeshr and Alrefaei, 2019) and less frequent among turkeys (Mirzaei *et al.,* 2016; Albeshr and Alrefaei, 2019). Based on a study by Mirzaei *et al.* (2016), yellowish-white masses of caseous necrotic material were seen in the oral cavity, esophagus, crop and proventriculus of the infected turkey.

Blastocystis sp. is one of the most frequent protozoan parasites found wild and livestock birds (Zenetti *et al.,* 2020). Due to its low host specificity and zoonotic potential, animals might serve as possible reservoirs for transmission of this protozoan parasite (Alfellani *et al.,* 2013; Cian *et al.,* 2017; Greige *et al.,* 2019; Mohammadpour *et al.,* 2020; Rauff-Adedotun *et al.,* 2020). A comprehensive information which includes the prevalence, morphology, ultrastructure, and genetic

diversity of *Blastocystis* in turkey populations was not well studied, although zoonotic importance has been reported in other bird species (Greige *et al.,* 2018). Presently, several studies were reported on *Blastocystis* in turkeys' worldwide (Lee, 1970; Belova, 1992a; Noel *et al.,* 2003; Sreekumar *et al.,* 2014; Mokhtar and Youssef, 2018; Maloney *et al.,* 2020).

2.3 A neglected zoonotic protozoan, *Blastocystis* sp.

2.3.1 Classification

2.3.1(a) Taxonomic status

The taxonomic status of *Blastocystis* is unique and it was first identified over a century ago by Alexeieff (1911) and Brumpt (1912) who initially classified it as a new harmless saprophytic yeast species known as *Blastocystis hominis*. However, over five decades later, this organism was reclassified as a protist based on several characteristics of protists namely, the presence of one or more nuclei, smooth and rough endoplasmic reticulum, Golgi apparatus, and mitochondrion-like organelles. Unfortunately, this organism was failure to grow on fungal media, resistance to antifungal agents but it was susceptible to antiprotozoal drugs (Zierdt *et al.,* 1967).

Although the taxonomic status of this organism has continuously been confusing, but it was now clear with the advent of molecular tools. Over three decades later, a successful taxonomical home for this enigmatic organism was accomplished by the molecular analysis of small subunit ribosomal RNA gene (SSU-rRNA) and elongation factor 1α conducted by Silberman *et al*. (1996) who found that this organism was not a monophyletic to yeast (*Saccharomyces*), fungi

(Neurospora), sporozoans (*Sarcocystis* and *Toxoplasma*) or sarcodines (*Naegleria*, *Acanthamoeba*, and *Dictyostelium*) and it was classified within the Stramenopiles, a diverse group of mostly unicellular or multicellular eukaryotes which includes diatoms, brown algae, slime nets and water moulds. Nevertheless, this clade also consists of several lineages of protozoa (Patterson, 1999; Massana *et al.,* 2014). However, there is a discrepancy in the morphological features between *Blastocystis* and other Stramenopiles. An important characteristic of Stramenopiles is the presence of at least one flagellum permitting motility which is characteristically absent in *Blastocystis*. Thus, there is an argument to revise the current classification and indicated this organism in a separate sixth kingdom known as Chromista by Cavalier-Smith (1998) who considered Stramenopiles to be identical to the infrakingdom Heterokonta under the kingdom Chromista.

The Stramenopiles synonymous with Chromista is a complex collection of protists comprising heterotrophic and photosynthetic representatives. Based on molecular phylogenetic studies, this organism is most closely related to *Proteromonas lacertae*, a flagellate of the hindgut of lizards and amphibians (Arisue *et al.,* 2002; Hoevers & Snowden (2005). As *Blastocystis* sp. does not possess flagella and nonmotile, hence it was placed in a newly created classification by Tan (2008) as indicated below:

Scientific classification of *Blastocystis* spp.

Kingdom: Stramenopiles/Chromista

Infrakingdom: Heterokonta

Subkingdom: Chromobiota

Infrakingdom: Heterokonta

Subphylum: Opalinata

Class: Blastocystea

Order: Blastocystida

Family: Blastocystidae

Genus: *Blastocystis*

2.3.1(b) Speciation and terminology

There had been a small number of specific scientific names for *Blastocystis* published previously (Zierdt, 1991). The genus name, *Blastocystis* was initially assigned by Alexeieff (1911) whereas the species name, *hominis* was provided by Brumpt (1912) for *Blastocystis* isolated from human. Nevertheless, there had been several scientific names for *Blastocystis* isolates from specific animal exclusively for rat, chicken, duck, geese and reptilian. Chen *et al.* (1997) concluded that the *Blastocystis* isolated from rat was a distinct species, and therefore *B. ratti* was proposed whereas reptilian *Blastocystis* isolates from sea-snakes was known as *B. lapemi*, isolate from reticulated python as *B. phythoni*, rhino iguana isolate as *B. cycluri* and red-footed tortoise isolate as *B. geocheloni* since these isolates have shown singular phenotypic characteristics which differentiated them from human and other homeothermic animals (Teow *et al.,* 1991; Singh *et al.,* 1996). Besides,

there were several *Blastocystis* species of bird origin namely, *B. galli* from chickens (Belova and Kostenko, 1990) and turkey (Belova, 1992a), *B. anatis* from ducks (Belova, 1991) and *B. anseri* from geese (Belova, 1992b) which were reported based on the morphological and host differences.

However, due to the poor host specificity of *Blastocystis* demonstrated in the published small-subunit ribosomal RNA gene (SSU-rRNA) analyses, the previous denominations restricted to species have proven to be ineffective (Stensvold *et al.,* 2007). It was demonstrated that some reptilian and amphibian species fall within the range of variation covered by the mammalian and avian clades (Yoshikawa *et al.,* 2004a, Noël *et al.,* 2005). Therefore, the more appropriate nomenclature was proposed by Stensvold *et al*. (2007) in which *Blastocystis* isolated from a variety of animals as well as humans were designated as *Blastocystis* sp. and assigned to the specific subtype *n* in which *n* is a number (Table 2.1 and 2.2).

Table 2.1: Correlation of *Blastocystis* subtype designations and suggestion for consensus terminology by Stensvold *et al.* (2007).

Clade[a]	Subtype[b]	Group and subtype[c]	Subtype[d]	Ribodeme[e,f]	Subgroup[g]	Cluster[h]	Subtype[i]	Consensus
I	I	I/1	1	1, 8[j]	III	E	1, 1 variant	*Blastocystis* sp. subtype 1
II	II	II/5	5	6	V	C, D	-[k]	*Blastocystis* sp. subtype 2
-	X	I+II/I+5 outlier	-	-	-	-	-	Chimaeric sequence
III	III	III/3	3	2, 7, 4?[l], 5?	1, II	A	3	*Blastocystis* sp. subtype 3
IV	IV	IV/7	7	3	IV	B	-	*Blastocystis* sp. subtype 4
-	IVa	IV/7 outliers	-	-	-	-	-	*Blastocystis* sp. subtype 8
V	V	V/6	6	-	-	-	-	*Blastocystis* sp. subtype 5
VI	VI	VI/4	4	9[j]	-	-	4	*Blastocystis* sp. subtype 6
-	VIa	VI/4 outliers	-	-	-	-	-	*Blastocystis* sp. subtype 9
VII	V11	VII/2	2	10	VI[m]	-	2	*Blastocystis* sp. subtype 7
-	VII	VII/2 outliers	-	-	-	-	-	*Blastocystis* sp. subtype 7

[a]Clades described by Arisue *et al.* (2003) and Yoshikawa *et al.* (2004b).
[b]Subtypes described by Scicluna *et al.* (2006)
[c]Groups and subtypes described by Noel *et al.* (2005)
[d]Subtypes described by Yoshikawa *et al.* (2000, 1998)
[e]Ribodemes are groups that share the same SSU-rDNA PCR-RFLP patterns and are described by Clark (1997) and Yoshikawa *et al.* (2000).
[f]Ribodemes in bold are those originally described by Clark (1997).
[g]Subgroups described by Bohm-Gloning *et al.* (1997) based on PCR-RFLP analysis and partial SSU-rDNA sequences.
[h]Clusters described by Stensvold *et al.* (2006) based on PCR and sequencing analysis of partial SSU-rDNA sequences.
[i]Subtypes described by Yoshikawa *et al.* (2000) using PCR-STS
[j]Ribodemes 8 and 9 described by Yoshikawa *et al.* (2000) differ from those described by Kaneda *et al.* (2001).
[k]'–' symbols indicate no equivalent described.
[l]Question mark indicates that the subtype equivalent is probable but not proven.
[m]Subgroup VI described by Thathaisong *et al.* (2003) equals ribodeme 10 described by Yoshikawa *et al*. (2000).

Table 2.2: List of previous and latest classification of commonly studied *Blastocystis* isolates based on consensus terminology (Tan, 2008).

Species	Isolate (s)	Culture type	Host	New designation	References
B. hominis	Nand II	Axenic	Human	*Blastocystis* sp. subtype 1	Noel *et al.* (2005); Silberman *et al.* (1996)
B. hominis	Si	Axenic	Human	*Blastocystis* sp. subtype 1	Ng and Tan (1999); Noel *et al.* (2005)
B. hominis	B, C, E, G, H	Axenic	Human	*Blastocystis* sp. subtype 7	Ho *et al.* (1993); Noel *et al.* (2005)
B. ratti	S1, WR1, WR2	Axenic	Rat	*Blastocystis* sp. subtype 4	Chen *et al.* (1997); Noel *et al.* (2005)
Blastocystis sp.	NIH:1295:1	Xenic	Guinea pig	*Blastocystis* sp. subtype 4	Noel *et al.* (2005); Yoshikawa *et al.* (2004c)

2.3.2 Current subtypes

Based on the epidemiological studies, it was revealed that the extensive diversity of *Blastocystis* ST was found in domestic and wildlife animals (Oliveira-Arbex *et al.,* 2020; Valença-Barbosa *et al.,* 2019; Rauff-Adedotun *et al.,* 2020), insects (Suresh *et al.,* 1997; Yoshikawa *et al.,* 2016; Farah Haziqah *et al.,* 2017) as well as humans globally (Sanpool *et al.,* 2017; Oliveira-Arbex *et al.,* 2018; Lhotská *et al.,* 2020). At present, there are 26 ST of which several STs namely, ST18 - ST20, and ST22 are speculated to be experimental artefacts such as chimeras which may arise during PCR amplification (Stensvold and Clark, 2020; Maloney and Santin, 2021; Maloney *et al.,* 2021).

To date, the number of subtypes found in humans has remained constant with 10 ST namely, ST1 - ST9 and ST12 (Parkar *et al.,* 2010; Wawrzyniak *et al.,* 2013; Ramírez *et al.,* 2016; Greige *et al.,* 2019). ST1 - ST4 were primarily found in human as well as several animal hosts such as avian, canines, felines, hoofed animals, primates, and rodents (Wawrzyniak *et al.,* 2013; Wang *et al.,* 2014; Ruaux and Stang, 2014; Anderson and Stensvold, 2016; Chai *et al.,* 2020). On the other hand, ST5 - ST8 are rarely detected in human as ST5 is prevalent in ungulates (Shams *et al.,* 2021; Oliveira-Arbex *et al.,* 2020) and non-human primates (Menu *et al.,* 2021), ST6 and ST7 among avian hosts (Stensvold *et al.,* 2009; Farah Haziqah *et al.,* 2018; Greige *et al.,* 2018) whereas ST8 in non-human primates (Alfellani *et al.,* 2013; Stensvold and Clark, 2016; Oliveira-Arbex *et al.,* 2018). It is noteworthy that ST1 - ST8 and ST12 are of zoonotic concern for humans as ST9 is being only reported in human (Ramírez *et al.,* 2016; Yoshikawa *et al.,* 2016; Asghari *et al.,* 2020).

Furthermore, ST10 - ST17 are exclusively found in animal with ST10 and ST15 present among ungulates and non-human primates, ST11 among proboscidea, ST12 among ungulates and marsupials, ST13 among non-human primates and marsupials, ST14 among ungulates, ST16 among marsupials and ST17 among rodents and ungulates (Stensvold *et al.,* 2009; Parkar *et al.,* 2010; Alfellani *et al.,* 2013; Jiménez *et al.,* 2019; Maloney *et al.,* 2019) whereas ST21, ST23, ST24 and ST26 were also been found among ungulates (Maloney *et al.,* 2019). However, Stensvold and Clark (2020) speculated that the accuracy of the novel proposed ST namely, ST18 - ST26 are still argumentative and need to be confirmed.

Table 2.3: List of current ST.

SUBTYPES	HOSTS	REFERENCES
ST1 – ST9 and ST12	Human	Parkar *et al.* (2010); Wawrzyniak *et al.* (2013); Ramírez *et al.* (2016); Greige *et al.* (2019)
ST1 – ST4	Human and several animal hosts such as avian, canines, felines, hoofed animals, primates, and rodents	Wawrzyniak *et al.* (2013); Wang *et al.* (2014); Ruaux and Stang (2014); Anderson and Stensvold (2016); Chai *et al.* (2020).
ST5 – ST8	Detected in human as ST5 is prevalent in ungulates and non-human primates	Shams *et al.* (2021); Oliveira-Arbex *et al.* (2020); Menu *et al.* (2021)
ST6 and ST7	Avian hosts	Stensvold *et al.* (2009); Farah Haziqah *et al.* (2018); Greige *et al.* (2018)
ST10 - ST17	ST10 and ST15 among ungulates and non-human primates, ST11 among proboscidea, ST12 among ungulates and marsupials, ST13 among non-human primates and marsupials, ST14 among ungulates, ST16 among marsupials and ST17 among rodents and ungulates	Stensvold *et al.* (2009); Parkar *et al.* (2010); Alfellani *et al.* (2013); Jiménez *et al.*, (2019); Maloney *et al.* (2019)
ST21, ST23, ST24 and ST26	Ungulates	Maloney *et al.* (2019)

2.3.3 Morphology

Blastocystis exists in various morphotypes such as vacuolar, avacuolar, multivacuolar, granular, amoeboid and cysts form (Tan, 2008; Suresh *et al.,* 2009; Parija and Jeremiah, 2013) in which the morphological variety was probably due to cell physiology or the external environment (Zierdt, 1991). This organism might be easily missed on microscopic methodologies due to its small size and irregular shape especially in a slow-growing subtype. Nevertheless, *Blastocystis* is also more likely to be misidentified as debris, yeast or lipid (Tan, 2008).

The vacuolar form also known as central body form is the most frequently observed form found during the examination of samples either in direct examination or *in vitro* cultivation. This form displays a wide size variation with the average cell diameter of 4 to 15 µm (Stenzel and Boreham, 1996; Tan, 2008). However, certain cells isolated from animal such as chickens may revealed up to 200 µm in diameter (Stenzel and Boreham, 1996; Yamada and Yoshikawa, 2012; De la Cruz and Stensvold, 2017; Farah Haziqah *et al.,* 2018). The vacuolar form is circular to ovoid in shape, characterized by a large central vacuole that occupies almost 90% of the whole cytoplasmic space and a thin peripheral rim contains the organelles (Tan, 2008) (Figure 2.9a and b).

Meanwhile, granular forms structurally resemble the vacuolar form except for the presence of granules within the central vacuole with a diameter size ranging from 4 and 15 µm (Tan, 2008). It is hardly seen in direct examination, but it is commonly observed in old and non-axenized culture samples (Tan, 2004) (Figure 2.9c and d). According to Taylor-Brown and Hurd (2013), the formation on granules