# OCCURRENCE, STRUCTURAL MORPHOLOGY AND GENOTYPING OF *Blastocystis* FROM WATER SOURCES IN NORTHERN STATES OF PENINSULAR MALAYSIA

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**by**

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## LIST OF SYMBOLS

| | |
|---|---|
| $^{o}$ | Degrees |
| = | Equal to |
| > | Greater than |
| km | Kilometre |
| $km^2$ | Kilometre squared |
| < | Less than |
| µl | Microlitre |
| µm | Micrometre |
| mg | Milligrams |
| mA | Milli Amperes |
| ml | Milli litre |
| mM | Milli Molar |
| - | Minus |
| M | Molar |
| nm | Nanometre |
| % | Percentage |
| ± | Plus or minus |
| ′ | Prime |
| P | Probability |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| BIC | Bayesian Information Criterion |
| BLAST | Basic Local Search Alignment Tool |
| bp | Base Pair |
| C | Celsius |
| COD | Chemical Oxygen Demand |
| DAPI | 4'-6-diamidino-2- phenylindole |
| DO | Dissolved Oxygen |
| DOE | Department of Environment |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| GIS | Geographic Information System |
| GNB | Government of New Brunswick |
| HDMS | Hexamethyldisilazane |
| HKY | Hasegawa-Kishino-Yano |
| IBD | Inflammatory Bowel Disease |
| IBS | Irritable Bowel Syndrome |
| IL | Illinois |
| Inc. | Incorporated |
| M | Metres |
| $MgCl_2$ | Magnesium Chloride |
| ML | Maximum Likelihood |
| MLST | Multilocus Sequence Typing |
| MOH | Ministry of Health |
| NCBI | National Centre for Biotechnology Information |
| NGS | Next Generation Sequencing |
| ORP | Oxidation Reduction Potential |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| pH | Potential of Hydrogen |
| qPCR | Real-time Polymerase Chain Reaction |
| RAPD | Random Amplified Polymorphic DNA |
| RFLP | Restriction Fragment Length Polymorphism |
| rpm | Revolutions Per Minute |
| REDAC | River Engineering and Urban Drainage Research Centre |
| SAR | Stramenopiles, Alveolata, and Rhizaria |
| SEM | Scanning Electron Microscopy |
| SPSS | Statistical Package for Social Science |
| SSU rDNA | Small Sub-Unit Ribosomal Deoxyribonucleic Acid |
| SSU rRNA | Small Sub-Unit Ribosomal Ribonucleic Acid |
| sp. | Species |
| ST | Subtype |
| STS | Sequence Tagged Site |
| Taq | Thermus aquaticus |
| TEM | Transmission Electron Microscopy |

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**KEJADIAN, STRUKTUR MORFOLOGI DAN PENGENOTIPAN *Blastocystis* DARIPADA SUMBER AIR DI NEGERI UTARA SEMENANJUNG MALAYSIA**

## ABSTRAK

*Blastocystis* adalah parasit bawaan air yang meluas dan telah dikaitkan dengan beberapa keadaan penyakit termasuk kanser kolorektal, sindrom usus terganggu, cirit-birit, dan kecelaan kulit. Malaysia dikurniakan dengan banyak sumber air yang digunakan sebagai minuman, memancing, dan tujuan rekreasi lain. Bersentuhan atau meminum air yang telah tercemar dengan *Blastocystis* boleh berpotensi menyebabkan jangkitan dan penularan antara manusia dan haiwan. Walaupun berdepan dengan risiko yang jelas ini, terdapat kekurangan data mengenai kejadian dan kepelbagaian *Blastocystis* dalam sumber air, terutamanya di utara Semenanjung Malaysia. Oleh itu, kajian ini bertujuan untuk menentukan kewujudan, ciri morfologi, dan taburan subjenis (ST) *Blastocystis* dalam beberapa sumber air terpilih di utara Semenanjung Malaysia, serta menentukan kaitan parasit tersebut dengan parameter fizikokimia dan kiraan koliform najis. Sebanyak 85 sampel air dikumpulkan dari 43 tapak pensampelan yang terdiri daripada 13 sungai, enam tasik, sembilan kolam, 10 kolam renang, dan 11 air paip. Pensampelan dilakukan di negeri Kedah, Pulau Pinang, Perak, dan Perlis. Sampel-sampel tersebut telah menjalani kaedah sentrifugasi, pengkulturan *in vitro*, dan pengekodan bar DNA. Sampel dari kolam renang, air paip, kolam, dan tasik yang diperiksa semuanya negatif untuk *Blastocystis*. *Blastocystis* dikesan di empat sungai termasuk Sungai Pinang, George Town (100%), Sungai Air Terjun (Taman Botani Pulau Pinang) (33.3%), Sungai Air Terjun (Taman Belia Pulau Pinang) (25%), dan Sungai Perai, Seberang Jaya (100%) dimana peratus keseluruhan kewujudan

*Blastocystis* dalam sungai yang tercemar ialah 22.2%. Selain itu, analisis mikroskop cahaya telah mengenal pasti banyak bentuk vakuolar dan sebilangan kecil bentuk granular *Blastocystis* dalam sungai tersebut yang berukuran antara 2.01 µm dan 35.94 µm. Analisis bagi struktur permukaan dengan mikroskop elektron imbasan mendedahkan ciri permukaan yang pelbagai, termasuk permukaan licin, penampilan seperti jejaring dan berserat, serta lekukan dalam dan cetek dengan lekatan bakteria. Juga kelihatan adalah pencilan dengan permukaan kasar yang telah dikaitkan dengan kepatogenan. Begitu juga, mikrograf elektron transmisi bagi sel tersebut mendedahkan lapisan permukaan yang tidak seragam dengan vakuol pusat yang menonjol dan bahan elektron yang kurang padat. Analisis subjenis mendedahkan *Blastocystis* ST1 (3), ST2 (3), dan ST yang tidak diketahui (2). Analisis filogenetik menyokong pengenalan subjenis pencilan kerana ia dikelompokkan pada klad yang sama dengan subjenis yang serupa manakala ST yang tidak diketahui dikelompokkan pada klad yang berbeza. Tiada korelasi antara kewujudan *Blastocystis* dalam sumber air dengan parameter fisikokimia. Koliform tinja berada di atas paras ambang dalam semua sampel air sungai dan tasik yang menunjukkan bahawa sampel air tersebut sangat tercemar dengan bahan tinja. Kewujudan parasit yang rendah dalam persekitaran akuatik dalam kajian ini mungkin disebabkan oleh keterbatasan kaedah yang digunakan. Kajian ini telah mengenal pasti pencemaran beberapa sungai di Pulau Pinang dengan subjenis *Blastocystis*, sekali gus, meningkatkan pemahaman tentang dinamik penularan organisma tersebut. Pemahaman ini akan membantu dalam pembangunan dan pelaksanaan langkah-langkah pencegahan dan strategi kawalan yang cekap untuk peningkatan kesihatan awam.

**OCCURRENCE, STRUCTURAL MORPHOLOGY AND GENOTYPING OF *Blastocystis* FROM WATER SOURCES IN NORTHERN STATES OF PENINSULAR MALAYSIA**

## ABSTRACT

*Blastocystis* is a ubiquitous waterborne parasite that has been implicated in some disease conditions including colorectal cancer, irritable bowel syndrome, diarrhea and skin defects. Malaysia is endowed with many water sources that are utilized for drinking, fishing and other recreational purposes. Contacting or drinking water that has been contaminated with *Blastocystis* could potentially lead to infection and subsequent transmission between humans and animals. Notwithstanding this obvious risk, there is a dearth of data on *Blastocystis* occurrence and diversity in water sources, particularly, in the northern region of Peninsular Malaysia. Consequently, this study aims to determine the occurrence, morphological characteristics, and subtype (ST) distribution of *Blastocystis* in several selected water sources in Northern Peninsular Malaysia, as well as determine the association of the parasite with physicochemical parameters and faecal coliform counts. A total of 85 water samples were collected from 43 sampling sites comprising 13 rivers, six lakes, nine ponds, 10 swimming pools, and 11 tap waters. The sampling was done in the States of Kedah, Pulau Pinang, Perak, and Perlis. The samples were subjected to centrifugation *in vitro* cultivation and DNA barcoding. Samples from swimming pools, tap water, ponds, and lakes examined were all negative for *Blastocystis*. *Blastocystis* was detected in four rivers including Pinang River, George Town (100%), Air Terjun River (Penang Botanical Gardens) (33.3%), Air Terjun river (Penang Youth Park) (25%), and Perai River, Seberang Jaya (100%) in which the overall percentage of *Blastocystis*

occurrence in the contaminated rivers was 22.2%. Additionally, light microscopy analyses identified many vacuolar and a limited number of granular forms of *Blastocystis* in those rivers measuring between 2.01 µm and 35.94 µm. Analysis of the surface structure by scanning electron microscopy revealed diverse surface features, including smooth surfaces, mesh-like and fibrous appearances, as well as deep and shallow indentations with bacterial adhesion. Also noticeable were isolates with rough surfaces which has been associated with pathogenicity. Similarly, the transmission electron micrograph of the cells revealed non-uniformed surface coats with prominent central vacuoles and slightly dense electron materials. The subtype analysis revealed *Blastocystis* ST1 (3), ST2 (3), and unknown STs (2). Phylogenetic analysis supported the subtype identification of the isolates as clustered on the same clades with similar subtypes while the unknown STs clustered on different clades. There was no correlation between *Blastocystis* occurrence in water sources with physicochemical parameters. Faecal coliforms were present above the threshold level in all rivers and lakes water samples indicating that the water samples were highly contaminated with faecal materials. The low occurrence of the parasite in the aquatic environment in this study might be due to the limitation of the method employed. This study has identified the contamination of several rivers in Pulau Pinang with *Blastocystis* subtypes, hence, enhancing the understanding of the organism's transmission dynamics. This comprehension will aid in the development and efficient execution of preventive measures and control strategies for the enhancement of public health.

# CHAPTER 1

# INTRODUCTION

## 1.1 Research background

*Blastocystis* is a single-celled parasitic microorganism that belongs to the Stramenopile group (Angelici *et al.,* 2018). It is the most encountered protozoan in the intestinal tract and the stool samples of humans and many animals with a high prevalence in areas with inadequate sanitary conditions, and occurrence in environmental sources including water sources across the world (Kumarasamy *et al.,* 2018; Rudzińska *et al.,* 2022; Attah *et al.,* 2023). Besides, it has also been widely reported in tropical and sub-tropical countries (Fahim *et al.,* 2021) and has been associated with two out of 325 outbreaks (0.6%) of waterborne diseases caused by parasites worldwide (Karanis *et al*., 2007). Although *Blastocystis* was discovered more than a century ago, it has recently become a subject of intense attention due to its potential to cause public health issues to the host (Petrášová *et al*., 2011). The first recorded documentation of *Blastocystis* may be traced back to the early 1900s. However, it was not until the late 1900s that significant advancements were made in understanding its biological properties of this protozoan (Tan, 2008).

*Blastocystis* is classified among the Stramenopiles, particularly due to its anaerobic characteristics and absence of flagella or tubular hairs (Clark *et al.,* 2013; Ajjampur & Tan, 2016; Stensvold & Clark, 2016a). *Blastocystis* is highly polymorphic, exhibiting very distinctive morphological variation. The described morphological forms include the cyst, vacuolar, granular, and amoeboid forms (Suresh *et al.,* 2005; de la Cruz & Stensvold, 2017) with each morphological form differing in size from the others. The cyst form is the only transmissible form of *Blastocystis* (Lee & Stenzel,

1999; Parija & Jeremiah, 2013). The ability of the cyst to persist in different environments including water sources may play a role in the extensive dissemination of *Blastocystis* (Lee & Stenzel, 1999; Parija & Jeremiah, 2013; Chen *et al.,* 2021; Hublin *et al*., 2021). The vacuolar and granular forms are the most frequently encountered morphological forms in *in vitro* cultivation. Other less frequently encountered forms are the multivacuolar and avacuolar (Stenzel & Boreham, 1996; Ahmed & Karanis, 2018). However, the amoeboid form which is not common is considered as more pathogenic than the other forms as it is mostly encountered in the samples of symptomatic individuals and old cultures (Zierdt, 1991; Tan, 2008; Moosavi *et al*., 2012). The capacity of this protozoan to transition between several morphologies may play a vital role in its adaptability to different environments and ability to cause disease (Ajjampur & Tan, 2016). *Blastocystis* isolates from varying hosts have been reported to have very similar morphology (Beghini *et al.*, 2017). As a result of these morphological variations, it is difficult to distinguish between subtypes or identify novel subtypes based on the morphological features (Stensvold *et al*., 2007).

Consequently, the genetic polymorphism through variations in nucleotide sequences of the small subunit ribosomal RNA (SSU rRNA) gene has emerged as the most reliable and precise method for *Blastocystis* classification (Clark, 1997; Stensvold *et al.,* 2007; Alfellani *et al.,* 2013a; Yoshikawa *et al.,* 2016; Liu *et al.,* 2022). Yoshikawa *et al.* (2016) have reported the isolation of *Blastocystis* organisms exhibiting substantial genetic heterogeneity within the same host species. Additionally, genetically identical isolates have also been recovered from different host species. The assignment of species epithets to *Blastocystis* isolates based on their host origin is deemed impracticable and misleading (Stensvold *et al.,* 2007; Tan, 2008;

Clark *et al*., 2013). To resolve this challenge, Stensvold *et al.* (2007) presented a nomenclature approach that involved the classification of genomic variations as subtypes (ST) of *Blastocystis* with each subtype being designated a numerical value. *Blastocystis* has infected at least one billion individuals globally, making it the most often found micro-eukaryote in the human large intestine (Scanlan *et al.,* 2014; Stensvold, 2015; Ramírez *et al.,* 2017). The prevalence of *Blastocystis* in Europe is approximately 20%, and it is anticipated to exceed 50% in numerous developing nations (Bart *et al.,* 2013; El Safadi *et al.,* 2016). Infection of *Blastocystis* occurs through ingesting food or drinking water that has been contaminated with the cyst form (Ajjampur & Tan, 2016). Identifying the transmission route will aid the interruption and subsequent control of the spread of this parasite.

Water contamination has been identified as a risk factor for acquiring *Blastocystis*. Presence of *Blastocystis* in both water and humans that use it has been studied leading to the detection of overlapping subtypes (Leelayoova *et al*., 2008; Angelici *et al*., 2018; Pawestri *et al.,* 2021). *Blastocystis* has been detected in drinking water (Leelayoova *et al*., 2008), tap water (Eroglu & Koltas, 2010; Jinatham *et al.,* 2022), rainwater tanks (Noradilah *et al*., 2017; Waters *et al*., 2019), bodies of freshwater (Ithoi *et al*., 2011; Khalifa *et al*., 2014), as well as drinking water treatment facilities (Richard *et al*., 2016). These investigations which have demonstrated the presence of *Blastocystis* in water sources indicate that humans and other animals can contract *Blastocystis* by consuming or using water that has been contaminated by the parasite (Ithoi *et al.,* 2011). Varying concentrations of *Blastocystis* cysts can lead to infectivity rates ranging from 10% to 100%. According to a paper by Yoshikawa *et al.* (2004), *Blastocystis* cysts can remain viable for a period of 19 days to 1 month in water at a temperature of 25°C, and for 2 months at 4°C. This robustness of *Blastocystis* is a

thing of concern. Thus, the ingestion of polluted water can result in the infection of humans or animals by *Blastocystis* (Tan, 2008).

Furthermore, researchers have found viable cysts of *Blastocystis* in sewage samples from Scotland and Malaysia (Suresh *et al.,* 2005). This indicates that if septic tanks are not properly handled or raw sewage enters a watershed, this parasite could contaminate the environment. Molecular approaches were effectively employed to identify *Blastocystis* that were found in wastewater samples collected in the Philippines (Banaticla & Rivera, 2011). These investigations are crucial as they establish that *Blastocystis* cysts may be identified in sewage and wastewater, indicating the potential for this parasite to live in water and potentially cause infection if consumed.

To date, there are about five previous studies on *Blastocystis* occurrence in water sources in Malaysia (Suresh *et al.,* 2005, 2009; Ithoi *et al.,* 2011; Noradilah *et al.,* 2016, 2017). These studies opened the discourse on the detection of *Blastocystis* in water samples while focusing on, sewage, rivers, lakes, tap water, water in tanks, wells, a small stream, fish ponds, and water stored outside a house. Molecular approaches were also employed using the sequence-tagged site (STS) and DNA barcoding methods (Suresh *et al*., 2009; Noradilah *et al*., 2016). Of the 11 *Blastocystis* ST so far isolated from various water sources across the world, seven STs including ST1 - ST5, ST8 and ST10 have been detected in water sources in Malaysia (Attah *et al.,* 2023). Some of the studies also beamed light on the association of physicochemical parameters with *Blastocystis* occurrence in water sources (Ithoi *et al*., 2011; Noradilah *et al*., 2016, 2017).

## 1.2 Justification of the study

Water sources are significant in every society, including Malaysia. However, waterborne parasitic diseases threaten human and animal lives worldwide (Bourli *et al.,* 2023). Available data on outbreaks of waterborne parasitic protozoa reveal that *Blastocystis* accounted for 0.6% of global outbreaks in an early study by Karanis *et al*. (2007); 8.1% between 2004 and 2010, and 0.6% between 2017 and 2022 (Baldurson & Karanis, 2011; Bourli *et al.*, 2023). It is estimated that 58% of diarrhoeal diseases can be attributed to unsafe water supply, sanitation and hygiene (World Health Organisation - WHO, 2017). Studies have been carried out on *Blastocystis* occurrence in some water sources in Pahang, Selangor and Kuala Lumpur (Suresh *et al.,* 2005, 2009; Ithoi *et al.,* 2011; Noradilah *et al.,* 2016, 2017; Lee *et al.,* 2017). However, there is a paucity of data on *Blastocystis* occurrence in water sources in Malaysia, as a larger part of Peninsular Malaysia have not been covered. The perspective of the Sustainable Development Goal makes it imperative to screen water sources for their safety, both for human consumption and use (Weststrate *et al.*, 2018). Among the 17 SDGs, goal six focuses on clean water and sanitation. Consuming water that is contaminated with waterborne pathogens like *Blastocystis* could result in a range of health complications. Consequently, it is crucial to survey and determine the status of water sources with *Blastocystis* contamination and then implement a well-planned system to control it in the future. This is in the pursuit of Goal Six of the SDGs.

Ultrastructural studies on *Blastocystis* isolates from water sources are lacking. Earlier ultrastructural studies of the parasite focused on isolates from humans and animals (Teow *et al*., 1992; Cassidy *et al*., 1994; Stenzel & Boreham, 1996; Singh *et al*., 1996; Yoshikawa *et al*., 2004; Chandrasekaran *et al*., 2014; Sanggari *et al.*, 2023). The surface characteristics of *Blastocystis* are thought to be important for cellular

nutrition, pathogenicity, and the immune system by the parasite (Cassidy *et al.,* 1994; Stenzel & Boreham, 1994; Zaman *et al.,* 1997, 1999; Sanggari *et al.*, 2023). However, ultrastructural studies on *Blastocystis* isolates from water sources are lacking. Therefore, it is essential to study the morphology, surface characteristics and ultrastructure of *Blastocystis* isolates from water sources.

Human and potentially zoonotic STs of *Blastocystis* have been reported in water sources in Malaysia, and it has been observed that users of such water and the animals that drink them are at risk of *Blastocystis* infection (Tan, 2008; Lee *et al*., 2012; Jinatham *et al.*, 2021, 2022; Attah *et al*., 2023). There is, however, a paucity of considerable data on the subtype distribution and predictors of *Blastocystis* in water sources in Malaysia. Therefore, genetic characterisation of *Blastocystis* isolates recovered from water sources using the DNA barcoding method and evaluation of the evolutionary relationships among *Blastocystis* found in water sources with animals or human isolates will suggest possible sources of *Blastocystis* contamination in water sources and enhance possible control measures.

Physicochemical parameters and biological indicators such as faecal coliform are known to either facilitate or inhibit the occurrence of parasites in water sources or have no effect at all on their occurrence. Faecal coliform and several physicochemical parameters such as dissolved oxygen, temperature, turbidity, conductivity, chemical oxygen demand (COD), total dissolved solids (TDS), and concentration of sulfate have been associated with the occurrence of *Blastocystis* in water sources (Ithoi *et al.*, 2011; Noradilah *et al*., 2016; 2017). A correlation between these parameters with *Blastocystis* occurrence will provide guidance in the prediction of *Blastocystis* occurrence in water sources.

## 1.3 Research objectives

This study embarks on the following objectives:

1. To determine the occurrence of *Blastocystis* in various selected water sources in northern states of Peninsular Malaysia.

2. To validate the morphological characteristics of *Blastocystis* isolated from several selected water sources in northern states of Peninsular Malaysia.

3. To characterize genetically positive *Blastocystis* isolates recovered from water sources using the DNA barcoding method and evaluate the evolutionary relationships among *Blastocystis* found in water sources with animals or humans isolates to postulate the possible source of *Blastocystis* contamination of the water source.

4. To determine the association between *Blastocystis* infection from water sources with the physicochemical parameters and faecal coliforms presence.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Taxonomy of *Blastocystis*

### 2.1.1 Historical background

The first documented reference of *Blastocystis* is believed to have occurred in 1849 from England (Brittan, 1849) and America (Swayne, 1849) during epidemics in their respective nations. The organisms that were found were recognised as annular bodies and cholera bodies, respectively. Nevertheless, their illustrations of the organisms have a resemblance to the eggs of *Ascaris lumbricoides* (Brittan, 1849) and *Trichuris trichiura* (Brittan, 1849; Swayne, 1849; Yakimoff, 1923; Zierdt, 1991a; Parija & Jeremiah, 2013). Since its discovery in the mid-nineteenth century, the taxonomy of *Blastocystis* has been a subject of controversy. This is because the organism's simple round shape resembles that of many other organisms, and the limited capabilities of microscopes at the time may have contributed to confusion in its identification and drawing. Several years after the discovery of *Blastocystis*, another scientist identified it as a coccidian (Perroncito, 1899). Though the accounts of these scientists showed variability in morphology, they agreed that the said organism was a parasite.

The characterization and identification of *Blastocystis* began in the mid-1800s. However, there were several periods when no reports of the organism were made from 1894 to 1899 (4 years), 1899 to 1911 (12 years), 1938 to 1967 (29 years), and 1967 to 1972 (5 years). The reasons for these gaps in reporting are not clear. Nonetheless, the confusing morphology of *Blastocystis* likely led to misidentifications, and the non-discovery of its pathogenic status during the period caused a lack of interest.

More progress was made in the early 1900s when Alexeieff (1911) described and named the organism *Blastocystis enterocola* and classified it as yeast. In the same study, it was hypothesized that the observed organism might also be a degenerated cyst form of *Trichomonas intestinalis*, *Chilomastix mesnili* or *Endolimax nana* (Alexieff, 1911). This was followed by the works of Brumpt (1912) who independently hypothesized that it was a harmless saprophytic yeast of the digestive system and called it *Blastocystis hominis* as the samples was obtained from human, changing the specific epithet. However, *Blastocystis* has no characteristics that strongly associate it with yeast. Unlike yeast, the organism did not grow on a fungal media and was not susceptible to antifungal drugs. Conversely, it responded to antiprotozoal drugs. Later, researchers conducting epidemiological studies in different places identified the organism they discovered as *B. hominis* (Lynch, 1917; Swellengrebel, 1917; Wenyon & O'Connor, 1917). Specifically, Wenyon & O'Connor (1917) were engaged in epidemiological work on British soldiers in Egypt when they identified *B. hominis*. Subsequently, the organism was identified as *B. hominis* or *Blastocystis* by other scientists during the 1920s with occasional modifications in the 1930s. However, the name *B. hominis* became widely accepted in the 1960s and continues to be commonly used today.

From 1922 to 1989, many significant findings and advancements were made in the studies conducted on *B. hominis*. The parasite was identified in several animals including flies, snakes, rodents, and cockroaches (Mazza, 1922). However, this discovery went unrecognised and was subsequently disregarded by other researchers. In the same year, Lynch successfully cultivated *B. hominis* and in the subsequent year, he documented that the organism could induce intestinal inflammation (Lynch, 1922; 1923). In 1927, a case of misidentification occurred where the disease known as

blackhead in turkeys, caused by the parasite *Histomonas meleagridis* was mistakenly labelled as *B. hominis* (Rosenbusch, 1927). This could be attributed to the consistent documentation of this organism over that specific period, as well as its comparable morphology to that of *B. hominis*.

In another development, Micheletti and Cicchitto identified a newly found organism as *Blastocystis jalinus* (Micheletti, 1932; Cicchitto, 1937) in their respective studies. Cicchitto (1937) hypothesized that the organism he discovered could potentially be a degenerated or cystic form of *T. intestinalis, C. mesnili, or E. nana*. The subsequent year proved to be a pivotal moment for *B. hominis*. The study conducted by Ciferri & Redalli (1938) identified *B. hominis* as a non-photosynthetic alga belonging to the genus *Prototheca*. This finding was widely accepted by the scientific community.

Furthermore, Zierdt (1967) classified the organism as a protozoan based on its distinctive protozoan features. Nevertheless, it was reclassified as a phycomycete fungus, a kind of yeast (Wolyńska & Soroczan, 1972). Subsequently, the organism was classified as a sporozoan, a type of protozoan that lacks any form of locomotion (Zierdt and Tan, 1976a & b). Zierdt (1983) established the protozoan characteristics of *B. hominis* through a sequence of experiments seven years after the initial discovery. During one of the tests, *B. hominis* was subjected to antiprotozoal drugs *in vitro* and the reaction of the organism was monitored. Based on this discovery, it was concluded that *B. hominis* is a protozoan parasite capable of infecting human intestinal tract. Later, *B. hominis* was categorised as a protozoan belonging to the Sarcodina subphylum (Zierdt, 1988). In the subsequent year, *B. hominis* was classified as a protozoan based on a comparison of ribosomal ribonucleic acid (rRNA) sequences conducted by Johnson and colleagues. Nevertheless, Arisue *et al*. (2002) classified the parasite within the

Stramenopiles group based on a study of the small subunit rRNA phylogeny. Further modifications to the classification or grouping of *Blastocystis* may still evolve. However, it is presently widely acknowledged that this parasite belongs to the Stramenopiles group, also known as heterokonts, which includes algae, diatoms, and water moulds (Keeling *et al*., 2005).

The phylum Heterokontophyta, otherwise referred to as Stramenopila, is a diverse and intricate evolutionary group comprising many organisms such as brown algae, golden-brown algae, diatoms, slime nets, water moulds, and oomycetes (Silberman *et al*., 1996; Arisue *et al*., 2003; Ahmed & Karanis, 2018). The taxonomic group Stramenopiles, in conjunction with Alveolata & Rhizaria, constitutes the eukaryotic supergroup SAR, according to Gentekaki *et al*. (2017). The Stramenopila is a highly diversified group of eukaryotes that encompasses both unicellular and multicellular protists. The membership of this group consists of heterotrophs, including flagellates that are free-living, parasites of plants (such as *Peronospora*), parasites of animals (such as *Phythium insidiosum*), and organisms that resemble fungi in terms of their morphology and ecological characteristics. Additionally, there are several autotrophs in this group, primarily algae (Derelle *et al*., 2016). The Stramenopiles exhibit distinctive features, including mitochondria with tubular cristae. Additionally, they possess tripartite tubular hairs, which are unique among eukaryotes, found either on their cell surface or predominantly on their elongated anterior flagellum.

*Blastocystis* is a highly peculiar Stramenopile. The organism has tubular cristae within its mitochondria, but it functions anaerobically. It does not possess flagella or flagella hairs, and its nucleus is distinguished by a crescent-shaped cap of heterochromatin (Silberman *et al.*, 1996; Arisue *et al.*, 2003; Denoeud *et al.*, 2011).

The delay in accurately classifying *Blastocystis* may be due to various factors, including the existence of multiple morphological forms inside its host (Silberman *et al*., 1996) in addition to the absence of typical Stramenopile characteristics (Stensvold & Clark, 2016a). Currently, the group known as Stramenopiles encompasses only one other eukaryotic organism that is capable of infecting humans, namely *Pythium* (Stensvold and Clark, 2016a). According to Stensvold *et al*. (2020), the latest taxonomy and classification of *Blastocystis* are as follows:

**Domain** Eukaryota

**Supergroup** SAR (Stramenopiles, Alveolates, and Rhizarians)

**Group** Stramenopila

**Class** Opalinea

**Order** Blastocystida

**Family** Blastocystidae

**Genus** *Blastocystis*

### 2.1.2 Speciation

The taxonomic classification of *Blastocystis* at the genus level is still to be conclusively defined. Previously, *Blastocystis* isolates obtained from humans were designated as *Blastocystis hominis* (a term which is now obsolete) while isolates from other animal hosts were either identified as *Blastocystis* sp. or in some cases named after their respective hosts, such as *Blastocystis anatis* from domestic ducks, *Blastocystis anseri* from domestic geese, *Blastocystis galli* from chickens, *Blastocystis lapemi* from sea snakes, *Blastocystis pythoni* from reticulated pythons, and *Blastocystis ratti* from rat (Belova & Kostenko, 1990; Belova, 1991; Belova, 1992b,

1992a, 1992c; Stenzel & Boreham, 1996). Nevertheless, a significant number of *Blastocystis* isolates obtained from various hostshave been observed to exhibit a high degree of similarity in terms of their morphological characteristics, as determined using both light and electron microscopy techniques (Noel *et al*., 2005). Therefore, the distinguishing between isolates based on only morphological characteristics has presented difficulties (Tan, 2008). Additionally, it has been discovered that *Blastocystis* isolates obtained from one host species exhibit a wide range of genetic diversity whereas isolates obtained from different host species display genetic homogeneity. Consequently, the taxonomic classification of *Blastocystis* has been challenging (Arisue *et al*., 2003), and the practice of assigning species names based on host specificity is deemed unreliable (Stensvold *et al*., 2007).

### 2.1.3 Subtyping of *Blastocystis*

The proposal of a *Blastocystis* subtype terminology was necessitated by the recognition that the previously indicated appellations were either invalid or contained numerous unique entities (Stensvold *et al*., 2020). The introduction of this subtype nomenclature was conducted by Stensvold *et al.* (2007). In their study, isolates were identified as *Blastocystis* sp. and subsequently classified into subtypes according to the presence of polymorphisms in the SSU rRNA gene. As of the present time, a total of 46 subtypes of *Blastocystis* have been isolated from different hosts and the aquatic environments (Higuera *et al*., 2021; Maloney *et al*., 2022; Santin *et al*., 2023; Attah *et al*., 2023).

While it has not been feasible to distinguish between *Blastocystis* isolates based on their morphological characteristics, there is significant genetic diversity among many isolates from both humans and animals (Noel *et al*., 2005; Tan, 2008). The

nucleotide sequences of the SSU rRNA gene and the elongation factor-1a gene of *Blastocystis* isolates have been shown to have significant genetic variety (Noel *et al*., 2005; Parkar *et al*., 2007; Stensvold *et al*., 2007; Stensvold and Clark, 2020). Therefore, an individual organism is designated as *Blastocystis* subtype *n*, with *n* representing the assigned numerical identifier for the specific subtype it belongs to. According to the phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene, at least 46 subtypes (ST1-ST46) have been found in a variety of hosts namely, arthropods, Aves, amphibians, reptiles, and mammals, and the environment (Alfellani *et al*., 2013a, b, c; Andersen & Stensvold 2016; Asghari *et al*., 2019; Rauff-Adedotun *et al*., 2020; 2021; Hernández-Castro *et al.,* 2023). This was occasioned by the recent division of ST10 into three new STs (ST42-ST44) (Santin *et al.,* 2023).

Nevertheless, four subtypes ST18, ST19, ST20, and ST22, have been queried because of the possibility that they came up from recollections and therefore their improbable appearance (Stensvold *et al.,* 2020). Only ST1-17, 21, and 23-46 met the required criteria for characteristic subtype submissions (Maloney & Santin, 2021). The *Blastocystis* subtyping system does not currently include *Blastocystis* SSU rRNA gene sequence variants isolated from poikilothermic species (Stensvold *et al.,* 2007). Whereas humans have been found to harbour ten different *Blastocystis* subtypes, namely STs 1 - 9, and ST12 (Clark *et al.,* 2013; Ahmed & Karanis, 2018; Lhotská *et al.,* 2020; Stensvold *et al.,* 2020). Among these subtypes, ST9 had been thought to be exclusively found in humans. Conversely, ST10, ST11, and ST13 to ST32 have only been identified in non-human hosts as documented in studies by Stensvold *et al.* (2009), Parkar *et al.* (2010), and Higuera *et al.* (2021). Nonetheless, a study conducted by Noradilah *et al.* (2017) documented the identification of ST9 in chickens in Malaysia. Similarly, Ma *et al.* (2020a) reported the isolation of ST9 from ring-tailed

lemurs in China. Furthermore, recent research by Khaled *et al.* (2021) revealed the presence of ST10 and ST14 in healthy school children in Senegal, while ST16 was identified in children in Colombia by Osorio-Pulgarin *et al.* (2021). Interestingly, some of these subtypes including ST1 - 8, ST10, ST23 and ST26 have been detected in various water environment (Attah *et al.,* 2023).

Various molecular methods have been designed worldwide to detect the genetic diversity of *Blastocystis*. These include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) analyses of PCR-amplified small-subunit (SSU) rRNA, PCR followed by dideoxy sequencing, single-strand conformational polymorphism, and PCR with subtype-specific (sequence-tagged site [STS]) primers (Stensvold *et al*., 2007; Clark *et al*., 2013). As a result, multiple naming patterns were developed to classify the different molecular types of *Blastocystis* that were found. This has led to a confusing range of terminologies for identifying and categorising *Blastocystis* variants/groups (Stensvold *et al*., 2007; Clark *et al*., 2013; Stensvold and Clark, 2020).

A few articles have reported the development of quantitative PCR (qPCR) for *Blastocystis* detection. The initial investigation identified the presence of ST1, ST3, and ST4 (Souppart *et al.*, 2010). Nevertheless, the precise measurement of the assay's specificity and sensitivity, as well as its potential for detecting other subtypes, remains uncertain. The second study identified a genus-specific PCR assay that could detect all known subtypes seen in humans to date (Poirier *et al*., 2012). Regrettably, the specificity of this test was only 95%, and the amplicon size of 339 bp is significantly greater than the optimum size for qPCR sensitivity. A very sensitive and specific TaqMan test for subtypes 1-9 of *Blastocystis* was developed in the most current study conducted by Stensvold and Clark (2020). Despite its high sensitivity and specificity, the current

qPCR method is unable to distinguish between distinct subtypes. Currently, qPCR is the most effective technique for screening *Blastocystis*. However, the only method available for detecting subtypes is conventional PCR isolate subtyping. Creating quantitative polymerase chain reaction (qPCR) techniques that can differentiate between the multiple subcategories present in a sample will be advantageous for the purpose of identifying and studying *Blastocystis*, as well as for conducting epidemiological research. Extraction can enhance the efficiency of qPCR, making it more time-consuming but cost-effective. Theoretically, qPCR can be completed within a few hours (Poirier *et al*., 2012).

The proposed nomenclature by Stensvold *et al*. (2007) for categorising *Blastocystis* variants/groups as "subtypes" provided a useful tool for comparing and linking existing results. The classifications were mostly determined by variations in the small-subunit ribosomal RNA gene sequences (Stensvold *et al*., 2007; Hublin *et al*., 2021; Stensvold and Clark, 2020). *Blastocystis* subtypes 1 - 9 were identified by consolidating the several designations used in various studies, such as subtype, (sub)group, clade, and cluster into a consensus number system/terminology as shown in Table 2.1. Subsequently, numerous other subtypes have been suggested. The assignment of subtype labels has been carried out using a numbering scheme that is chronological and relies on the chronology of publication (Maloney & Santin, 2021). The update of *Blastocystis* subtypes from 2007 till date is shown in Table 2.1.

Table 2.1 Correlation of *Blastocystis* subtype designations and suggestion for consensus terminology (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/I | 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? | I, 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 | 9j | - | - | 4 | *Blastocystis* sp. subtype 6 |
| - | Via | VI/4 outliers | - | - | - | - | - | *Blastocystis* sp. subtype 9 |
| VII | VII | 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.*, 2004c).
[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.*, 1998b, 2000).
[e]Ribodemes are groups that share the same SSU-rDNA PCR–RFLP patterns and are described by (Clark, 1997; Yoshikawa *et al.*, 2000).
[f]Ribodemes in bold are those originally described by Clark (Yoshikawa *et al.*, 2004c).
[g]Subgroups described by (Böhm-Gloning *et al.*, 1997) on the basis of PCR–RFLP analysis and partial SSU-rDNA sequences.[h]Clusters described by (Stensvold *et al.*, 2006) on the basis of 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 equivalence is probable but not proven. [m]Subgroup VI described by (Thathaisong *et al.*, 2003) equals ribodeme 10 described by (Yoshikawa *et al.*, 2000).

## 2.2 Biology of *Blastocystis*

### 2.2.1 Morphology of *Blastocystis*

*Blastocystis* has been reported to show morphological polymorphism. This has been described by various authors based on light and electron microscopy. Each morphological form varies in size and certain features (Suresh *et al.,* 2009; Jeremiah & Parija, 2013). These forms have been isolated and described from different hosts and the environment (Suresh *et al.,* 2005, 2009; Lee *et al.,* 2019). In initial accounts, *Blastocystis* was primarily characterised as spherical cells with a diameter ranging from 5 to 20 μm. These cells exhibited a prominent central body, also referred to as a central vacuole, internal body, or reserve body. Additionally, they possessed a thin outer layer of cytoplasm, multiple nuclei with condensed chromatin caps, cytochrome-free mitochondria, and a thick, mucilaginous coating enveloping the organism. Boreham and Stenzel (1993a) as well as Stenzel and Boreham (1996) have also documented the presence of smaller cells containing glycogen and lipid inclusions, characterized by thick walls and the absence of vacuoles. These cells are believed to represent a resilient stage.

The morphology of *Blastocystis* varies depending on the environment of the parasite, and ultrastructural investigations reveal the existence of a range of different forms of *Blastocystis* (Stenzel & Boreham, 1996; Tan & Suresh, 2006). Additional research has demonstrated that the shape and structure of *Blastocystis* can be influenced by several physical factors, including changes in osmotic conditions, the presence of specific medications, and the metabolic state. These effects have been observed both in living organisms (*in vivo*) and in laboratory settings (*in vitro*) (Boreham & Stenzel, 1993; Stenzel & Boreham, 1996).

### 2.2.1(a) Cyst form

The cyst (Figure 2.1) is the smallest of the morphological form of *Blastocystis,* and it is believed to be the infective stage of the parasite due to its robustness and viability (Suresh *et al.,* 2009 Poirier *et al.,* 2011; Ahmed & Karanis, 2018; Attah *et al.,* 2023). It measures between 3 - 10µm, is mostly spherical or ovoid in shape and is protected by a thick wall and condensed cytoplasm like what is obtainable in other protozoan cysts (Stenzel & Boreham, 1996; Mehlhorn, 1998). It is surrounded with a fibrillar layer which is usually shed upon maturation (Zaman *et al*., 1997). The cyst lacks a central vacuole and possesses one to four nuclei (Stenzel & Boreham, 1996; Zaman *et al.,* 1997).

It exhibits resilience against stressors linked to osmotic and temperature conditions. Specifically, it has been observed to endure for a duration of 19 days when exposed to water, one month at a temperature of 25°C, and two months at a temperature of 4°C (Tan, 2008; Jeremiah & Parija, 2013; Adao & Rivera, 2018). They are resistant to chlorine and may be able to withstand gastric juices (Sighn *et al.,* 1995; Zaki *et al.,* 1996). Notwithstanding this viability of the cyst, they are unstable when exposed to extreme temperatures such as -196, -70, -20, 4, 40, 50, 60 and $70^0$C, and some disinfectant solutions (Moe *et al*., 1996). Although the cysts of *Blastocystis* are commonly found in fresh faeces, long-term culture and stored faecal specimens, they have also been isolated from sewage samples (Stenzel & Boreham, 1991; Suresh *et al.,* 2005).

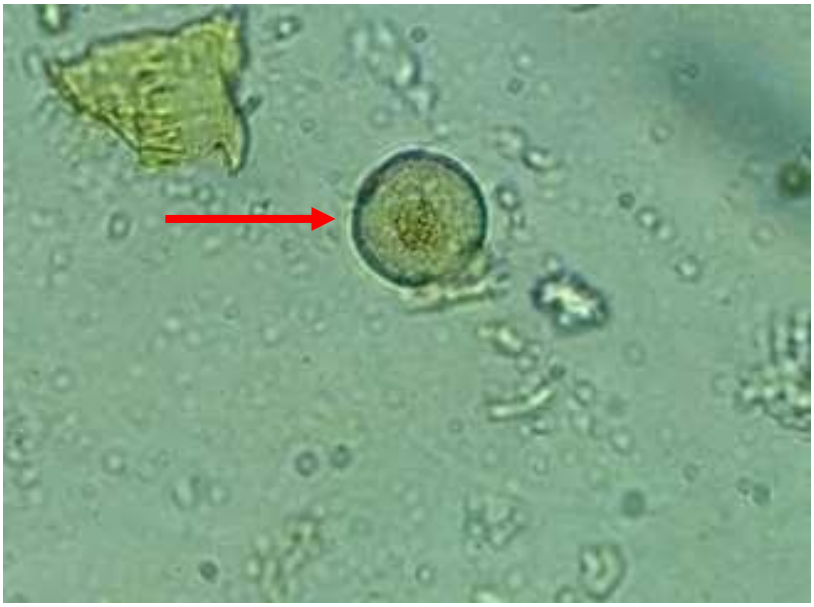

Figure 2.1 Light microscopic view of unstained cyst form of *Blastocystis* (red arrow) (400x magnification) (Ifqiyyah *et al.*, 2021).

### 2.2.1(b) Vacuolar form

The vacuolar form (Figure 2.2) is the most easily recognizable and frequently encountered form of *Blastocystis* in *in vitro* cultivation and stool (Stenzel & Boreham, 1996; Tan, 2008; Wawrzyniak *et al.,* 2013). This morphological form is characterised by the presence of a prominent central vacuole that fills a significant portion of the cellular volume, hence restricting the cytoplasm and other intracellular constituents to a narrow peripheral region. Cellular organelles like the nucleus, golgi apparatus, and mitochondria-like structures (MLS) are also found in the peripheral cytoplasm. The observed morphological shape exhibits a wide range of sizes, with diameters spanning from 2 µm to more than 200 µm (Stenzel & Boreham, 1996; Jeremiah & Parija, 2013). According to some studies, vacuolar forms that have been isolated from humans can vary in size from 4 µm to 63 µm, with the bulk of the isolates having a diameter of 5 to 15 µm (Dunn *et al.,* 1989; MacPherson & MacQueen, 1994). There have also been reports of size variation between and within certain *Blastocystis* subtypes isolated from

humans (Karamati *et al.,* 2019). However, there is no data on the sizes of vacuolar forms of *Blastocystis* isolated from water sources.

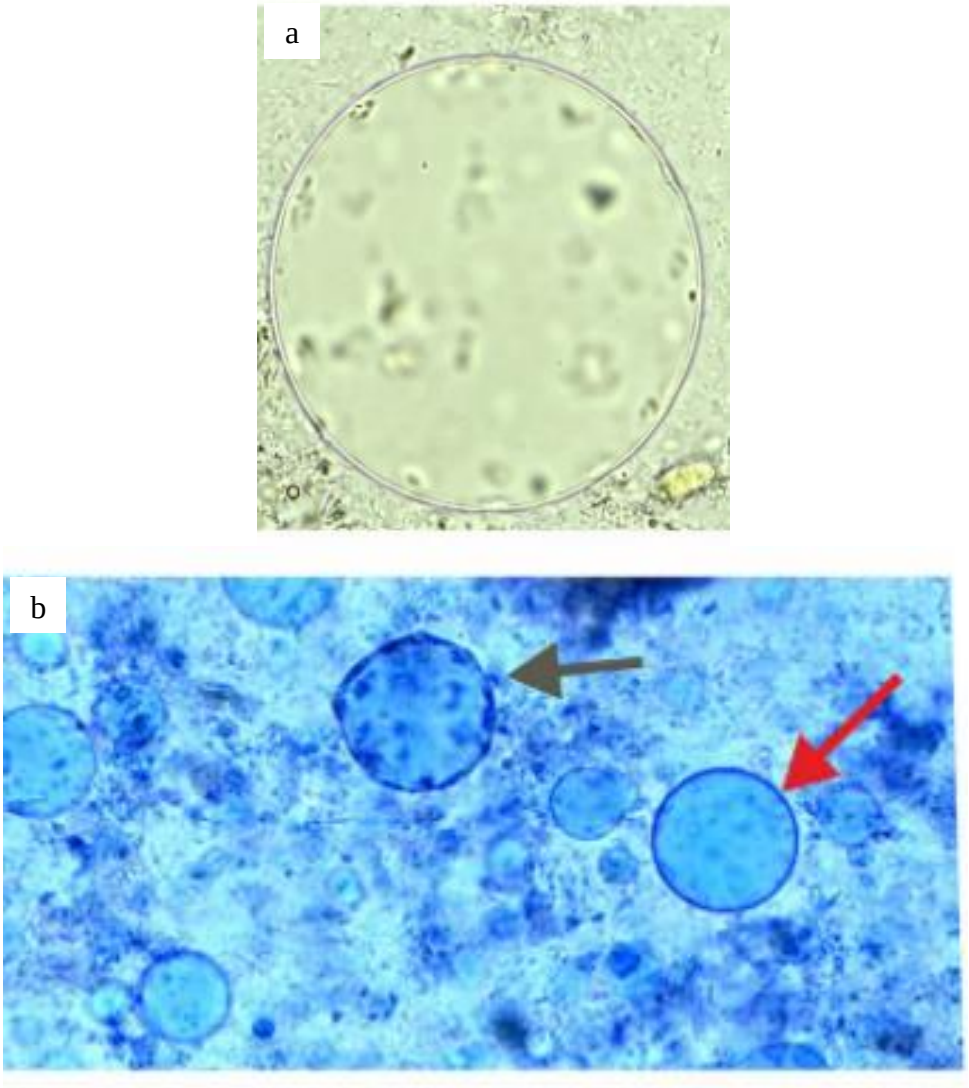


Figure 2.2 Vacuolar form of *Blastocystis* (a) unstained and (b) stained with methylene blue (red arrow) as viewed under a light microscope (x400 magnification) (Boutahar *et al*., 2023).

### 2.2.1(c) Granular form

The granular form of *Blastocystis* (Figure 2.3) is also usually seen in faecal samples and cultures (Ajjampur & Tan, 2016). It is similar in structure to the vacuolar form except that it possesses morphologically and cytochemically different central vacuole contents making some authors suggest that the granular form of *Blastocystis* is

more or less a vacuolar form with granules (Stenzel & Boreham, 1996). Homogeneous material within the central vacuole of the vacuolar form becomes more compact and denser in the granular form (Suresh *et al.,* 2009; Adao & Rivera, 2018;). The central vacuoles of the granular form have granules which are sometimes observed in its cytoplasm (Rojas-Velázquez *et al.,* 2022). Three types of granules including metabolic granules which are found exclusively in the cytoplasm; lipid granules which are seen in both the cytoplasm and the central body, and reproductive granules which are found only within the central body, have been reported by electron microscopic studies (Tan & Zierdt, 1973; Jeremiah & Parija, 2013). These forms are somewhat larger than the common vacuolar forms, and diameters of 10 to 60 µm, 15 to 25 µm, 3 to 80 µm, and 6.5 to 19.5 µm have been observed (Zierdt, 1991; Stenzel & Boreham, 1996).

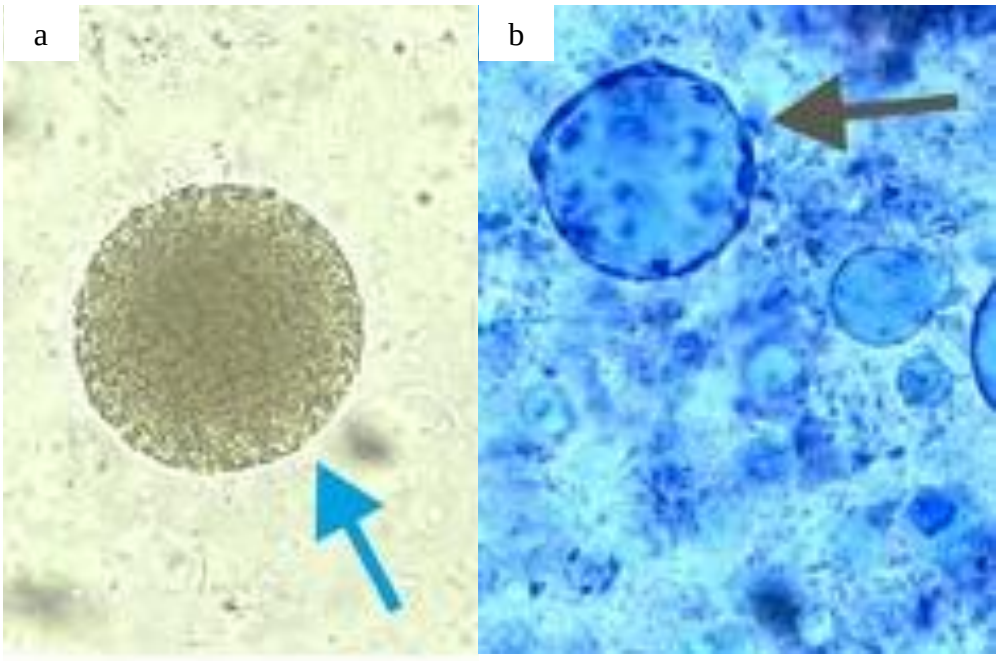


Figure 2.3 Granular forms of *Blastocystis* under the light microscope (a) observation of unstained (blue arrow) and (b) methylene blue stained (black arrow) (x400 magnification) (Boutahar *et al*., 2023).

### 2.2.1(d) Amoeboid form

The amoeboid form (Figure 2.4) is less frequently encountered in cultures compared to the vacuolar and granular forms. They have irregular shapes and measure about 10 µm in size (Tan & Zierdt, 1973; Jeremiah & Parija, 2013). Although this form of *Blastocystis* possesses one or two pseudopodia, they are not motile (Tan, 2008). According to Suresh *et al.,* (2009), this form of the parasite lacks certain organelles including the Golgi complex, surface coat, coated pits, and mitochondria. The ameboid forms are often seen in the faecal samples of symptomatic patients suggesting that it is pathogenic (Adao & Rivera, 2018; Jeremiah & Parija, 2013). According to some scholars, the amoeboid forms are mostly reported in patients with urticarial (Katsarou-Katsari *et al.,* 2008; Karamati *et al.,* 2019). The presence of the amoeboid form in the culture colony growths of drug-treated samples signifies that it is a form of self-protective structural adaptation (Suresh *et al*., 2009, Attah *et al*., 2023).

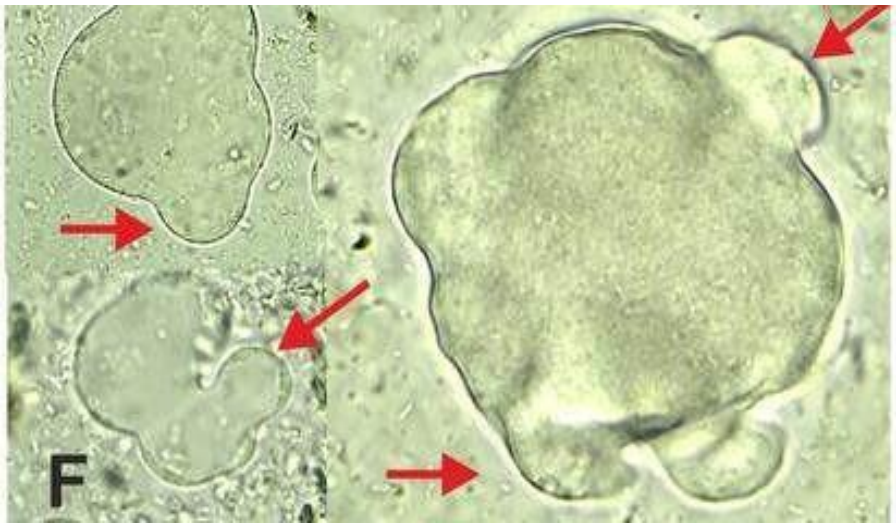


Figure 2.4 Light microscopic view (x400 magnification) of unstained amoeboid forms of *Blastocystis* with two or multiple pseudopodia (red arrows) (Boutahar *et al*., 2023).

### 2.2.2 Mode of transmission and life cycle of *Blastocystis*

The full understanding of the life cycle of *Blastocystis* remains unclear. However, recent research has provided more insight into the transmission and morphological transformation of the parasite (Adao & Rivera, 2018). It is now widely accepted that the cyst form of *Blastocystis*, which is water and environmentally resistant, is the transmissible form of the organism (Tan, 2008; Wawrzyniak *et al.,* 2013). *Blastocystis* is mainly transmitted through the oral-faecal route (Adao & Rivera, 2018) although a potential host can acquire the parasite through other means including human-to-human, animal-to-animal, animal-to-human, and human-to-animal routes or through consumption of contaminated food or water (Ithoi *et al.,* 2011; Hublin *et al.,* 2020).

Aquatic environments have been reported as possible transmission routes for *Blastocystis.* According to Abdulsalam *et al*. (2013), prevalence of *Blastocystis* among school children in rural Malaysia significantly correlated with absence of piped water supply. *Blastocystis* ST was detected in human, animal, and water samples from a river in a village in Nepal by Lee *et al.* (2012). This, among many other reports, confirms the status of water sources as a channel for *Blastocystis* transmission.

The suggested life cycle of *Blastocystis* commences when a vulnerable host (human or animal) consumes the cyst form. Within the large intestine, the organism undergoes excystation and transforms into the vacuolar form, which reproduces through binary fission (Tan, 2008). The transformation from vacuolar forms to either amoeboid or granular forms remains unknown, as the process of transitioning between these forms is not yet fully understood (Tan, 2008; Hublin *et al.,* 2020).

Although different modes of reproduction including binary division, budding,