Tropical Biomedicine 41(3): 370-376 (2024)
https://doi.org/10.47665/tb.41.3.018

RESEARCH ARTICLE

Occurrence and ultrastructural surface of *Blastocystis* isolated from water sources in Kedah and Penang, Malaysia

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ARTICLE HISTORY

Received: 25 March 2024
Revised: 14 May 2024
Accepted: 14 May 2024
Published: 30 September 2024

ABSTRACT

Blastocystis is a ubiquitous waterborne parasite that has been implicated in some disease conditions including colorectal cancer and irritable bowel syndrome, and its surface coat characteristics have been associated with its pathogenicity. Although the morphology of *Blastocystis* isolates from human and animal sources have been studied, there is a paucity of data on the surface ultrastructure of *Blastocystis* isolated from water sources. Therefore, this study aimed to determine the occurrence and the ultrastructural surface of *Blastocystis* isolates from several water sources in Kedah and Penang, Malaysia. A total of 12 water samples were collected, namely, Pinang River and USM Harapan Lake all in Penang, whereas Lata Bayu Waterfall in Baling and UniSHAMS Lake, Kuala Ketil in Kedah. These were examined for *Blastocystis* by centrifugation and *in vitro* cultivation. Scanning electron microscopy (SEM) and light microscopy were employed to study the morphological characteristics and the surface ultrastructure of the parasite. Polymerase chain reaction (PCR) was carried out to obtain the subtypes (ST) of the positive *Blastocystis* isolates. The result revealed 25.0% (3/12) contamination with *Blastocystis* in which ST1, ST2, and an unknown ST (with a high similarity to ST1) were detected in water samples from the upstream, downstream, and midstream, respectively of Pinang River. Our study also revealed similarities in the sizes of the isolates from different river points, which were notably more diminutive compared to the sizes of the parasites observed in existing data from human and animal isolates. The surface characteristics showed a collection of single and dividing cells with smooth, folded surfaces enclosed in a film-like layer. Additionally, there were roundish, irregularly shaped cells with rough surfaces, and a woolly appearance. This study has added to our knowledge of the surface ultrastructure of *Blastocystis* and its possible contribution to the pathogenicity of the parasite.

Keywords: *Blastocystis*; contamination; rivers; ultrastructure; water.

INTRODUCTION

Blastocystis is a ubiquitous anaerobic parasite that causes gastrointestinal symptoms in humans, and it is being studied for its potential role in waterborne transmission (Anuar *et al*., 2013; Yu *et al*., 2023). It has been detected in sewage treatment plants and drinking water, raising concerns about waterborne transmission and contamination. Inadequate treatment processes can lead to contaminated water discharge into rivers, lakes, or other bodies of water, potentially affecting water quality (Suresh *et al*., 2009; Rangel-Martínez *et al*., 2015; Javanmard *et al*., 2019; Stensvold *et al*., 2020; Jinatham *et al*., 2021). *Blastocystis* is primarily transmitted through the oral-faecal route and its infection has been associated with poor hygiene and unsanitary conditions, contact with animals, and eating and drinking contaminated food and water (Abdulsalam *et al*., 2012; Lee *et al*., 2012; Rudziɒska *et al*., 2022). It is one of the protozoan pathogens included in the World Health Organization's Guidelines for Drinking-water Quality (World Health Organization - WHO, 2022). *Blastocystis* has been detected in natural freshwater bodies such as rivers and lakes, and in marine environments like seas and lagoons (Attah *et al*., 2023).

Scanning electron microscopy has emerged as a powerful tool to visualize the details of the surface ultrastructure, topography, and distinctions in the surface coats of microorganisms (Widisuputri *et al*., 2021). *Blastocystis* isolates are known for having a fibrillar surface coat, which is thought to be important for cellular nutrition, pathogenicity, and immune system evasion (Cassidy *et al*., 1994; Stenzel & Boreham, 1994; Zaman *et al*., 1997, 1999; Tan, 2008). However, several studies have noted variations in the surface coat characteristics across several isolates of *Blastocystis* and these variations have been found to be a probable cause of the differences in the pathogenic potentials of *Blastocystis* subtypes (Yason & Tan, 2015; Farah Haziqah *et al*., 2018a, 2018b; Sanggari *et al*., 2023). The surface coat has also been found to play a critical role in the interaction between *Blastocystis* and the host's immune system, being the first point of contact between the parasite and the host. It has been observed that essential surface antigens of the parasite are protected against the host's immune system by the surface

coat. Therefore, understanding the structure and composition of the surface coat of *Blastocystis* could provide insights into how the parasite evades the host's immune responses and establishes infection (Tan *et al*., 1997; Yason & Tan, 2018). The ultrastructure of *Blastocystis* has been studied in humans and a variety of animal species using electron microscopy. These investigations have shed light on *Blastocystis's* surface coat and organelles (Cassidy *et al*., 1994; Farah Haziqah *et al*., 2018a, 2018b; Yason & Tan, 2018; Widisuputri *et al*., 2021). However, this has not been extended to *Blastocystis* isolates from water sources.

Also, *Blastocystis* has been detected in animals in northern Peninsular Malaysia (Farah Haziqah *et al*., 2014; Rauff-Adedotun *et al*., 2022). Conversely, there are no records of *Blastocystis* occurrence in water sources in this region. Therefore, this present study aims to analyse the occurrence, morphology, and surface ultrastructure of *Blastocystis* STs in water sources in Kedah and Penang, Malaysia. It is hoped that the findings will contribute to the comprehension of the biology of *Blastocystis* isolates from water sources while also assisting relevant authorities in initiating a control programme against the spread of *Blastocystis* to humans and other animals through water sources.

MATERIALS AND METHODS

Study areas

Water sampling was conducted in Penang (Pinang River, George Town and USM Harapan Lake, Gelugor) and Kedah (Lata Bayu Waterfall, Baling and UniSHAMS Lake, Kuala Ketil). Pinang River located on 5°24N100°19E, is a naturally occurring river situated in the northeastern region of Penang Island. It is regarded as one of the most polluted rivers in Malaysia (Ismail & Hamid, 2023). The river traverses a densely inhabited and extensively developed area of George Town, the capital city of Pulau Pinang State. Meanwhile, USM Harapan Lake situated at coordinates 5.3535°N, 100.3005°E, is a compact artificial tropical lake located at the main campus of Universiti Sains Malaysia, Gelugor. The lake's surface area measures around 6070 square metres, with an average depth of 1.0 – 1.5 meters (Teh *et al*., 2008; Tay *et al*., 2022). The lake exhibits a high concentration of algae and is inhabited by monitor lizards which are also commonly sighted in the vicinity of the campus. Lata Bayu Waterfall is a recreational waterfall located at coordinates 5.7175°N, 100.8140°E just a few kilometers outside Baling in the State of Kedah, Malaysia. The park is well maintained, attracting many visitors on public holidays and weekends whereas UniSHAMS Lake is a man-made lake within the campus of Universiti Islam Antarabangsa Sultan Abdul Halim Mu'adzam Shah (UniSHAMS), Kuala Ketil, Kedah, Malaysia. The lake is also utilised for recreational activities such as boating.

Water samples collection

A sterile container was used to collect one liter of water from three (3) sampling points in each of Pinang River, USM Harapan Lake, Lata Bayu Waterfall and UniSHAMS Lake. The three-sampling points collection was adopted to capture variability in the distribution of *Blastocystis* cysts in the water body. The sampling points were determined by considering the anthropogenic activities around the rivers.

Cultivation and detection of *Blastocystis*

The water samples were poured into 50 ml falcon tubes and were centrifuged at 1400 x g for 10 minutes. The supernatant was discarded, leaving about 1 ml. The pellet was then inoculated in a tube containing 3 ml of Jones' Medium supplemented with 10% heat-activated horse serum and incubated at 37°C for 24-72 hours (Jones, 1946; Suresh *et al*., 2005; Lee *et al*., 2012). A drop of the cultured sample was examined microscopically under 100x and 400x magnification for the presence of *Blastocystis* and if negative, it was subcultured into a new medium and incubated for 24 hours. If there were no *Blastocystis* forms thereafter, the samples were considered negative.

Scanning Electron Microscopy examination

Positive samples by *in vitro* cultivation were sub-cultured for 24 hours and then centrifuged at 500 x g for 5 minutes. Each sediment was resuspended in 1 ml of 2.5% glutaraldehyde and preserved in the refrigerator till further procedure. Subsequently, each sample was centrifuged at 1400 x g for post-fixed with 1% osmium tetroxide. The isolated cells were then mounted on a polycarbonate membrane and dehydrated using a sequential ethanol series of 50%, 75%, 95%, and 100%, and Hexamethyldisilazane (HMDS). Each ethanol step and the HMDS lasted for 10 minutes. Carbon dioxide was employed for critical point drying (CPD), followed by coating the specimen with a layer of gold. The observations were conducted using the Scanning Electron Microscope (SEM) at the Electron Microscopy Unit, School of Biological Sciences, Universiti Science Malaysia as described by Ragavan *et al*. (2014). The electron micrographs were captured by using Zeiss Supra 50vp.

DNA extraction and subtyping of *Blastocystis* isolates

The DNA extraction from the positive culture was performed using the Nucleospin DNA Stool Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. DNA extracted were screened for the presence of *Blastocystis* in a single-step PCR. Based on recommendations by Stensvold (2013), the broad-specificity eukaryote-specific primer RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') was used as the forward primer; and the *Blastocystis*-specific primer BhRDr (5'-GAGCTTTTTAACTGCAACAACG-3'), targeting the small subunit (SSU) ribosomal RNA (rRNA) gene of *Blastocystis* was used as the reverse primer (Scicluna *et al*., 2006). These primers amplify a 600 bp region of the SSU rDNA that contains sufficient information for unambiguous assignment of STs to samples (Scicluna *et al*., 2006). The PCR was performed in a 50 µl reaction volume containing 25 µl of Vivantis 2X Taq Master Mix, 2.5mM $MgCl_2$, 0.5 µl of each primer and 2 µl of DNA. PCR conditions consisted of an initial denaturing step of 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, 56.3°C for 1 minute 30 seconds and 72°C for 1 minute, then followed by a final elongation step of 72°C at 10 minutes. All PCR amplifications were performed using the Bio-Rad Thermo Cycler (USA). The PCR products were observed on a 1.5% agarose gel, purified and then submitted for Sanger sequencing processes. Later, the nucleotide sequences were analysed using BioEdit software. Phylogenetic tree was constructed with MEGA 11 software based on the obtained multiple alignment by using the maximum likelihood method (ML heuristic method: Nearest-Neighbor-Interchange – NNI, initial tree for ML: neighbour-joining, branch swap filter: very strong, rates among sites: uniform rates, gaps/missing data treatment: complete deletion) and the Tamura-Nei model with 500 bootstrap sampling (Kumar *et al*., 2018). The sequence of *Proteromonas lacertae* (U37108) was used as the outgroup to root the tree. The categorization of subtypes for every *Blastocystis* isolate was established based on the established terminology.

RESULTS

Occurrence of *Blastocystis* in several selected water sources

Blastocystis was detected in all the sampling points in Pinang River in Penang, 100% (3/3). There was zero occurrence of *Blastocystis* in USM Harapan Lake, Penang, and from Lata Bayu recreational waterfall and UniSHAMS Lake in Kedah State (Table 1).

Table 1. Occurrence of *Blastocystis* in several selected water sources in relation to location

Location	Type of water source	No. of sampling points	No. positive (%)	Subtype (ST)
Penang				
Pinang River	Natural river	3	3 (100)	ST1, ST2, Unknown
USM Harapan Lake	Man-made lake	3	0 (0)	–
Kedah				
Lata Bayu Waterfall	Recreational waterfall/river	3	0	–
UniSHAMS Lake	Man-made lake	3	0	–
Total	**4**	**12**	**3 (25)**	**3**

Morphological characteristics of *Blastocystis* isolated from water

In this investigation, the light microscopy analysis of *Blastocystis* isolates showed predominantly vacuolar forms (Figure 2A) with a minority of granular forms. Cells were typically spherical, with a prominent central vacuole displacing the cytoplasm towards the cell's outer edge. Cell sizes differed even within isolates from the same sampling point. The cells were also found to be undergoing binary fission, which is the typical reproduction mode for *Blastocystis* (Figure 2B).

Surface feature of *Blastocystis*-positive isolates

The surfaces of *Blastocystis* cells isolated from Pinang River vary according to the sampling points and within the sampling points. From the upstream was a roundish cell with a somewhat rough surface and a protrusion; dividing cells of the parasite with a smooth surface and a film-like structure which appeared to hold the cells together, a single cell with folds and a collection of cells with smooth surfaces and held together by a film-like layer with a set undergoing binary fission (Figure 3A-C). From the midstream was an oval-shaped isolate with an extreme surface, an irregularly shaped cell with a rough surface, a woolly appearance, and a kind of depression and a collection of cells with smooth surfaces, and held together by a film-like layer, with two (2) sets undergoing division (Figure 4A-C). All the cells had no bacteria attached to them. Isolates from the downstream could not be viewed on the SEM possibly due to cell distortions during processing.

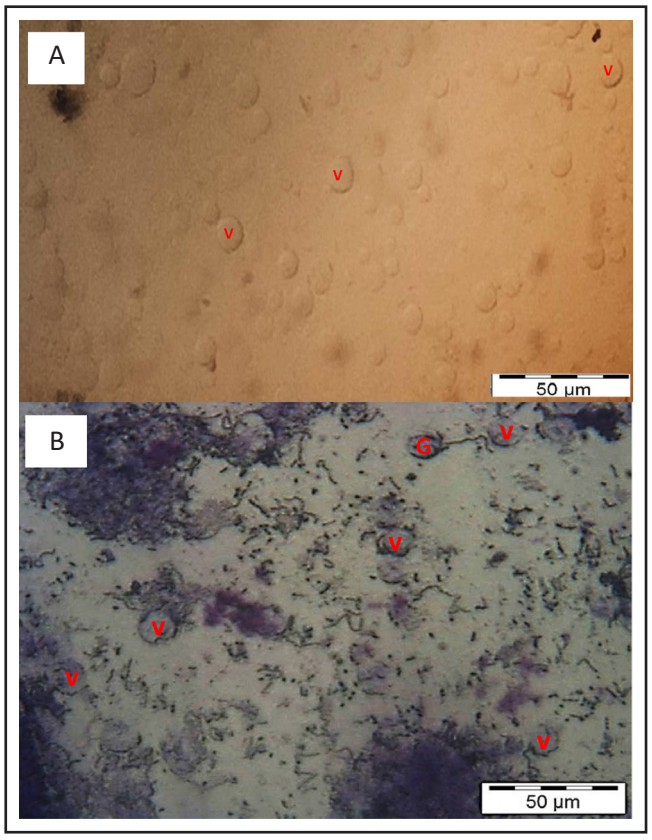

Figure 2. Morphology of *Blastocystis* isolated from Pinang River (A) unstained vacuolar forms; (B) stained vacuolar and granular forms. (V = vacuolar; G = Granular).

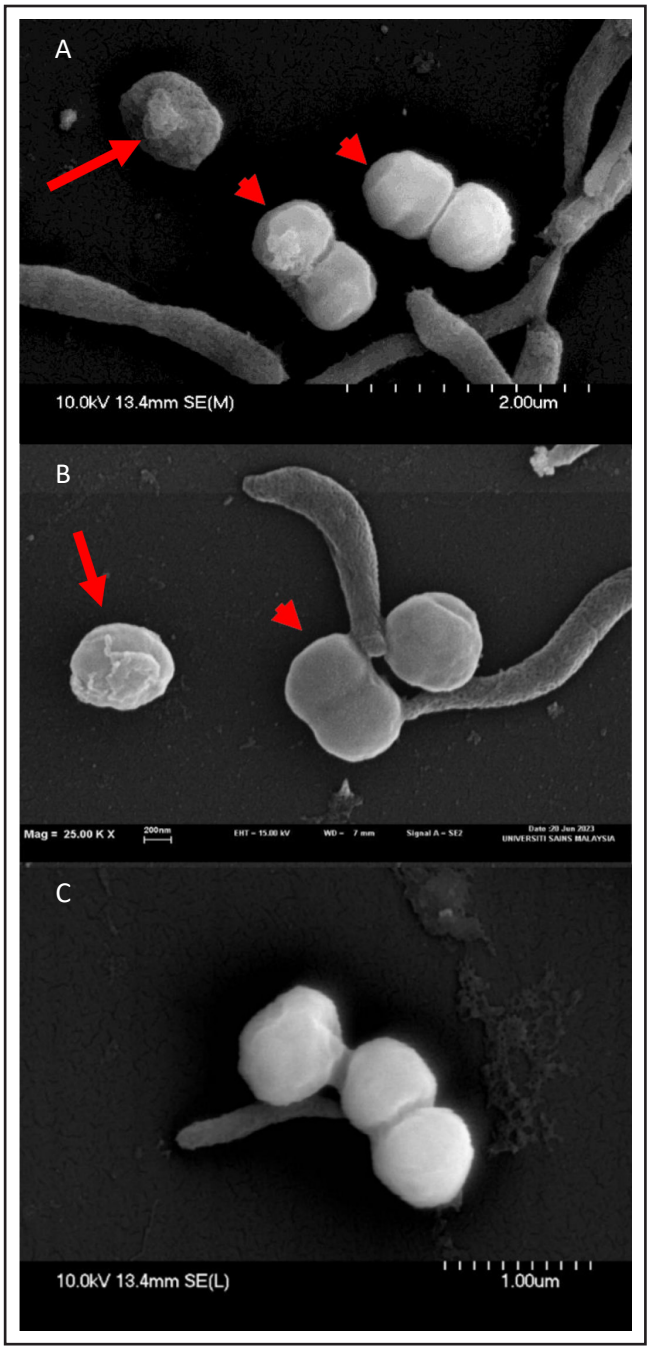

Figure 3. Scanning electron micrograph of *Blastocystis* recovered from Pinang River – Station 1 (upstream). (A) Roundish cell with a somewhat rough surface and a protrusion (arrow). Dividing cells of the parasite with a smooth surface and a film-like structure holding the cells together (arrowhead); (b) smooth surface with a thick fold (arrow). Dividing cells with a smooth surface and a film-like cover (arrowhead); (C) A collection of cells with smooth surface and held together by a film-like layer, with a set undergoing binary fission.

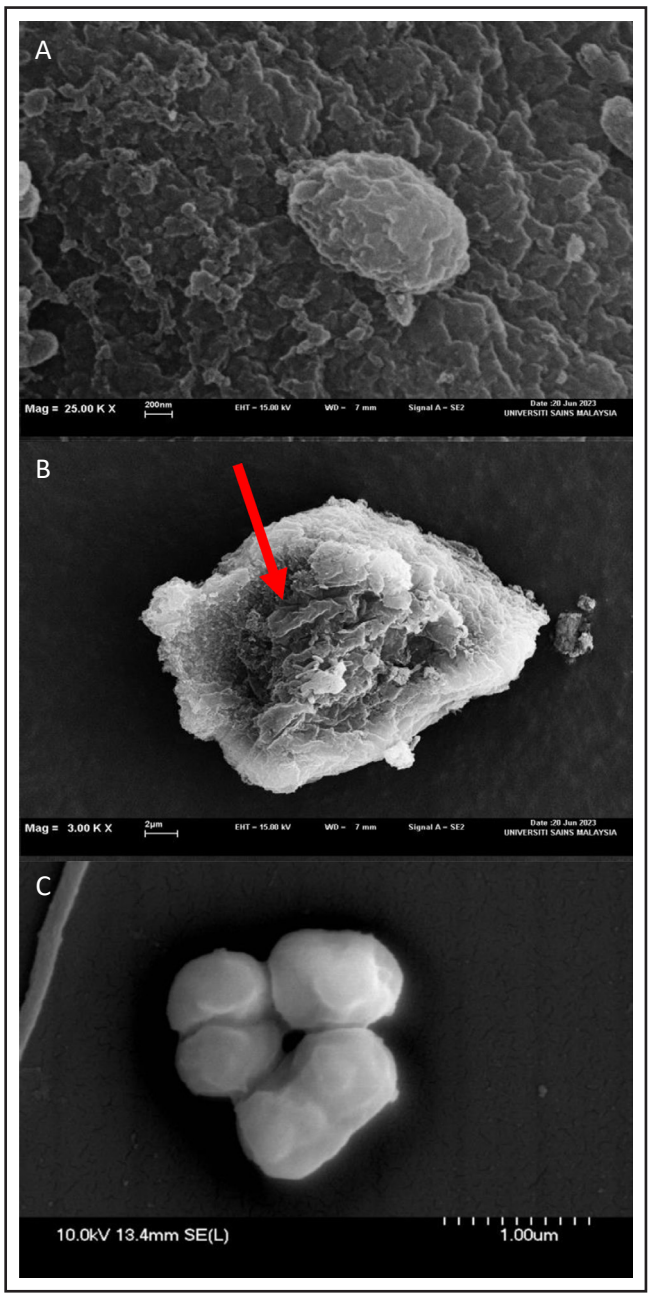

Figure 4. Scanning electron micrograph of *Blastocystis* recovered from Pinang River – Station 2 (midstream) (A) Oval cell with a rough surface and extreme folds; (B) Irregular-shaped cell with a rough surface, a woolly appearance, and a kind of depression (arrow); (C) collection of cells with smooth surface and held together by a film-like layer, with two (2) sets undergoing division.

Subtype identification, alignment and phylogenetic analysis

Based on the amplification of the PCR products using the DNA barcoding primers and sequencing, three subtypes were detected among the isolates from Pinang River. The sequences were subjected to BLAST queries on *Blastocystis* Sequence Typing Databases (https://www.pubmlst.org/blastocystis) and at GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 2). The sequence of the sample SGT1 isolated from the upstream corresponded 100% with a ST1 sequence obtained from GenBank, SGT3 sequences matched with a ST2 sequence while SGT2 sequence matched with a ST1 sequence by 96.13%. The three sequences obtained were deposited in the NCBI GenBank database with the accession numbers OR135774, OR135777 and OR135778. From the phylogenetic tree (Figure 5), the isolate with accession number OR135777 was identical with ST1 sequences MF186699 (goat, England), MH332372 (human, Egypt), MG011609 and MG011607 (human, Iran), MK719675 (human, India), and OM065817 (rabbit, China). The sequence with accession number OR135774 shows a close relationship with ST1 isolates from pig in the Philippines (KY610194) and human isolates from Colombia (MZ396329). On the other hand, the sequence OR135778 closely matched with *Blastocystis* ST2 isolates from humans and rhesus monkeys (*Macaca mulatta*) from Iran and Bangladesh respectively (ON955855 and MN338076).

DISCUSSION

This study has revealed the occurrence of *Blastocystis* in a polluted river in Penang, Malaysia. All the sampling points were positive for the parasite. This confirms the possibility of isolating *Blastocystis* from environmental samples (Puthia *et al*., 2008). Having been noted as one of the most polluted rivers in Malaysia, the detection of *Blastocystis* in Pinang River also validates the association of the parasite with poor environmental sanitation and hygiene (Jiménez *et al*., 2019; Ruzdzińska *et al*., 2022; Attah *et al*., 2023). Although efforts are being made to improve the water quality of the Pinang River, the present detection of *Blastocystis* in it shows that the river is still contaminated and can be a source of *Blastocystis* transmission to animals and humans (Ithoi *et al*., 2011). *Blastocystis* has been detected in other rivers in Malaysia (Ithoi *et al*., 2011; Noradilah *et al.,* 2016, 2017) and elsewhere in the world (Elshazly *et al*., 2007; Eroglu & Koltas, 2010; Lee *et al*., 2012; Koloren *et al*., 2018; Adamska, 2022). However, this is the first detection of the parasite in a water source in Penang and northern Peninsular Malaysia at large.

Scanning electron micrographs showed varying cell shapes and surface textures. Most of the cells were oval and roundish in shape while one had an irregular shape. Dividing cells with smooth cell surfaces like asymptomatic isolates obtained from humans and isolates from invertebrates like cockroaches were also observed (Ragavan *et al*., 2014; Farah Haziqah *et al*., 2017). Coarse, rough, and folded surfaces observed in isolates from individuals with

Table 2. SSU rDNA sequence similarities between *Blastocystis* isolated from Pinang River and GenBank reference sequences

No.	Isolate	GenBank reference sequence	Percentage sequence similarity (%)	GenBank Accession No.
1.	SGT1 (Upstream)	*Blastocystis* sp. subtype 1 isolate ELB_WW_Goat 2 clone 2 small subunit ribosomal RNA gene, partial sequence	100.00	OR135777
2.	SGT2 (Midstream)	*Blastocystis* sp. subtype 1 isolate BF46 small subunit ribosomal RNA gene, partial sequence from Pig	96.13	OR135774
3.	SGT3 (Downstream)	*Blastocystis* sp. subtype 2 isolate ZP-152 small subunit ribosomal RNA gene, partial sequence fromMacaca mulatta	100.00	OR135778

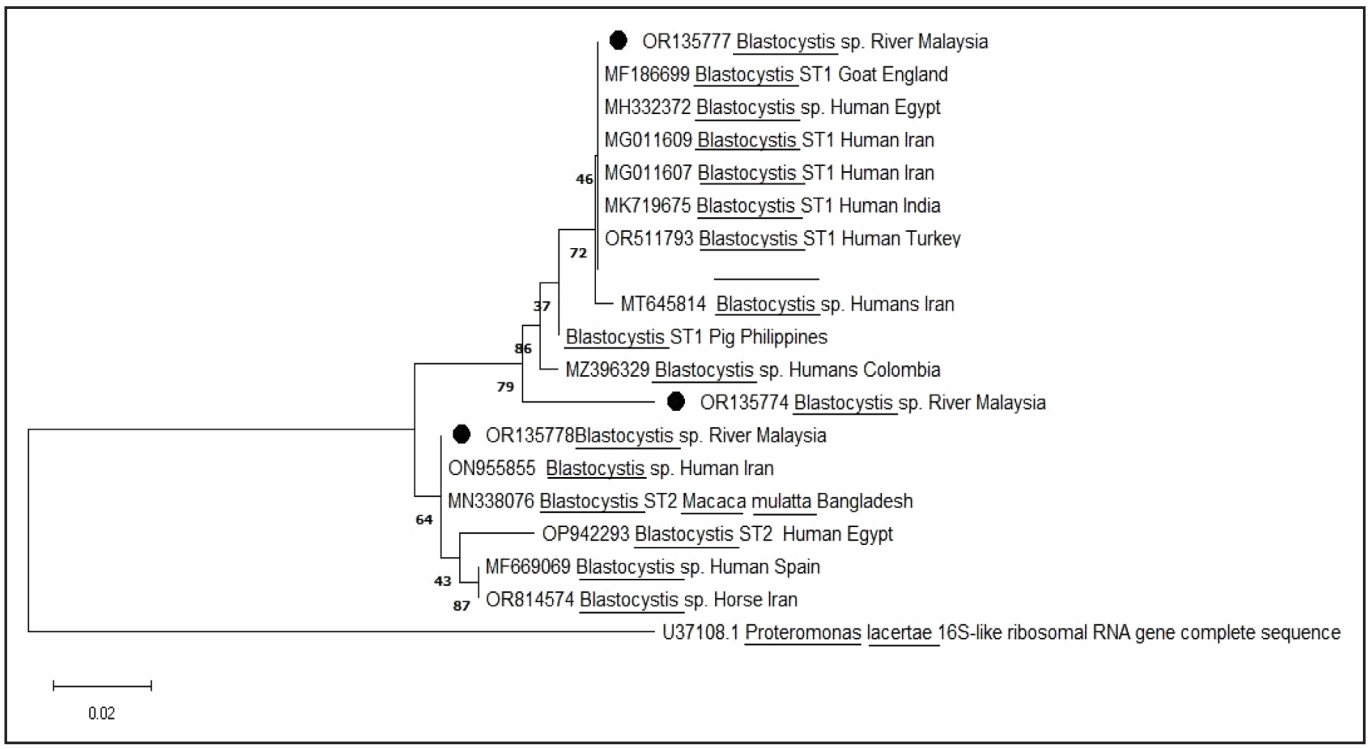

Figure 5. Phylogenetic tree constructed by the Maximum Likelihood approach and the Tamura-Nei Model with 500 bootstrap sampling based on 18 SSU-rRNA gene sequences from GenBank. The tree was rooted from *Proteromonas lacertae* as an outgroup. The Malaysian sequences described in this study are indicated by the black circles.

IBS were also noticed among the cells (Ragavan *et al*., 2014). An irregular-shaped cell with a rough surface, a woolly appearance, and a kind of depression was also observed. The variation in the surface ultrastructure of the isolates suggests that the source of contamination of the river with *Blastocystis* may be from different animals as it has been noted that the surface structure of *Blastocystis* from different hosts shows variability (Sanggari *et al*., 2023). This is the first study on the surface ultrastructure of *Blastocystis* isolates from water sources. The surface of the cells may be an adaptation against the physicochemical parameters of the water.

Blastocystis transmission to people significantly depends on water sources, especially surface water. In a study on the incidence of *Blastocystis* in schoolchildren in a rural community in Thailand, Leelayoova *et al*. (2008) suggested that the water supply inside a school was most likely the cause of the infection. According to Abdulsalam *et al*. (2012), water sources are a significant predictor in the transmission of *Blastocystis.* In a study carried out in Nepal, Lee *et al*. (2012) found *Blastocystis* in both animals and river water. They suggested the likelihood of a zoonotic transmission of *Blastocystis* through the water sources. Many rivers are used as irrigation water for agricultural purposes. When a river contaminated with *Blastocystis* is used for irrigating a vegetable farm, for instance, it could lead to the contamination of the vegetable and subsequent transmission to humans or other animals. Scholars in Iran have detected *Blastocystis* in treated wastewater used for irrigation purposes (Javanmard *et al*., 2019). Besides, *Blastocystis* has also been detected in vegetables from street markets in northern Thailand by Jinatham *et al*. (2023).

The sequences of *Blastocystis* ST in this study fell on the same clade as sequences of *Blastocystis* ST1 and ST2 obtained from humans and animals. In contrast, the third one (OR135774) stands on a different clade while sharing a common ancestor with ST1 suggesting that it might be a novel subtype. This suggests that these isolates are zoonotic aligning with previous findings that animals pose as reservoirs of many subtypes of *Blastocystis* (Banaticla & Rivera, 2011; Koloren *et al*., 2018; Adamska, 2022). This is not surprising as some cattle were seen around the bank of Pinang River where the water samples were collected. The faecal materials from the cattle could be washed into the river upon rainfall and thereby contaminate the river with *Blastocystis* especially if the animals were infected. Moreover, the detection of *Blastocystis* ST1 followed previous findings that ST1 is the most prevalent *Blastocystis* subtype generally in water sources. According to several studies (Leelayoova *et al*., 2008; Eroglu & Koltas, 2010; Banaticla & Rivera, 2011; Lee *et al*., 2012; Noradilah *et al*., 2016; Angelici *et al*., 2018; Koloren *et al*., 2018; Adamska, 2022), it has been found in a variety of water sources in Italy, Turkey, Nepal, Thailand, Philippines, Malaysia, and Poland. However, the revelation from the phylogenetic tree suggests difficulty in determining the exact source of contamination of this river with *Blastocystis*. Notwithstanding, it is evident that poor sanitation and unhygienic conditions promote the spread of *Blastocystis*. While the pathogenic potential of the *Blastocystis* subtypes identified in this study remains unclear, it is important to note that ST1 is considered the most virulent ST based on previous studies (Kaczmarek *et al*., 2017). Additionally, ST2 is commonly associated with human isolates. A phylogenetic analysis conducted by Javanmard *et al*. (2019) revealed that ST2 forms a cluster with other subtype 2 isolates, which have been previously identified in human subjects. However, the ST2 found in the present study forms a cluster with other ST2 isolates from monkeys and horses in addition to humans. This observation strongly suggests a possible link between the presence of ST2 in the water sample collected from Sungai Pinang with human and animal sources. Essentially, this correlation with human sources is also supported by the observation of household wastewater being discharged into the river.

CONCLUSION

This study has presented data on the occurrence and surface ultrastructure of *Blastocystis* STs in a polluted river that passes through a densely populated area in Penang, Malaysia. The variation in the surface ultrastructure of the parasite suggests that the

river was contaminated with *Blastocystis* from different animals and that the strain of the subtypes may be pathogenic. The STs identified in this study have been associated with some pathological conditions. Because of the strategic location of this river, protecting it from organic and faecal contaminants is vital to control further contamination with *Blastocystis* and prevent its transmission to other humans, wild animals or even livestock.

ACKNOWLEDGEMENTS

The authors would like to thank the undergraduate students of the Veterinary Parasitology Laboratory of the Universiti Sains Malaysia, Penang for their contribution to the water samples collection for this study. This work was supported by Sultan Abdul Halim Mu'adzam Shah International Islamic University, Kuala Ketil, Kedah [100-161(RMC)01GPU2023 Bil. (41)].

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