Tropical Biomedicine 43(1): 76-82 (2026)
https://doi.org/10.47665/tb.43.1.010
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RESEARCH ARTICLE

HSP70 gene variants and subtype linked to thermal stress adaptation in *Blastocystis* sp.

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ARTICLE HISTORY

Received: 12 October 2025
Revised: 26 January 2026
Accepted: 16 February 2026
Published: 31 March 2026

ABSTRACT

Blastocystis sp. is the most common enteric protist colonizing a wide range of host and is characterized by wide genotypic diversity. Heat Shock Protein 70 (HSP70), a common stress-response protein has been associated with increased proliferation in *Blastocystis* sp. under thermal stress. However, the extent of HSP70 genetic variation and its link to thermostability remains unexplored. Our study aims to elucidate HSP70 gene variants among different *Blastocystis* sp. subtypes, analyze their responses to thermal stress, and evaluate their protease activity following the thermal stress. Heat Shock Protein 70 gene from eighteen *Blastocystis* sp. isolates of varying subtype – ST1, ST3, ST4, ST6, and ST7 – were amplified, sequenced and phylogenetically analyzed. The cells were then subjected to thermal stress at 42°C for 24 hours and the cell viability was monitored for seven days. Protease activity in solubilized antigens (SA) from purified *Blastocystis* sp. cells was evaluated following exposure to thermal stress. Phylogenetic analysis revealed two distinct clades: ST4 and ST7 (Clade 1) which are mostly thermotolerant, while ST1, ST3, and ST6 (Clade 2) exhibited lower tolerance. Total protease activity was similar across subtypes, suggesting need for further sub-class specific analysis. Our findings suggests that thermal stress adaptation is subtype dependent and may be influenced by HSP70 variation. This insight highlights the importance of HSP70 as a potential molecular marker for stress adaptation in *Blastocystis* sp. and underscore the need for deeper investigation of its functional role in parasite persistence and pathogenic potential.

Keywords: *Blastocystis* sp.; subtype; HSP70 gene; genetic variation; thermal stress.

INTRODUCTION

Blastocystis sp. is a unicellular protozoan commonly found in human stool samples. It colonizes the gastrointestinal tract of wide range of hosts (Salehi Sangani *et al*., 2025), often resulting in either symptomatic, commonly diarrhea, flatulence and nausea or asymptomatic presentations. The variation in clinical outcomes is believed to be associated with the specific *Blastocystis* sp. strain or subtype involved. To date, more than 44 genetically distinct subtypes have been identified in both human and animals (Matovelle *et al*., 2024).

Within human hosts, *Blastocystis* sp. cells which usually establishes persistent colonization, is exposed to diverse environmental stressors, including thermal stress, nutritional deprivation, osmotic stress, and oxidative stress (Gaythri *et al*., 2014). Among these, thermal stress is of particular relevance, as it reflects febrile conditions associated with infection such as dengue or malaria which are often accompanied by gastrointestinal disturbances. However, the mechanisms by which *Blastocystis* sp. adapts to this kind of thermal stress, especially in the context of its long-term colonization, remain poorly understood.

Heat shock proteins (HSPs) are reportedly upregulated during response mechanism to thermal stress (Rakib *et al*., 2024). They essentially function as a molecular chaperone in stabilizing proteins, preventing misfolding, and facilitating the refolding or degradation of damaged polypeptides. These functions are vital for cell survival during exposure to stressors such as elevated temperatures, oxidative damage, or nutrient deprivation (Sun *et al*., 2024). In *Blastocystis* sp., 70 kDa heat shock proteins (HSP70) have been implicated in enabling survival under thermally stressed conditions (Evans *et al*., 2010; Gaythri *et al*., 2014). Nevertheless, it remains unclear whether the HSP70 gene sequence is conserved across *Blastocystis* sp. subtypes or exhibits subtype-specific variations. Whether such genetic variations may influence thermotolerance and potentially contribute to differences in pathogenic potentials remains unexplored.

Proteases in *Blastocystis* sp. have been widely studied for their role in host–parasite interactions, particularly in degrading host immunoglobulins, disrupting epithelial barrier integrity, and modulation of inflammatory responses (Puthia *et al*., 2005; Ajjampur & Tan, 2016). These enzymes are regarded as potential virulence factors due to their ability to induce cytopathic effects and facilitate

Published by Malaysian Society of Parasitology and Tropical Medicine.

parasite persistence in the gut. Our previous work demonstrated that metronidazole-resistant isolates exhibit elevated protease activity, suggesting a possible link between drug pressure enhanced pathogenic potential (Rajamanikam *et al*., 2019). Whether thermal stress adaptation similarly modulate protease activity and thereby lead to enhanced pathogenic potential remains underexplored.

Despite the advances in understanding of *Blastocystis* sp., the variation in thermal stress adaptation among different subtypes and its potential links to pathogenicity is still insufficiently defined. The present study aims to determine whether variation in HSP70 gene contributes to thermotolerance and whether such adaptation influences protease activity, thereby providing insights into persistent colonization and variability of pathogenic potential.

MATERIALS AND METHOD

Blastocystis sp. isolates

A total of 18 archived *Blastocystis* sp. isolates representing 5 distinct subtypes (ST1, ST3, ST4, ST6, and ST7) were used in this study (Table 1). These isolates were obtained from previous surveys conducted among individuals attending University Malaya Medical Centre (UMMC). Table 1 describes the source of *Blastocystis* sp. isolates used in this study.

Table 1. *Blastocystis* sp. subtypes, isolate names, and associated clinical symptoms

Subtype	Isolates Name	Clinical Symptoms
ST1	BP03	Asymptomatic
	BP22	Asymptomatic
	20	Asymptomatic
ST3	JEG	Symptomatic
	FR17	Asymptomatic
	FR13	Asymptomatic
	BP2	Symptomatic
	BP18	Symptomatic
	BP16	Symptomatic
ST4	EM10	Symptomatic
	EM11	Symptomatic
	EM12	Symptomatic
	GH	Symptomatic
ST6	NC51	Symptomatic
	21	Asymptomatic
	BP10	Symptomatic
ST7	FP74	Asymptomatic
	BP11	Symptomatic

Genomic DNA extraction and amplification of HSP70 gene

Blastocystis sp. cell suspensions were collected and centrifuged. The resulting pellet was then washed with Phosphate Buffered Saline (PBS). DNA extraction was performed using Instagene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following manufacturers protocol. Briefly, 20 µl of cell suspension was mixed with 200 µl Instagene Matrix and incubated at 56°C for 15 to 30 minutes according to manufacturer's protocol. The sample was vortexed at high speed and incubated at 100°C for 8 minutes. Following incubation, the sample was centrifuged to pellet the Instagene resins and cellular debris. DNA in the supernatant was used for the downstream analysis.

The heat shock protein 70 (HSP70) gene of *Blastocystis* sp. ST7, a gene known for its conserved nature was amplified using the forward and reverse primers (HSP70_F): 5'-ATTTCGATGAGGCGCTTCTG-3' and (HSP70_R): 5'-CCTCGTTGATGTCCGTCTTG-3') as described before (Gaythri *et al*., 2014). The primers are specific to compatible isolates for sequencing the HSP70 gene and thus unsuitable for other Blastocystis subtypes. The PCR amplification was done on Bio-Rad thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions consisted of one cycle of initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 1 min and extension at 72°C for 3 min; one cycle of final extension at 72°C for 10 min and final infinite holding at 4°C. Following PCR amplification, the products were visualized using 1.5% agarose gel electrophoresis and the gel was viewed under UV light to confirm the presence of the expected 318 bp amplicon.

Sequencing and Phylogenetic Analysis

Amplicons were then sequenced using Sanger sequencing. The resulting sequences were blasted in NCBI nucleotide database to confirm its identity. Subsequently, the sequences were trimmed to remove low quality reads using Mega 4.0 software to a final average length of 200bp and were further analysed phylogenetically. A phylogenetic tree was constructed using the Neighbor-Joining method implemented in MEGA (Karimi *et al*., 2025). The robustness of the inferred tree topology was assessed by bootstrap analysis with 1000 replicates, and bootstrap support values were mapped onto the corresponding nodes. This analysis assists to identify genetic pattern specific to each subtype (ST1, ST3, ST4, ST6, and ST7) and assess their genetic diversity – providing insights into the evolutionary relationships among isolates.

Thermal stressing *Blastocystis* sp. to determine thermotolerance.

Blastocystis sp. isolates confirmed positive for HSP70 genes amplification was cultured and adjusted to a standardized concentration of 10^5 cells/ml. For each isolate, thermal stress was induced by incubating at temperature beyond 40°C (42°C – 44°C) for 24 hours on a dry incubator while the controls were maintained at the optimal growth temperature of 37°C. This experiment was performed in triplicate for each isolate to ensure reproducibility. After the 24-hour exposure period, all tubes were transferred to 37°C and were assessed for their viability and proliferation using hemocytometer and trypan blue exclusion assay. Assessment was performed immediately following thermal stress exposure (day 0) and subsequently on days 3, 5, and 7 post-exposures. The data collected from triplicate experiments were used to construct growth curves, enabling comparison of proliferation rates and viability between heat-stressed and control groups across the different *Blastocystis* sp. subtypes. Morphometry was not conducted in this study.

Assessment of total protease activity

Purification of Blastocystis sp. cells

A standardized number of cells (100,000 cells/ml) in 3 ml was centrifuged at 1,500 rpm for 5 minutes. The pellets were washed in sterile PBS, resuspended to 5 ml cell suspension and layered onto Lymphoprep solution in a 15 ml centrifuge tube. The tubes were centrifuged at 1,800 rpm for 20 minutes in room temperature and the purified cells with minimal bacterial contamination were isolated as described previously (Franklin *et al*., 2022). The purified cells were stored in serum-free media at -20°C.

Isolation and quantification of Blastocystis sp. solubilized proteins

Total soluble proteins from the purified *Blastocystis* sp. were isolated by 20 freeze-thaw cycles followed by overnight incubation at 4°C. Protein concentration was determined using Bradford Assay with BSA standards (0.5 – 0.05 mg/ml) (Bio-Rad Laboratories, USA).

Colorimetric assessment of protease activity

Protease activity were assessed using azocasein colorimetric assay. Samples (100 ul) pre-treated with 2 mm DTT at 37°C for 10 minutes then incubated with 100 ul of pre-warmed azocasein for 1 hour

at 37°C. Reactions were stopped by adding 300 µl of ice-cold TCA and incubating on ice for 30 minutes. Samples were centrifuged at 8000 x g for 5 minutes and the supernatant were mixed with 500 µl of NaOH. The absorbance was measured at 440 nm. Trypsin and a no-antigen mixture served as the positive and negative control, respectively. All assays were performed in triplicates.

RESULT AND DISCUSSION

PCR amplification of the HSP70 gene were confirmed in 10 out of 18 *Blastocystis* sp. isolates, yielding the expected size of 318 bp. These includes isolates from subtypes ST1 (isolates 20 and BP22), ST3 (FR13 and FR17), ST4 (EM10, EM11, EM12), ST6 (NC51), and ST7 (FP74 and BP11). However, the amplification failure in remaining 8 isolates suggest potential primer-template mismatch due to sequence variability within the HSP70 gene, a phenomenon that has been previously reported in many genetically diverse protozoan parasites, including *Blastocystis* sp. (Stensvold *et al*., 2012; Ramsøe *et al*., 2020). Only the ten isolates yielding visible bands on gel electrophoresis proceeded to DNA sequencing. Experimental repeats from DNA extraction to amplification confirmed the reproducibility of this outcome, thus ruling out technical errors as shown in Figure 1.

Blastocystis sp. is known for its high inter-subtype and intra-subtype sequence variability, particularly within highly conserved genes such as SSU rRNA and HSP70 (Gaythri *et al*., 2014, Hernández *et al*., 2023). In the present study, successful amplification was observed for all five STs used. However, amplification failure was observed in some isolates, likely attributable to pronounced sequence divergence within the target regions. Phylogenetic studies have demonstrated that sequence identity across subtypes (e.g., ST1, ST3, ST5, ST7) can be as low as 88%, increasing the likelihood of mismatches in a 318-bp target region and reducing the primer annealing efficiency (Gaythri *et al*., 2014). Studies in the past have also highlighted the limitations of using a single primer set across divergent subtypes, as extensive heterogeneity undermines reliability of universal amplification (Stensvold *et al*., 2012; Srichaipon *et al*., 2019). This underscores an important consideration in molecular investigations of *Blastocystis* sp. for subtype-specific studies where degenerate primers that can account for genetic variability should be used (Li *et al*., 2015). Future studies should focus on designing multiple primer sets tailored to individuals subtype based on known sequence alignments or incorporating degenerate bases to enhance primer flexibility (Najafabadi *et al*., 2008). Alternatively, whole-genome or targeted next-generation sequencing approaches offer promising avenue for capturing HSP70 and other polymorphic genes across *Blastocystis* sp. subtype spectrum.

The HSP70 gene sequences from 10 PCR-positive *Blastocystis* sp. isolates were aligned using W to evaluate intra- and inter-subtype genetic variation (Figure 2). The alignment revealed numerous nucleotide polymorphisms across the sequence region, indicating significant genetic heterogeneity. Notably, consistent patterns were observed within certain subtypes, especially among ST4 isolates (EM10, EM11, EM12), while more pronounced variations appeared between ST4/ST7 and ST1/ST3/ST6 groups.

The alignment of sequences using ClustalW in MEGA software resulted in the construction of phylogenetic tree using Neighbor-Joining (NJ) method (Zou *et al*., 2024). The NJ method was chosen for this study due to its computational efficiency and suitability for analyzing evolutionary distances among closely related sequences, particularly when the sample size is relatively small (Zou *et al*., 2024). NJ is a widely used distance-based method that does not assume a constant rate of evolution, making it ideal for examining heterogenous genes such as HSP70 that may evolve at different rates across subtypes (Saitou & Nei, 1987). Furthermore, NJ provides robust phylogenies when working with protein-coding genes, which often show varying substitution rates due to selective pressures.

Based on the phylogenetic tree in Figure 2, we noted the formation of two distinct clades: Clade 1, consisting of subtypes ST4 and ST7, and Clade 2, comprising ST1, ST3, and ST6. This subdivision reflects underlying genetic divergence in the HSP70 gene and supports previous findings on inter-subtype variability observed in SSU-rRNA studies (Alfellani *et al*., 2013). Notably, Clade 1 clustered with high bootstrap value of 94, indicating strong sequence similarity and potential functional conservation in the heat shock response. In contrast, Clade 2 exhibited broader diversity, incorporating subtypes ST1, ST3, and ST6, which may suggest more variable adaptation mechanisms at the HSP70 level. This clustering may point towards potential subtype specific evolutionary pressures, acting on stress response pathways, possibly conferring altered resilience under host

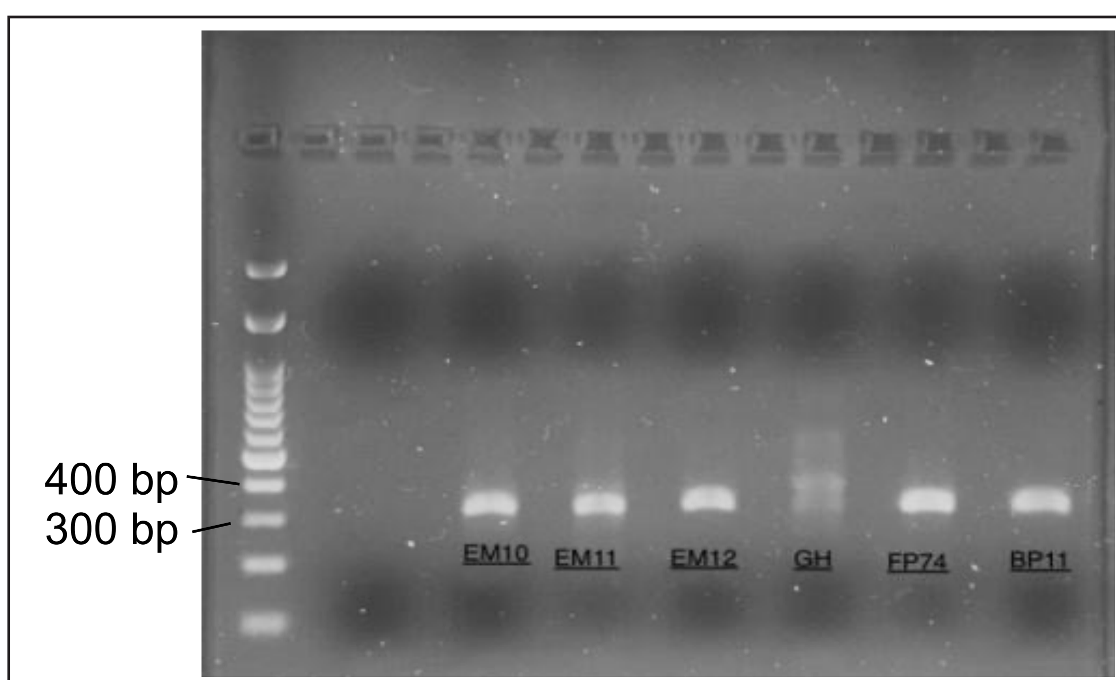

Figure 1. Gel electrophoresis image showing successful amplification of the HSP70 gene in *Blastocystis* isolates.

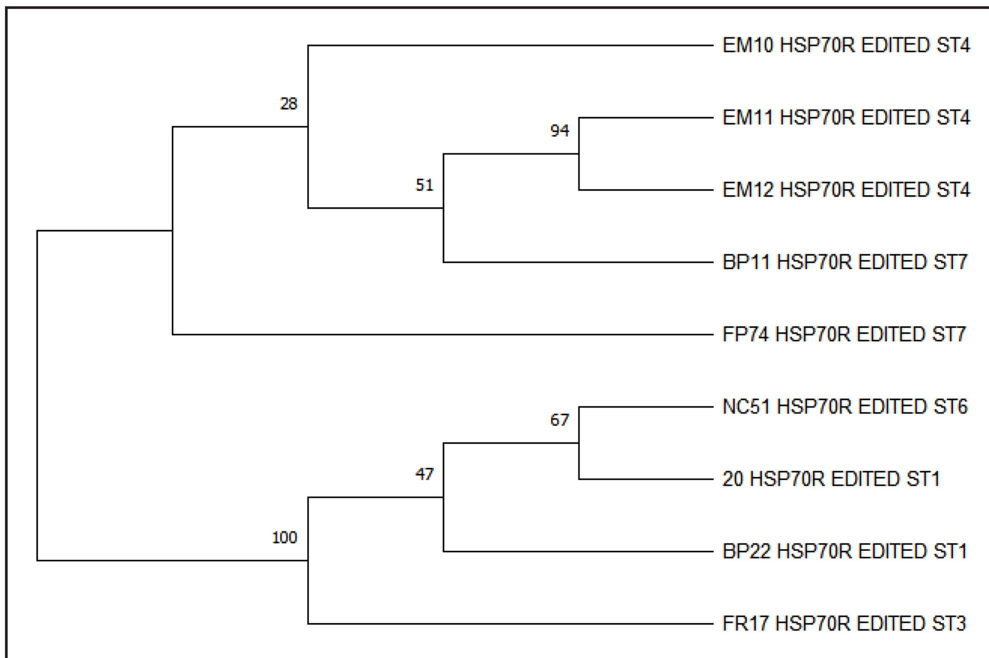

Figure 2. Phylogenetic tree constructed using the Neighbor-Joining method based on the HSP70 gene sequences of nine *Blastocystis* sp. isolates.

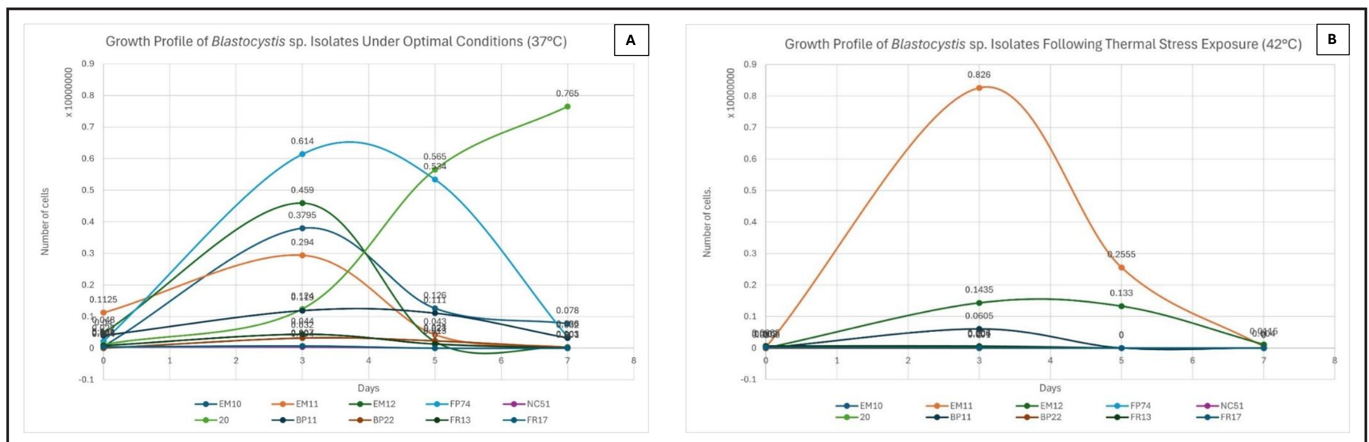

Figure 3. A) Growth curve of *Blastocystis* sp. isolates maintained at 37°C (optimal condition) over 7 days (Control). All isolates showed consistent proliferation patterns, indicating normal viability and baseline growth capacity in the absence of thermal stress. **B**) Growth curve of *Blastocystis* sp. isolates following 24-hour exposure to thermal stress, monitored over 7 days. Among the 10 tested isolates, EM11, EM12, and BP11 demonstrated the most notable post-stress proliferation.

derived environments. However, the present study is limited to only 5 subtypes and further studies incorporating wider subtype diversity are necessary to clarify whether the observed genetic divergence is seen in other subtypes as well.

An important methodological consideration was the exclusion of one ST3 isolate (FR13) from the final phylogenetic as it displayed unusually high sequence divergence, which caused it to behave as an outlier or unintentionally as outgroup. In the absence of a designated external outgroup sequence, its inclusion would have distorted the tree topology and masked the intra-subtype relationships that were the focus of this analysis. While the lack of an outgroup constrains our ability to definitively root the tree, the study's primary aim was to examine relative clustering and divergence among intra-species isolates. From these perspectives, the exclusion of FR13 preserves the interpretive clarity of the phylogeny and does not compromise the validity of observed subtype-specific patterns. We have, however, include the tree with FR13 as a supplementary figure.

In the present study, we examined whether genetic variants in the *Blastocystis* sp. HSP70 gene, as reflected in phylogenetic clustering, corresponds with differential responses to thermal stress. The parasite cells exposed to 37°C and thermally stressed at temperature beyond 40°C for 24 hours were evaluated by the growth pattern after the exposure (Figure 3A). Under 37°C, all isolates, regardless of subtype or clade, exhibited normal growth pattern with a characteristic peak on Day 3, as shown in Figure 3A. This growth pattern is consistent with previously reported *in vitro Blastocystis* sp. cultures and reflects the organism's ability to thrive at physiological human body temperature (Pei *et al*., 2020). However, when subjected to elevated temperature (42°C – 44°C), intended to mimic stressed conditions experienced in host infection such as dengue or malaria (Liu *et al*., 2023), a notable shift in growth dynamics of *Blastocystis* sp. was observed. The majority of isolates displayed suppressed proliferation (Figure 3B), indicative of heat sensitivity and cellular stress under thermal challenge.

Interestingly, only three isolates – EM11, EM12, and BP11 – maintained significant peak cell growth following exposure to high temperature, with growth peaking on Day 3 ($P < 0.05$, t-test; df=7). All three isolates belonged to HSP70 variants within Clade 1, suggesting that these variants may possess a greater resilience to thermal stress compared to other isolates, which showed no substantial growth following heat exposure. We found 60% of Clade 1 isolates demonstrated the capacity to tolerate and adapt to elevated temperatures, supporting the hypothesis that subtype-specific differences in thermotolerance may be driven, at least in part by genetic variation in HSP70 genes (Hassan *et al*., 2019). Such enhanced resilience could potentially provide a survival advantage under host- or environment-stress, prolonging colonization and sustaining host-parasite interaction. A previous study reported that heat stress in *Blastocystis* sp. promotes granular formation within the central body, surrounded by electron dense material, with subsequent rupture of these granules producing viable progenies (Thergarajan *et al*., 2018). This process represents a survival strategy and could also account for the persistence of certain isolates in our study. However, whether variation in HSP70 gene is mechanistically linked to granular formation warrants further investigations.

The correlation between phylogenetic grouping and thermal stress resilience implies that *Blastocystis* sp. subtype diversity is not merely taxonomic but may also carry functional consequences relevant to survival and pathogenic potential (Skotarczak, 2018). The HSP70 gene, a key molecular chaperone involved in maintaining protein homeostasis under stress, may play a central role in this process (Singh *et al*., 2025). It is plausible that Clade 1 isolates have either upregulated HSP70 expression or possess sequence variants that confer enhanced stress adaptation. This finding aligns with previous research showing that HSP70 is upregulated in *Blastocystis* sp. under thermal stress and supports the idea that subtype-specific responses may influence parasite persistence and disease outcomes (Gaythri *et al*., 2014).

Furthermore, from a clinical perspective, the ability of certain *Blastocystis* sp. subtypes to survive under febrile conditions may have implications for their pathogenicity (Pawelec-Peciak *et al*., 2025). Patients with infections that cause fever often experience gastrointestinal symptoms, including diarrhea, which raises the question of whether thermotolerant *Blastocystis* sp. subtypes contribute to gut disruption during febrile illness (Thergarajan *et al*., 2019; Robles-Cabrera *et al*., 2021). While this study did not directly measure virulence, the association between thermal tolerance and phylogenetic clustering provides a foundation for future investigations into the relationship between genetic diversity, environmental resilience, and pathogenic potential (Mildenberger *et al*., 2025).

In this study, total protease activity was assessed to compare functional variation in terms of proteolytic activity between the distinct clades. Proteases were evaluated using azocasein-based colorimetric assay, which measures total proteolytic degradation by detecting the release of azo-dye-labeled casein peptides (Coêlho *et al*., 2016). As shown in Figure 4, both Clade 1 and Clade 2 isolates exhibited comparable levels of total protease activity, with mean values of 5.77 and 6.03 azocasein units, respectively. This suggests that, despite the genetic differences observed between clades, the total protease activity did not significantly vary between them under the conditions tested. While total protease activity provides an overview of general enzymatic function, it does not capture the complexity of protease subtypes that may contribute differentially to pathogenesis. Protozoan parasites are known to secrete a range of protease classes, including serine, cysteine, aspartic, and metalloproteases (Sibley, 2013), all of which play diverse roles in host tissue invasion, immune evasion, and nutrient acquisition. Among these, cysteine proteases have been most frequently implicated in virulence (Verma *et al*., 2016), particularly in other protozoa such as *Entamoeba histolytica*, where they are directly associated with tissue damage and immune modulation (Moncada *et al*., 2003; Serrano-Luna *et al*., 2013). Future work should focus on characterizing specific protease classes, particularly cysteine proteases, to elucidate their roles in pathogenicity and stress adaptation.

In this study, the use of a total protease assay precludes differentiation between specific protease types. Therefore, it remains unclear whether the activity measured includes virulence-associated enzymes such as cysteine proteases. This limitation is important to note, as isolates may exhibit similar total activity yet differ in pathogenic potential depending on the specific protease profile expressed. Consequently, future studies should include substrate-specific assays or utilize protease inhibitors to distinguish between serine, cysteine, and other protease classes. This way, a more precise assessment of the relationship between protease activity and the virulence potential of different *Blastocystis* sp. subtypes and clades.

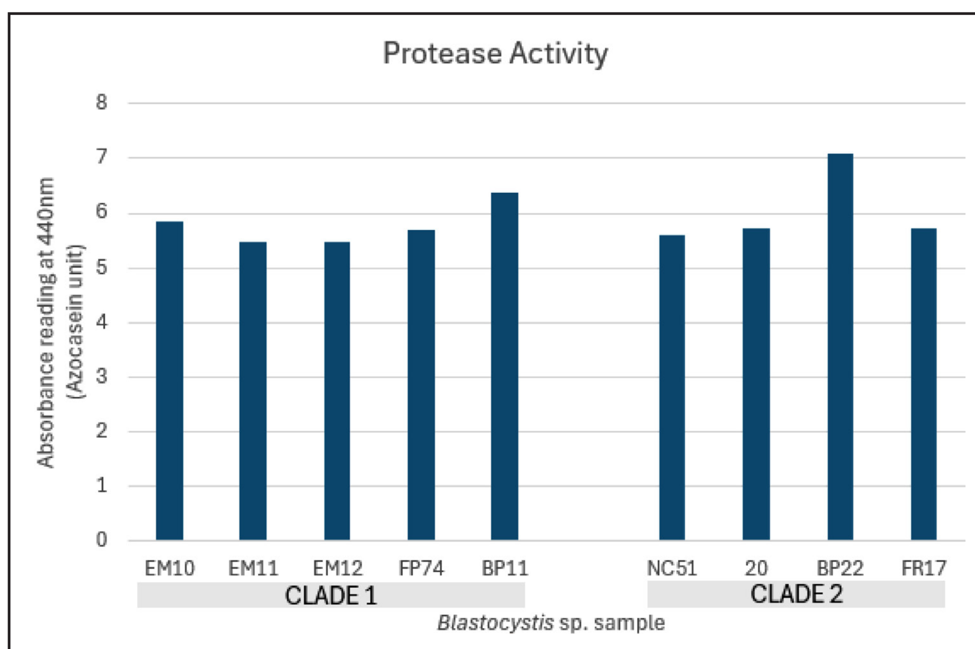

Figure 4. Total protease activity in *Blastocystis* sp. isolates.

Moreover, only a single primer set specific for the HSP70 gene of *Blastocystis* sp. ST7 was used. Consequently, some isolates may not have been detected due to sequence variation in the HSP70 gene across different subtypes. Given the genetic diversity of the HSP70 gene among *Blastocystis* sp. subtypes, designing multiple primer sets targeting conserved regions across subtypes is necessary to improve PCR amplification success in future studies. Different *Blastocystis* subtypes may require subtype-specific primers for the *HSP70* gene due to inter-subtype sequence variations.

Secondly, our resources were restricted in such a way that achieving a fixed high temperature was not possible. We have however, achieved a thermal stress state in *Blastocystis* sp. cells. Our findings clearly demonstrated that HSP70 gene variants may have different thermal resilience. Future studies should employ a fixed temperature in stress condition to ensure precise effects are documented.

CONCLUSION

This study demonstrated that *Blastocystis* sp. subtypes exhibit distinct thermal tolerance and variation in HSP70 gene. Specifically, ST4 and ST7 showed notable resilience to thermal stress, maintaining growth after exposure to temperature beyond 40°C, while ST1, ST3, and ST6 did not. These findings suggest that certain subtypes possess enhanced thermotolerance, potentially supporting prolonged colonization in the host. Although total protease activity was similar across subtypes, further research is needed to clarify the roles of specific proteases in thermal stress adaptation and pathogenicity.

Conflict of Interest

The author declares that they have no conflict of interests.

Ethical Statement

The live cultures of *Blastocystis* sp. used in this study was isolated from Universiti Malaya Medical Centre patients in a previous study. The study was ethically approved by Medical Ethics Committee of University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia (20191226-8107). The patients consented to participate in the study before samples can be obtained.

ACKNOWLEDGEMENT

The author wishes to express her sincere gratitude to the Department of Parasitology and Department of Biomedical Science, Faculty of Medicine, University Malaya, for providing research facilities and financial support through the Final Year Project grant.

REFERENCES

Ajjampur, S.S.R. & Tan, K.S.W. (2016). Pathogenic mechanisms in Blastocystis spp. – Interpreting results from *in vitro* and *in vivo* studies. *Parasitology International* **65**: 772-779. https://doi.org/10.1016/j.parint.2016.05.007

Alfellani, M.A., Stensvold, C.R., Vidal-Lapiedra, A., Onuoha, E.S.U., Fagbenro-Beyioku, A.F. & Clark, C.G. (2013). Variable geographic distribution of Blastocystis subtypes and its potential implications. *Acta Tropica* **126**: 11-18. https://doi.org/10.1016/j.actatropica.2012.12.011

Coêlho, D.F., Saturnino, T.P., Fernandes, F.F., Mazzola, P.G., Silveira, E. & Tambourgi, E.B. (2016). Azocasein substrate for determination of proteolytic activity: reexamining a traditional method using bromelain samples. *BioMed Research International* **2016**: 8409183. https://doi.org/10.1155/2016/8409183

Evans, C.G., Chang, L. & Gestwicki, J.E. (2010). Heat shock protein 70 (hsp70) as an emerging drug target. *Journal of Medicinal Chemistry* **53**: 4585-4602. https://doi.org/10.1021/jm100054f

Hernández, P.C., Maloney, J.G., Molokin, A., George, N.S., Morales, L., Chaparro-Olaya, J. & Santin, M. (2023). Exploring Blastocystis genetic diversity in rural schoolchildren from Colombia using next-generation amplicon sequencing reveals significant associations between contact with animals and infection risk. *Parasitology Research* **122**: 1451-1462. https://doi.org/10.1007/s00436-023-07841-3

Franklin, F., Rajamanikam, A., Raju, C.S., Gill, J.S., Francis, B., Sy-Cherng, L.W. & Kumar, S. (2022). Higher amoebic and metronidazole resistant forms of Blastocystis sp. seen in schizophrenic patients. *Parasites & Vectors* **15**: 313. https://doi.org/10.1186/s13071-022-05418-0

Gaythri, T., Suresh, K., Subha, B. & Kalyani, R. (2014). Identification and characterisation of heat shock protein 70 in thermal stressed Blastocystis sp. *Plos One* **9**: e95608. https://doi.org/10.1371/journal.pone.0095608

Hassan, F.-U., Nawaz, A., Rehman, M.S., Ali, M.A., Dilshad, S.M.R. & Yang, C. (2019). Prospects of HSP70 as a genetic marker for thermo-tolerance and immuno-modulation in animals under climate change scenario. *Animal Nutrition* **5**: 340-350. https://doi.org/10.1016/j.aninu.2019.06.005

Karimi, E., Momeni, Z., Nasiri, V. & Sabokbar, A. (2025). Genetic diversity and prevalence of Blastocystis subtypes in Alborz Province, Iran: A molecular epidemiological study. *Acta Tropica* **262**: 107537.

Li, K., Shrivastava, S. & Stockwell, T.B. (2015). Degenerate Primer Design for Highly Variable Genomes. In C. Basu (Ed.), *PCR Primer Design* (pp. 103-115). Springer New York. https://doi.org/10.1007/978-1-4939-2365-6_7

Liu, Z., Zhang, Q., Li, L., He, J., Guo, J., Wang, Z., Huang, Y., Xi, Z., Yuan, F., Li, Y. & Li, T. (2023). The effect of temperature on dengue virus transmission by Aedes mosquitoes. *Frontiers in Cellular and Infection Microbiology* **13**: 1242173. https://doi.org/10.3389/fcimb.2023.1242173

Matovelle, C., Quílez, J., Tejedor, M.T., Beltrán, A., Chueca, P. & Monteagudo, L.V. (2024). Subtype Distribution of Blastocystis spp. in patients with gastrointestinal symptoms in Northern Spain. *Microorganisms* **12**: 1084. https://doi.org/10.3390/microorganisms12061084

Mildenberger, D.P., Bonner, T.H., Corbett, T., Robertson, C.R., Lopez, R.R. & Randklev, C.R. (2025). Evaluating lethal thermal tolerances and vulnerability of three east Texas freshwater mussels. *Freshwater Science* **44**: 45-60. https://doi.org/10.1086/734177

Moncada, D., Keller, K. & Chadee, K. (2003). Entamoeba histolytica cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. *Infection and Immunity* **71**: 838-844. https://doi.org/10.1128/iai.71.2.838-844.2003

Najafabadi, H.S., Torabi, N. & Chamankhah, M. (2008). Designing multiple degenerate primers via consecutive pairwise alignments. *BMC Bioinformatics* **9**: 55. https://doi.org/10.1186/1471-2105-9-55

Pawelec-Peciak, O., Lanocha-Arendarczyk, N., Grzeszczak, K. & Kosik-Bogacka, D. (2025). The role of Blastocystis spp. in the etiology of gastrointestinal and autoimmune diseases. *Pathogens* **14**: 313. https://doi.org/10.3390/pathogens14040313

Pei, W., Fuqiang, Z., Bo, H., Xuefang, M., Zhenchao, Z., Xiangrui, L. & Shuai, W. (2020). Advances in *in vitro* culture of Blastocystis hominis. *China Tropical Medicine* **20**: 1207-1211.

Puthia, M.K., Vaithilingam, A., Lu, J. & Tan, K.S.W. (2005). Degradation of human secretory immunoglobulin A by Blastocystis. *Parasitology Research* **97**: 386-389. https://doi.org/10.1007/s00436-005-1461-0

Rajamanikam, A., Hooi, H.S., Kudva, M., Samudi, C. & Kumar, S. (2019). Resistance towards metronidazole in Blastocystis sp.: A pathogenic consequence. *Plos One* **14**: e0212542.

Rakib, M.R.H., Messina, V., Gargiulo, J.I., Lyons, N.A. & Garcia, S.C. (2024). Graduate student literature review: potential use of HSP70 as an indicator of heat stress in dairy cows – A review. *Journal of Dairy Science* **107**: 11597-11610. https://doi.org/10.3168/jds.2024-24947

Ramsøe, A., Clark, M.S. & Sleight, V.A. (2020). Gene network analyses support subfunctionalization hypothesis for duplicated hsp70 genes in the Antarctic clam. *Cell Stress and Chaperones* **25**: 1111-1116. https://doi.org/10.1007/s12192-020-01118-9

Robles-Cabrera, M.X., Maguiña, J.L., Gonzales-Huerta, L., Panduro-Correa, V., Dámaso-Mata, B., Pecho-Silva, S., Navarro-Solsol, A.C., Rabaan, A.A., Rodríguez-Morales, A.J. & Arteaga-Livias, K. (2021). Blastocystis species and gastrointestinal symptoms in Peruvian adults attended in a public hospital. *Infection & Chemotherapy* **53**: 374-380. https://doi.org/10.3947/ic.2021.0004

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Salehi Sangani, G., Hosseini-Safa, A., Hosseini Farash, B.R., Salehi Sangani, P., Zarean, M., Moghaddas, E., Zoghdar Moghadam, S., Jarahi, L., Gholizadeh, A. & Berenji, F. (2025). Detection and subtyping of Blastocystis sp. in human and animal stool samples using high-resolution melting analysis. *BMC Infectious Diseases* **25**: 171. https://doi.org/10.1186/s12879-024-10423-y

Serrano-Luna, J., Piña-Vázquez, C., Reyes-López, M., Ortiz-Estrada, G. & de la Garza, M. (2013). Proteases from Entamoeba spp. and pathogenic free-living amoebae as virulence factors. *Journal of Tropical Medicine* **2013**: 890603. https://doi.org/10.1155/2013/890603

Sibley, L.D. (2013). The roles of intramembrane proteases in protozoan parasites. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1828**: 2908-2915. https://doi.org/10.1016/j.bbamem.2013.04.017

Singh, M.K., Han, S., Ju, S., Ranbhise, J.S., Ha, J., Yeo, S.G., Kim, S.S. & Kang, I. (2025). Hsp70: A multifunctional chaperone in maintaining proteostasis and its implications in human disease. *Cells* **14**: 509. https://doi.org/10.3390/cells14070509

Skotarczak, B. (2018). Genetic diversity and pathogenicity of Blastocystis. *Annals of Agricultural and Environmental Medicine* **25**: 411-416. https://doi.org/10.26444/aaem/81315

Srichaipon, N., Nuchprayoon, S., Charuchaibovorn, S., Sukkapan, P. & Sanprasert, V. (2019). A simple genotyping method for rapid differentiation of Blastocystis subtypes and subtype distribution of Blastocystis spp. in Thailand. *Pathogens* **8**: 38. https://doi.org/10.3390/pathogens8010038

Stensvold, C.R., Alfellani, M. & Clark, C.G. (2012). Levels of genetic diversity vary dramatically between Blastocystis subtypes. *Infection, Genetics and Evolution* **12**: 263-273. https://doi.org/10.1016/j.meegid.2011.11.002

Sun, Y.Y., Guo, H.Y., Liu, B.S., Zhang, N., Zhu, K.C., Xian, L., Zhao, P.H., Yang, H.Y. & Zhang, D.C. (2024). Genome-wide identification of heat shock protein gene family and their responses to pathogen challenge in Trachinotus ovatus. *Fish & Shellfish Immunology* **145**: 109309. https://doi.org/10.1016/j.fsi.2023.109309

Thergarajan, G., Govind, S. & Bhassu, S. (2018). *In vitro* and *in vivo* thermal stress induces proliferation of Blastocystis sp. *Parasitology Research* **117**: 177-187. https://doi.org/10.1007/s00436-017-5688-3

Thergarajan, G., Kumar, S., Bhassu, S., Omar, S. & Rampal, S. (2019). Effect of Blastocystis sp. in dengue patients-Increase in the treatment cost and exacerbation of symptoms. *Plos One* **14**: e0211034. https://doi.org/10.1371/journal.pone.0211034

Verma, S., Dixit, R. & Pandey, K.C. (2016). Cysteine proteases: modes of activation and future prospects as pharmacological targets [Review]. *Frontiers in Pharmacology* **7**: 107. https://doi.org/10.3389/fphar.2016.00107

Zou, Y., Zhang, Z., Zeng, Y., Hu, H., Hao, Y., Huang, S. & Li, B. (2024). Common Methods for phylogenetic tree construction and their implementation in R. *Bioengineering* **11**: 480. https://doi.org/10.3390/bioengineering11050480