APPLICATION OF *IN SILICO* AND *IN VITRO* APPROACHES ON REPURPOSED DRUGS TARGETING PUTATIVE RHO GTPASE AND RHOGAP OF *Giardia lamblia*

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by

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LIST OF SYMBOLS

%	Percentage
^{o}C	Degree Celsius
µg	Microgram
µL	Microliter
xg	Times gravity
Å	Armstrong
g	Gram
L	Litre
h	Hour
min	Minute
mL	Milliliter
mM	Milimolar
kJ/mol	Kilojoule per mole
kDA	Kilodalton
ns	Nanoseconds
K	Kelvin

LIST OF ABBREVIATIONS

3D	3-Dimensional
ANOVA	Analysis of Variance
BLASTp	DrugBank Protein Basic Local Alignment Tool
dH_2O	Distilled water
et al	*et alii* – "and others
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FASTA	Fast Approximation of Smith & waterman Algorithm
GlRac	*Giardia lamblia* Rac (Rho family GTPase)
GO	Gene Ontology
i.e.	For example,
KCI	Potassium Chloride
MD	Molecular Dynamic Simulation
MIC	Minimal Inhibitory Concentration
MTZ	Metronidazole
m-TYI-S-33	Modified Trypticase-Yeast Extract-Iron-Serum-33
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Rg	Radius of gyration
RhoGAP	Rho GTPase activating protein
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuations
UniprotKb	Universal Protein Knowledgebase

WHO	World Health Organization
YASARA	Yet Another Scientific Artificial Reality Application

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PENGGUNAAN KAEDAH *in silico* DAN *in vitro* TERHADAP DADAH GUNA SEMULA DALAM MENYASARKAN PUTATIF RHO GTPASE dan RhoGAP *Giardia lamblia*

ABSTRAK

Giardia lamblia ialah parasit protozoa yang tersebar secara global dan menyebabkan penyakit usus yang dikenali sebagai giardiasis. Rawatan utama bergantung kepada ubat nitroimidazole seperti metronidazole, tinidazole, dan albendazole. Walaubagaimanapun, kejadian kes- refraktori semakin meningkat menyebabkan berlakunya ketidakpatuhan terapeutik. Walaupun banyak kajian telah dijalankan untuk menangani isu ini, masalah ini masih belum dapat diselesaikan. Protein GTPase kecil, subkeluarga Rho dan pengawalatur positifnya, protein putatif pengaktifan Rho GTPase (RhoGAP) didapati terlibat dalam perbagai proses biologi dan selular. Kedua-dua protein ini didapati berfungsi bersama dalam mengawal pensistaan, pengangkutan membran, dan terlibat dalam proses metabolik parasit protozoa tersebut. Oleh itu, diandaikan dengan merencat protein ini boleh menyebabkan kematian parasit. Melalui analisis jujukan sasaran, dua dadah dipakai semula telah dipadankan dengan protein kecil GTPase subkeluarga Rho iaitu Dextromethorphan dan Azathioprine. Manakala, tiga dadah dipadankan dengan RhoGAP, iaitu Imatinib, Dasatinib dan Ponatinib. Analisis pendokan molekul menunjukkan tenaga pengikatan lima dadah tersebut kepada protein sasaran masing-masing ialah -8.5 kcal/mol, -8.0 kcal/mol, -7.0 kcal/mol, -5.3 kcal/mol, dan -6.8 kcal/mol, yang diwakili oleh Imatinib, Ponatinib, Dasatinib, Azathioprine dan Dextromethorphan. Simulasi dinamik molekul yang dijalankan pada 100 ns menunjukkan bahawa semua kompleks tersebut mempunyai pelbagai tahap kepadatan, keunikan dan kestabilan yang memuaskan. Ujian *in vitro* melalui penentuan kepekatan perencatan minimum (MIC) menunjukkan Dasatinib dan Imatinib dapat merencatkan RhoGAP pada nilai MIC masing-masing 12.5 µM dan 100 µM. Sebaliknya, Azathioprine dapat merencat protein kecil GTPase subkeluarga Rho pada kepekatan 500 µM dan Dextrometrophan tidak dapat merencatkan protein ini. Analisis statistik menggunakan ANOVA dan Ujian-t daripada keputusan LFQ menunjukkan bahawa kedua-dua protein; protein putatif RhoGAP dan protein yang dikawalaturnya iaitu protein kecil GTPase, subkeluarga Rho adalah antara protein yang dikawalatur secara berbeza akibat rawatan dengan Dasatinib. Analisis menggunakan perisian DAVID dan STRINGDB terhadap protein pengawalatur berbeza, khususnya memberi tumpuan kepada protein keluarga Rho GTPase dan RhoGAP, menunjukkan hubungan yang jelas antara ekspresi protein-protein pengawalatur tersebut dengan morfologi sel dan proses biologi yang terlibat apabila *Giardia* dirawat dengan Dasatinib. Kesimpulannya, kajian ini mendapati bahawa protein putatif RhoGAP berpotensi sebagai sasaran dadah dan Dasatinib merupakan dadah berpotensi digunakan dalam memerangi penyakit giardiasis.

APPLICATION OF *in silico* AND *in vitro* APPROACHES ON REPURPOSED DRUGS TARGETING PUTATIVE RHO GTPASE AND RHOGAP OF *Giardia lamblia*

ABSTRACT

Giardia lamblia is a globally distributed protozoan parasite that causes an intestinal disease named Giardiasis. The primary treatment relies on nitroimidazole drugs such as metronidazole, tinidazole, and albendazole. However, the incidence of refractory cases had increased, leading to therapeutic non-compliance. Although numerous research studies have been conducted to address these concerns, they remain unresolved. The small GTPase, Rho subfamily protein, and its positive regulator, putative Rho GTPase activating protein (RhoGAP), were found to be involved in various biological and cellular processes. They were found to work in tandem in regulating encystation and membrane trafficking and were involved in the metabolic processes of this protozoan parasite. Thus, it was hypothesized that inhibiting these proteins would lead to the killing of the parasite. The target sequence analysis showed that two repurposed drugs, Dextromethorphan and Azathioprine, were matched to the small GTPase, Rho subfamily proteins. On the other hand, three re-purposed drugs were matched to the putative RhoGAP, which were Imatinib, Dasatinib, and Ponatinib. Molecular docking analysis showed the binding energies of the five drugs to their respective proteins were -8.5 kcal/mol, -8.0 kcal/mol, -7.0 kcal/mol, -5.3 kcal/mol, and -6.8 kcal/mol, represented by Imatinib, Ponatinib, Dasatinib, Azathioprine, and Dextromethorphan, respectively. Molecular dynamics analysis performed at 100 ns showed that all the complexes exhibited various compactness, uniqueness, and satisfactory stability. *In vitro* study by the minimal inhibitory concentration (MIC) assay revealed that Dasatinib and Imatinib inhibited the putative RhoGAP at MIC values of 12.5 μM and 100 μM, respectively. On the other hand, Azathioprine inhibited the small GTPase, Rho subfamily protein at 500 μM, and Dextromethorphan was unable to inhibit this protein. Statistical analyses performed using ANOVA and t-test of the LFQ result revealed that the putative RhoGAP and its regulated protein, small GTPase, Rho subfamily protein, were among the differentially expressed proteins affected by the Dasatinib treatment. Analyses using DAVID and STRING of the differentially expressed proteins, mainly focusing on the Rho family GTPases and RhoGAP, demonstrated a clear connection between the expressions of the proteins, with the morphology and the biological process involved when the *Giardia* was treated with Dasatinib. Overall, this study concluded that the putative RhoGAP has potential as a drug target, and Dasatinib has the potential to be used to combat giardiasis.

CHAPTER 1

INTRODUCTION

1.1 Research background

Giardiasis is caused by *Giardia lamblia,* also known as *G. intestinalis* or *G. duodenalis*, a common pathogenic intestinal parasitic protozoan. Recently, it was reported that the giardiasis infection had affected an estimated 400 million people annually worldwide (Vicente *et al*., 2024). Giardiasis is a growing concern because of its ability to spread rapidly in areas with inadequate sanitation, posing significant risks to public health, particularly among children. Transmission occurs through the faecal-oral route or by ingestion of cysts from contaminated water or food (Cacciò *et al*., 2018; Resi *et al*., 2021). Despite being regarded as a prevalent disease in low-income and developing countries, current migratory flows have resulted in a rise in giardiasis cases in high-income countries (Gaona-López *et al.*, 2023). In Malaysia, the prevalence is about 0.5 % - 34.6 % were reported among schoolchildren of the aborigines (Roshidi *et al*., 2021). Infection occurs through the consumption of cysts in contaminated food or water, and a small number of cysts, between 10 to 100 cysts, is sufficient to cause an infection (Brightman, 2021). According to Brightman (2021), clinical symptoms can be difficult to recognize, as many patients are asymptomatic carriers. On the other hand, symptoms of giardiasis include watery diarrhoea (90 %), foul-smelling fatty stools (75 %), and abdominal cramps and bloating (71 %) (Brightman, 2021).

Apart from that, a vaccine for giardiasis prevention is not available for humans, but in animals, it helps in reducing symptoms and shortens the cyst-shedding durations (Aziz *et al*., 2022; Vargas-Villanueva *et al*., 2023). Treatment of giardiasis often involves nitroimidazoles such as metronidazole and tinidazole. Despite its effectiveness, treatment failures in giardiasis occur in up to 5 - 40 % of cases, and the mechanisms of resistance remain incompletely understood (Carter *et al.,* 2018; Escrig *et al.,* 2023). Although numerous research studies have been conducted to address these concerns, they remain unresolved. This happened due to inconsistent resistance-associated changes, incomplete phenotypic data, and temporary resistance of the parasite. Therefore, the search for new molecular targets that are more stable is essential.

Among multifunctional proteins in *G. lamblia*, Rho family GTPase and RhoGAP are crucial proteins that are involved in various cellular and biological processes. Such examples are that they were involved in regulating encystation, membrane trafficking, and modulating the association of Gl-14-3-3 and actin (Hardin *et al.*, 2022; Krtková *et al.*, 2016; Krtková *et al.*, 2017). Gene ontology analysis by Ng *et al.* (2018) reported that Rho family GTPase was shown to be involved in metabolic processes and catalytic activity, which targeting it might be promising in killing the parasite. Despite that, no study targeting *Giardia* Rho family GTPase proteins for the drug target has been conducted thus far.

1.2 Problem statement

Giardiasis is a prevalent parasitic infection caused by *G. lamblia*, with primary treatments including metronidazole and tinidazole. Unfortunately, treatment failures due to the emergence of drug resistance have been increasingly reported, highlighting the urgent need for alternative therapeutic strategies. Despite numerous resistance studies conducted from 1990 to 2022, the issues remain unresolved, largely because of inconsistent resistance-associated changes across different *Giardia* strains, incomplete phenotypic data, and the temporary nature of resistance, where parasites revert to sensitivity once the drug is removed (Emery *et al.*, 2018). These complications make it difficult to formulate effective treatment strategies and necessitate a more stable drug target. Given the challenges with current treatments, this study explores the potential of targeting the Rho family GTPase and its positive regulator, RhoGAP, which are crucial for cellular processes such as regulating encystation, membrane trafficking, and metabolic processes (Hardin *et al.*, 2022; Krtková *et al.*, 2016; Ng *et al*., 2018). This study postulates that inhibiting Rho family GTPase could disrupt these biological functions, potentially leading to the elimination of the parasite and offering a promising avenue for more effective treatment strategies against giardiasis. Therefore, this study focused on inhibiting small GTPase, Rho subfamily protein, and its positive regulator, putative RhoGAP, with the re-purposed drug candidates (Figure 1.1).

The general question of this project is to identify and evaluate drug compounds that can bind to and modulate the biological activity of small GTPase, Rho subfamily protein, and RhoGAP of *Giardia*. This study aims to investigate how repurposed drugs interact with these target proteins and influence their biological functions using a combination of *in silico, in vitro,* and proteomics, potentially paving the way for new therapeutic approaches that overcome the challenges of drug resistance seen with current treatments.

1.3 Research objectives

General objectives

The main objective of this study was to elucidate the potential of Rho family GTPase, including small GTPase, Rho subfamily protein, and its positive regulator, putative RhoGAP, as re-purposed drug targets of *G. lamblia*. The experimental workflow of this study is shown in Figure 1.2.

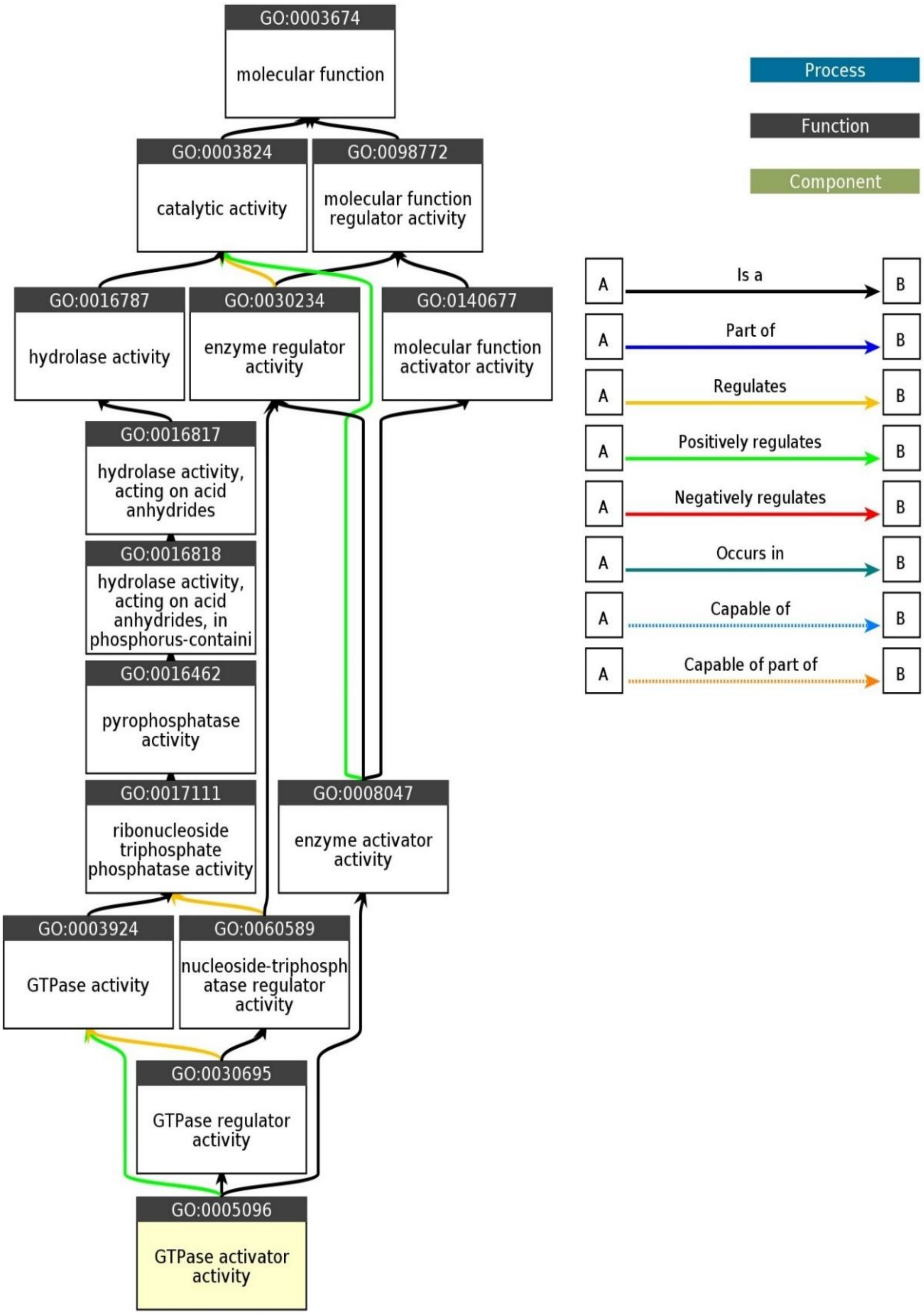

Figure 1.1 Gene ontology of the molecular function of *G. lamblia* putative RhoGAP (GO:0005096) and Rho GTPases including small GTPase, Rho subfamily protein (GO:0003924). The gene ontology shows that the putative RhoGAP positively regulates the small GTPase, Rho subfamily protein; thus, this indicates that both proteins work in tandem for proper function.

Objectives:

1. To identify potential re-purposed drugs targeting the small GTPase, Rho subfamily protein, and putative RhoGAP by conducting target sequence searches using the DrugBank database and evaluating the binding energies and other interactions using molecular docking.
2. To evaluate the binding affinity of re-purposed drug candidates using the DrugBank database on the small GTPase, Rho subfamily protein, and putative RhoGAP using the molecular dynamics simulation (MD).
3. To measure the effect of the re-purposed drug candidates for *G. lamblia* using the minimum inhibitory concentration (MIC) method.
4. To evaluate the expression of the putative RhoGAP and its regulated protein; small GTPase, Rho subfamily protein, using label-free-quantitative proteomics (LFQ) in *Giardia* trophozoites treated with the selected re-purposed drug.

1.4 Experimental work design

This study follows a systematic workflow to determine the suitability of both proteins as protein targets in combating G. *lamblia* (Figure 1.2). A sequence-based search using the DrugBank database was conducted to identify candidate compounds. Molecular docking was performed to predict drug-protein interactions, followed by MD simulations to assess the stability of these interactions. The compounds were tested *in vitro* using MIC assays to measure the effects on *Giardia* trophozoites. Trophozoites were collected at various time points to track time-dependent effects of the drug treatment. To understand the molecular effects of drug treatment, proteomic analysis was conducted using an LFQ approach, and differentially expressed proteins were analyzed through Gene Ontology to determine affected biological pathways.

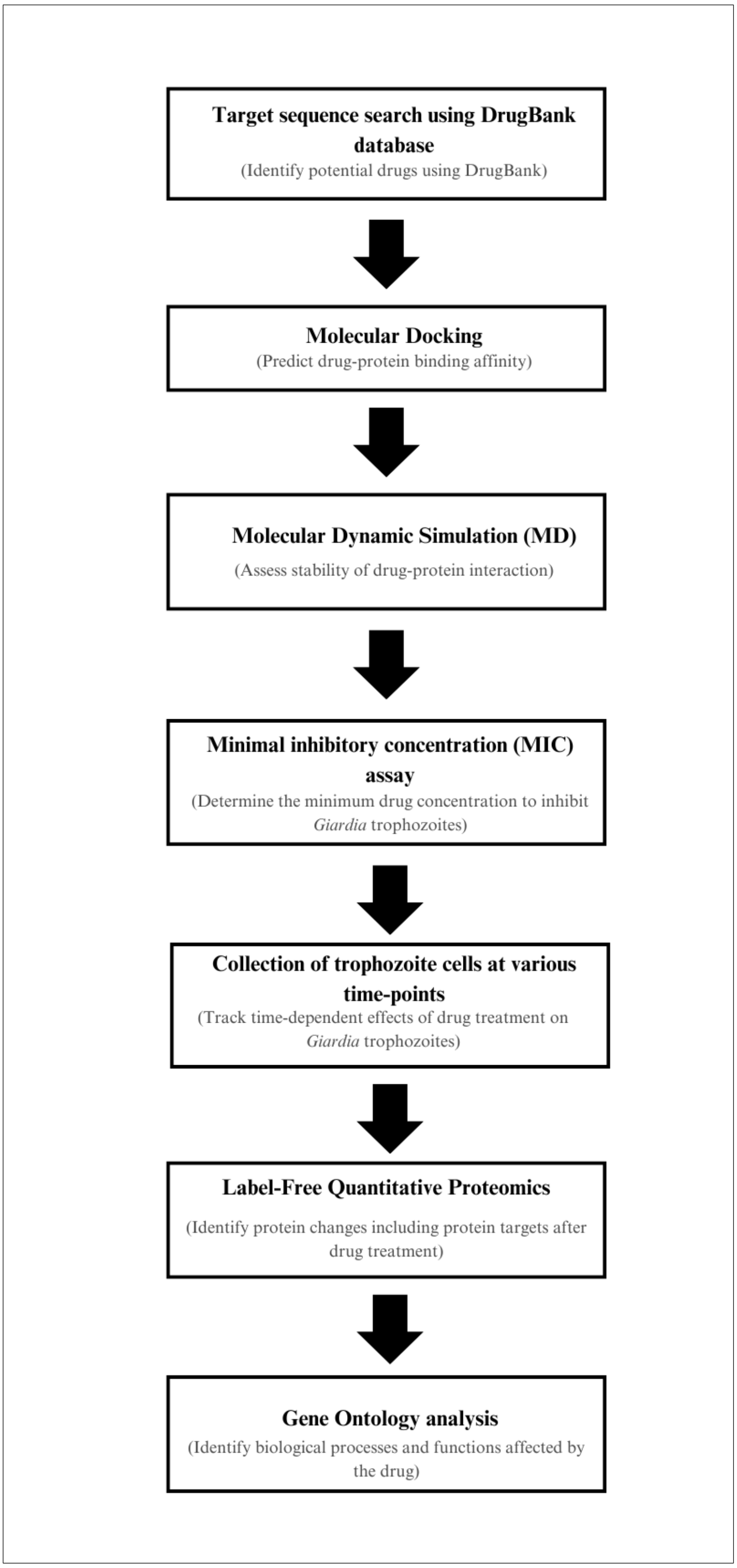

Figure 1.2 Flow-chart of this study

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of giardiasis and prevalence

Giardiasis is a neglected tropical disease caused by the enteric protozoan parasite *G. lamblia*. It is a worldwide health problem that requires public attention and was included in the "Neglected Diseases Initiative" in 2004 by the World Health Organization (WHO) (Albogami, 2023; Savioli *et al.*, 2006). This disease has been reported to be associated with poverty and to have contributed to negative impacts on development and socio-economic improvement (Savioli *et al.*, 2006; Šmigová *et al.*, 2022). It also affects livestock and pets, highlighting the need for a One Health approach to control the disease (Feng & Xiao, 2011; Roshidi *et al.*, 2021). One Health is a method to ensure the well-being of people, animals, and the environment through collaborative problem-solving at local, national, and global levels (Mackenzie & Jeggo, 2019). It includes designing and implementing programmes, policies, legislation, and research in which multiple sectors communicate and work together to achieve better public health outcomes.

More than 300 million cases are reported annually; however, there is no availability of approved vaccines for humans to prevent giardiasis (Garzon *et al*., 2021; Sengupta & Chakraborty, 2023). A vaccine has been developed for cats and dogs, but it only reduces symptoms and shortens the duration of cyst shedding (Aziz *et al*., 2022; Olson *et al*., 2000). Giardiasis has also been identified as a predictor of malnutrition in children as it is significantly linked with protein-energy malnutrition (PEM), micronutrient deficiency, particularly vitamin A deficiency (VAD), and iron deficiency anaemia (IDA). These deficiencies have been shown to lead to impaired cognitive functions and poor educational performance in children (Al-Mekhlafi *et al*., 2005).

As reported by Feng and Xiao (2011) and Roshidi *et al.* (2021), the worldwide prevalence of giardiasis was reported to be 20-40 % in low-income countries, with the highest risk populated by children under 5 years old. The high prevalence of giardiasis in low-income countries is primarily due to inadequate sanitation, limited access to clean water, poor hygiene practices, and lack of awareness (Hajare *et al*., 2022; Bekele, 2023). Many low-income countries lack proper sanitation facilities, leading to open defecation and indirectly contaminating the water sources with *Giardia* cysts. In addition, access to clean drinking water is limited, which forces people to rely on untreated or poorly treated water from rivers, lakes, or wells. On the other hand, the worldwide prevalence in high-income countries is about 2-7 %, and 40 % of the reported cases are due to travellers returning from highly endemic areas (Feng & Xiao, 2011; Roshidi *et al.*, 2021). The low prevalence in high-income countries is due to the countries' well-developed sanitation systems, which include broad sewage treatment facilities that can effectively remove contaminants, including *Giardia* cysts, from water supplies.

In Asia, the highest prevalence of giardiasis was reported in Nepal, about 73.4 %, which occurred due to poor sanitation (Roshidi et al., 2021). A study by Kalavani *et al*. (2023), reported that the pooled prevalence of *G. lamblia* infection among Asian children was estimated to be 15.1 %. Tajikistan and China had the highest and lowest pooled prevalence values of *G. lamblia* infection, which were 26.4 % and 0.6 %, respectively (Kalavani *et al*., 2023). Also, the prevalence of giardiasis tends to be higher in males than in females. This trend has been observed in various studies, which indicate that males are more frequently affected by giardiasis than females. Such examples could be observed from many independent studies performed in various countries such as Iraq (Khalid *et al*., 2023), Nigeria (Sikiru, 2020), Africa, Chad (Ndifor *et al.*, 2020), including Malaysia (Jeyaprakasam *et al.*, 2019). According to their studies, the higher prevalence in males was perhaps due to males tending to be involved more in outdoor activities such as swimming in the river, playing football, and eating outside (Jeyaprakasam *et al*., 2019; Khalid *et al.*, 2023; Sikiru, 2020). Despite that, there were still reported studies regarding the higher prevalence of giardiasis in females compared to males. However, most studies were not statistically significant (Abd Ghani & Musa, 2018; Haji & Bamarni, 2023).

In Malaysia (Figure 2.1), the prevalence of giardiasis could be underreported. Despite that, the recent rate of giardiasis in Malaysia is quite encouraging, which is at 13.7%, but no specific areas were mentioned (Roshidi *et al.*,2021). A previous report by Choy *et al*. (2014) reported that the prevalence of giardiasis was the highest among aboriginal communities (29.2 %), with higher cases diagnosed in West Malaysia compared to East Malaysia. In West Malaysia, the occurrence of *Giardia* infection was high among aboriginal communities, especially in children under 12 years old. Giardiasis was the highest among aboriginal communities in Pahang (15.9 %), followed by Negeri Sembilan (14.9 %) and Kedah (13.4 %), and the lowest in the community in Malacca (4.6 %). To conclude, giardiasis is a significant but neglected tropical disease, especially in low-income countries where poor sanitation and limited access to clean water enable it to spread. The disease particularly affects children, leading to serious health and developmental problems. With no effective human vaccination and developing treatment resistance, it is critical to manage giardiasis, particularly in underreported areas such as Malaysia.

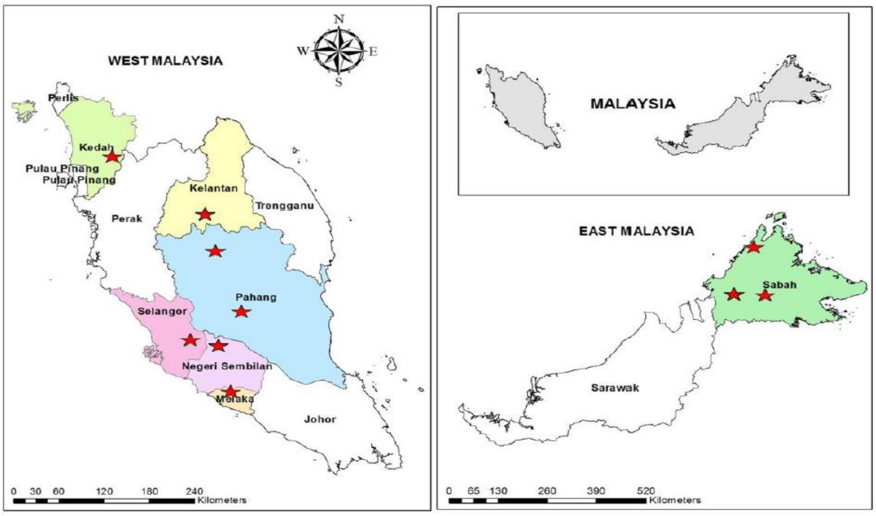

Figure 2.1 Occurrence of giardiasis in Malaysia (Choy *et al.*, 2014)

2.1.2 Symptoms and clinical manifestation of giardiasis

The clinical presentation of giardiasis can vary widely, ranging from asymptomatic cases to severe gastrointestinal symptoms. The most common symptoms include diarrhoea, loose or watery stools, abdominal cramps, bloating, and nausea. In some cases, the infection can lead to more severe complications, such as fatty stools, malabsorption, and significant weight loss, particularly in children. The symptoms usually begin 1 to 2 weeks after ingesting the cyst and can continue for 2-6 weeks in healthy individuals. An individual with a weakened immune system, such as persons with HIV/AIDS, cancer, and transplant patients, may have a more serious infection that can lead to severe illness or death (Angarano *et al.*, 1997; Huang *et al.*, 2023; Rosser & Blackburn, 2021). The patients often present with a history of a gradual onset of 2–5 loose stools per day and increasing fatigue (Roellig, 2023). While gastrointestinal symptoms are most associated with giardiasis, the infection can also present with dermatological symptoms. Olszak *et al.* (2023), have identified a range of skin conditions linked to giardiasis, including urticaria, angioedema, atopic dermatitis, erythema nodosum, and Wells syndrome, which is a rare form of eosinophilic cellulitis. Though these skin reactions are rare, they may occur as part of the body's immune response to the infection.

Furthermore, there are rare reports of neurological and cardiac complications arising from giardiasis. For instance, some patients have developed sensory neuropathy, a condition characterized by damage to the sensory nerves, leading to symptoms such as tingling, numbness, and pain (Elrefaey & Memon, 2023). Additionally, cases of myopericarditis, an inflammation of the heart muscle and its outer lining, have been documented following *Giardia* infection (Heredia *et al.*, 2024). Further complications may include polyneuropathy, which damages multiple peripheral nerves, and malabsorption syndrome, leading to significant nutritional deficiencies (Olszak *et al.*, 2023).

In paediatric patients, giardiasis poses a significant concern due to its potential impact on growth and development. The presence of *Giardia* has been linked to reduced weight gain and impaired linear growth (Riba *et al*., 2020). Riba *et al.* (2020) demonstrated in a neonatal mouse model that *G. lamblia* infection induces bile secretion and utilized bile constituents, such as phosphatidylcholine, as growth substrates. The infection also altered the gut microbiota, resulting in increased bile acid deconjugation and disrupted energy metabolism. These changes contributed to increased energy expenditure, reduced fat storage, and impaired weight gain, which led to stunted growth in infected subjects (Riba *et al.*, 2020). Further research by Giallourou *et al.* (2023), highlighted that giardiasis can cause growth restriction in children, accompanied by broad amino acid deficiencies and the overproduction of specific phenolic acids. These metabolic alterations were significant contributors to the impaired growth observed in affected children. Also, in other rare cases, giardiasis has been associated with reactive arthritis, which has been documented by Turner *et* *al.* (2021). To conclude, the symptoms and clinical manifestations of giardiasis are varied and can range from mild to severe conditions. By understanding the symptoms, the disease can be properly diagnosed, and the right treatment can be provided.

2.1.3 Treatment of giardiasis and its challenges

Giardiasis requires prompt and effective treatment to relieve symptoms and prevent complications. The treatment options for giardiasis include several classes of antiparasitic drugs, such as nitroimidazoles, benzimidazoles, aminoglycosides, quinacrine, furazolidone, and nitazoxanide (Gardner & Hill, 2001). For nitroimidazole, the class includes metronidazole, tinidazole, ornidazole, and secnidazole (Gardner & Hill, 2001). This class was identified in the year 1955 and proved to be quite efficient against various protozoan diseases (Gardner & Hill, 2001; Tracy, 1996). Metronidazole, also known as Flagyl, was discovered in the late 1950s and found to be effective against *Trichomonas vaginalis* and *Entamoeba histolytica* (Durel *et al*., 1960). In 1962, Darbon *et al.* discovered that it could be used to treat giardiasis (Gardner & Hill, 2001). Since this discovery, metronidazole and other nitroimidazoles have been employed as the basis of therapy for giardiasis.

Despite its widespread use, metronidazole has never been officially recognized by the United States Food and Drug Administration (FDA) specifically for treating giardiasis (Gardner & Hill, 2001). This has raised concerns about its regulatory status. Of the nitroimidazoles, metronidazole's mechanism of killing *Giardia* has been the most thoroughly studied, and metronidazole is often considered the first-line treatment due to its widespread use, established efficacy, and availability. The mechanism of how the drugs work involves the entrance of the drugs to the trophozoites of the parasite, and the drugs are reduced through anaerobic metabolic pathways, creating reactive metabolites. These metabolites bind to the DNA of the trophozoites, causing damage that disrupts the synthesis of nucleic acids and leads to cell death (Gardner & Hill, 2001). In addition, metronidazole inhibits the respiration of trophozoites and can produce toxic radicals that harm the cells (Gardner & Hill, 2001). For the dosage, the standard dosing for metronidazole is 250 to 500 mg 3 times a day for 5 to 10 days (Dunn, 2024). For children, the dosage is typically based on body weight, generally around 30 mg/kg to 50 mg/kg per day for 3 doses (Dunn, 2024).

While metronidazole is a preferred treatment for giardiasis, it has several drawbacks such as the side effects, not FDA approved for giardiasis, the emergence of resistance, the occurrence of drug interactions with other drugs or alcohol, and limited use in certain populations such as pregnant women. For example, the common side effects include gastrointestinal symptoms such as nausea, vomiting, and diarrhea. Some patients may also experience a metallic taste, dizziness, and headaches (Gardner & Hill, 2001).

Also, metronidazole can interact with other medications, particularly those that affect the liver, and it should not be taken with alcohol due to the risk of disulfiram-like reactions (Alonzo *et al.*, 2019; Miljkovic *et al.*, 2014). In addition, administering metronidazole to pregnant women, especially in the first trimester, requires caution because the drug safety in this group has not been thoroughly demonstrated (Gardner & Hill, 2001). These drawbacks highlight the need for careful consideration when using metronidazole for treating giardiasis and may necessitate alternative therapies in certain cases.

Although there are other alternative drugs present to combat giardiasis, there are still limitations that persist, such as the benzimidazoles group, which still requires further studies to confirm its efficacy and safety, as it is poorly absorbed and the excretion through the kidneys is negligible (Gardner & Hill, 2001). The aminoglycosides group, specifically paromomycin, is recommended for use in pregnancy but may have limitations in terms of effectiveness such as it achieves only a minimal concentration in the blood, though high dose of drugs was prescribed, and the side effects include ototoxicity and nephrotoxicity (Gardner & Hill, 2001). Furazolidone, although efficient, is not recommended as it causes mammary tumours in mice and mutations in bacteria (Gardner & Hill, 2001). To conclude, the drug treatments for giardiasis are various, but the drawbacks that exist in all the classes necessitate the findings of new drugs.

2.2 Overview of *G. lamblia*, taxonomy, and morphology

G. lamblia, also known as *G. intestinalis* or *G. duodenalis*, is a protozoan parasite that infects the gastrointestinal tract of humans and animals, causing a disease known as giardiasis. It is one of the leading causes of water and foodborne infections globally and has been overlooked by the World Health Organization (WHO) (Kılınç *et al*., 2023). Although *Giardia* is not classified as a primary bioterrorism agent, it is categorized as a Category B agent, which is the second highest priority of bioterrorism agents by the Centres for Disease Control and Prevention (CDC) (Bonilla-Santiago *et al.*, 2008). In addition, the Food and Agriculture Organization (FAO) also ranks *Giardia* as the 11th of the 24 most significant food-borne parasites, which highlights its impact on public health and the importance of monitoring and managing its presence in food and water supplies (Ryan *et al*., 2019).

Taxonomically, *Giardia* is a eukaryotic organism classified in the supergroup *Excavata*. It is placed within the phylum *Metamonada*, class *Diplomonadea*, order *Diplomonadida*, family *Hexamitidae,* and genus *Giardia*. The protozoan parasite was first described by Antonie van Leeuwenhoek in the year 1681, who discovered it in his stool samples using one of the microscopes (Adam, 2001; Dobell, 1920). It was later described in detail by a Czech physician named Vilem Lambl in the year 1859, who thought the organism belonged to the genus *Cercomonas* and named it as *Cercomonas intestinalis* (Adam, 2001). The significant contribution of Lambl was later acknowledged by Raphael Blanchard in the year 1888, who honoured him by naming the species Lamblia, leading to the full species name *Giardia lamblia* (Faubert, 2000; Meyer & Jarroll, 1980). The three names, which are *G. lamblia, G. intestinalis*, and *G. duodenalis*, are used interchangeably due to historical naming conventions and taxonomic revisions over time (Adam, 2001; Kulda *et al.*, 1978). According to Adam (2021), the name *G. lamblia* is the most used in medical writing, whereas *G. intestinalis* and *G. duodenalis* are used in scientific literature.

Morphologically, *G. lamblia* exists in two forms, which are the trophozoite and the cyst (Figure 2.2). Trophozoites are the vegetative forms, while cysts are the infective forms. The trophozoite is pear-shaped and about 12–15 μm long, 5–7 μm wide, and 1–2 μm thick (Carranza & Lujan, 2010). It has a median body with two symmetric nuclei at the anterior end of the body (Naeem *et al*., 2023). The two nuclei also contain a central nucleolus (Benchimol & De Souza, 2011). By light microscopy, the *Giardia* trophozoites look like a smiling face in which the two nuclei are the eyes, while the median body line is the mouth (Benchimol & De Souza, 2011). The surfaces of the median body are dorsally convex and ventrally flat (Naeem *et al*., 2023). The trophozoites also have four pairs of flagella emerging in four areas anterior, posterior, ventral, and caudal areas (Carranza & Lujan, 2010). The flagella are made of microtubules in a typically eukaryotic 9+2 pattern and are produced by basal bodies between both nuclei (Carranza & Lujan, 2010; Elmendorf *et al*., 2003). These complex cytoskeleton structures of *Giardia* are important for its attachment to the enterocytes of the host intestine (Carranza & Lujan, 2010; Luján *et al*., 1997).

In contrast, the cyst is a smooth-walled structure with an oval or ovoid shape (Carranza & Lujan, 2010; Naeem *et al.*, 2023). The size of the cyst ranges from 7 to 10 µm while its length is about 8 to 12 µm (Naeem *et al*., 2023). The thickness of the cyst wall varies from 0.3 to 0.5 µm and is composed of the outer filamentous layer and the inner membranous layer, which includes two membranes that enclose the periplasmic area (Adam, 2001; Carranza & Lujan, 2010). Compared to the trophozoites, the mature cyst contains four nuclei while the trophozoites only have two (Adam, 2021; Carpenter *et al.*, 2012). The cyst wall of the cyst is made of carbohydrates in the form of N-acetyl galactosamine polymers and cyst wall protein (CWP) (Carranza & Lujan, 2010; Luján *et al.,* 1995; Luján *et al.,* 1997; Sun *et al.*, 2003). Also, the metabolic rate of the cyst is only 10 % to 20 % of the trophozoites, which allows it to survive in the environment mainly in cool and moist areas. This perhaps explains the higher frequency of giardiasis in the northern part of the United States compared to the Southern areas (Adam, 2021; Coffey *et al.*, 2020).

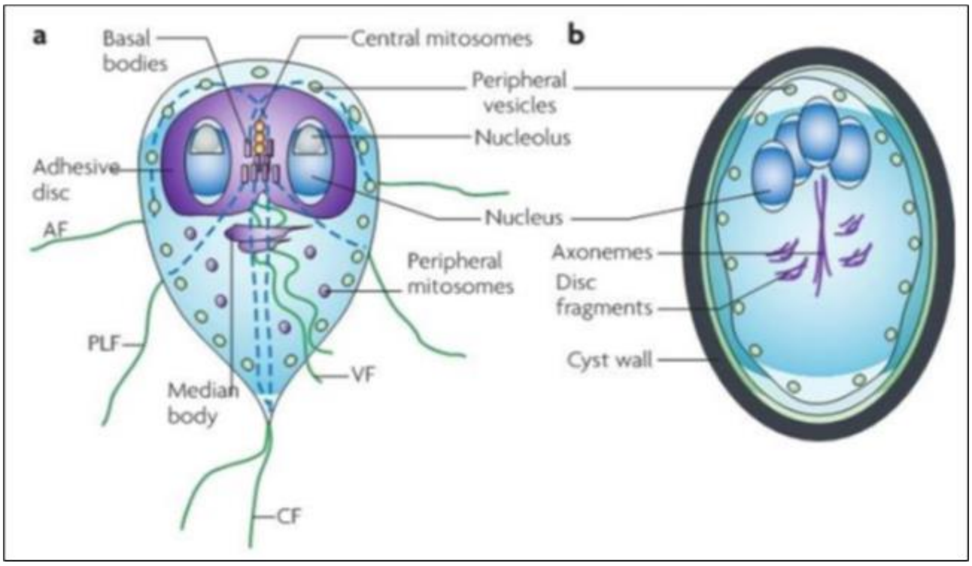

Figure 2.2 Morphological characteristics of the trophozoite (a) and cyst (b) of *G. lamblia* (Ankarklev *et al*., 2010).

2.2.1 Life cycle

G. lamblia has a simple life cycle that comprises two primary stages: the infectious cyst and the proliferating trophozoite (Zhang *et al*., 2023). According to Gaona-Lopez *et al.* (2023), the main form of reproduction is asexual, through binary fission; however, sexual reproduction is currently the subject of great research, which has been speculated would contribute to genetic variability, essential for the acquisition of resistance to antibiotics. The infection begins when a host ingests contaminated food or water contaminated with environmentally resistant cysts (Siddiq *et al.*, 2023). Studies indicated that the cysts remained viable for a few weeks or even months at warm temperatures and were resistant to chlorine concentrations typically used for water purification (Gaona-López *et al.*, 2023). Once inside the host, the cyst travels through the stomach, where an acidic environment encourages excystation occurring within the duodenum and jejunum of the small intestine (Wiggins, 2023).

An acidic environment occurred due to the presence of hydrochloric acid, bile, and trypsin (Benchimol *et al.*, 2023). Each *Giardia* cyst excysts into two *Giardia* trophozoites, and this trophozoite stage is the target of all chemotherapeutic therapies (Gaona-López *et al.*, 2023; Wiggins, 2023). The trophozoites dwell in the duodenum of the small intestine and firmly latch themselves onto enterocytes within the lumen and microvilli of the small intestine (Wiggins, 2023). The parasites use their anterior disc to connect to the intestinal wall to feed on the contents and replicate through binary fission within the small intestine lumen (Makawi & Yasin, 2023). Upon reaching the lower intestine, fluctuations in environmental conditions such as pH and cholesterol induce the trophozoites to convert into cysts through a process called encystation, which are then excreted through faeces, thus restarting the life cycle (Makawi & Yasin, 2023). The cysts are instantly infectious after excretion, and the life cycle can be completed in 72 hours, despite the prepatent period typically being 4-10 days (Makawi & Yasin, 2023).

2.2.2 Metronidazole-resistance *G. lamblia*

Metronidazole is the most used drug for the treatment of giardiasis and has been extensively used for more than 60 years. However, the emergence of resistance cases is becoming a problem across the globe. The study by Morch and Hanevik (2020) reported that the recent prevalence rates of refractory infections can be increased by up to 50 %. Though the causes of resistance are widely studied, the issues are not been completely resolved yet as the mechanism of resistance is not well understood. No specific genetic marker of metronidazole resistance has been discovered, and most studies utilize laboratory-derived sub-assemblage AI isolates, which rarely infect humans.

This mechanism of resistance is not well understood due to the metronidazole resistance in *Giardia* is multifactorial and associated with complex changes (Krakovka *et al.*, 2022). Despite that, a core set of pathways that involve the oxidoreductase, oxidative response, and DNA repair proteins becomes central to metronidazole resistance in bacteria and protozoa (Krakovka *et al.,* 2022). According to Ansell *et al.* (2017) and Ordoñez-Quiroz *et al.* (2018), the mechanism underlying the metronidazole resistance *Giardia* is associated with the decrease in the activity of the enzymes implicated with its activations such as the nitroreductase-1, thioredoxin reductase, and also pyruvate-ferredoxin oxidoreductase (PFOR), which resulted in the inactivation of metronidazole.

In addition, the low expression of the oxygen detoxification enzymes allows passive detoxification of the metronidazole via futile redox cycling (Ansell *et al*., 2017). Both mechanisms are called passive mechanisms of resistance. On the other hand, the active mechanism of resistance includes complete enzymatic detoxification of the pro-drug by nitroreductase-2 and enhanced repair of oxidized biomolecules via thioredoxin-dependent antioxidant enzymes (Ansell *et al.*, 2017). Another study by Faieq and Hraiga (2020), reported on the gene expression of drug-resistance genes, and their results showed the expression of the chaperone genes had increased up to 10 and 7-fold changes for both HSP 70 and 90, respectively. In the study by Ansell *et al.* (2017) the heat shock protein was one of the proteins that showed changes along with two other proteins, which were peroxidases and FMN-binding oxidoreductase. His study was supported by Ochoa-Maganda *et al.* (2020) reported that the expression of the genes was associated with the altered expression of genes involved in stress response, such as heat shock protein (Ochoa-Maganda *et al*., 2020). Though many studies have been performed to address this issue, the inconsistent resistance-associated changes between lines, incomplete phenotypic data, and the resistance that is rarely genetically fixed merit the identification of drug targets that are more stable to combat giardiasis (Emery *et al.*, 2018).

2.2.3 Role of Rho family GTPase proteins in *G. lamblia*

The Rho family GTPases of *G. lamblia*, including small GTPase, Rho subfamily protein, and its positive regulator, putative RhoGAP, are crucial proteins that work in tandem to facilitate the parasite's survival, transmission, and pathogenicity (Figure 2.3)(Consortium, 2025). These proteins belong to a family of small signalling G proteins and a subfamily of the Ras superfamily (Mott & Owen, 2010). They act as molecular switches that regulate various cellular processes by cycling between an active GTP-bound state and an inactive GDP-bound state (Mosaddeghzadeh & Ahmadian, 2021; Thomas *et al.*, 2021).

In *G. lamblia*, 11 members of the Rho GTPase family have been identified, a significantly lower number compared to humans, which possess more than 20 members (Benitah *et al*., 2004; Consortium, 2025c). The limited number of Rho GTPases in *G. lamblia* is likely due to its reduced genome and simplified signaling network. The size of the small GTPase, Rho subfamily protein, is 23.9 kDa, whereas RhoGAP is 24.7 kDa (Consortium, 2025b, 2025e). In terms of localization, both proteins are primarily cytosolic. Still, they can associate with membranes and can be found to localize at other organelles depending on activation and function, such as at the endoplasmic reticulum (ER) and the Golgi apparatus-like encystation-specific vesicles (ESVs), where they regulate the encystation process (Krtková *et al.*, 2016).

Currently, no experimentally determined 3D structures of *G. lamblia* small GTPase, Rho subfamily protein, and RhoGAP are available in the Protein Data Bank (PDB). However, a predicted structure is accessible through AlphaFold. This lack of experimental structures contrasts with the growing number of studies on human Rho GTPases, where multiple 3D structures have been elucidated; RhoB (Bery *et al.*, 2019), RhoA (Ihara *et al*., 1998), RhoC (Dias & Cerione, 2007), etc.

According to Hardin *et al.* (2022), the Rho family GTPases were found to be involved in the regulation of membrane protrusion of *G. lamblia,* also known as the ventrolateral flange. This structure is essential for the attachment of the parasite to the host intestine. The membrane protrusion of *G. lamblia* is a sheet-like membrane at the interface between the parasites and attached surfaces. The Rho family GTPases were reported to be located at the flange of *Giardia*. The study by Hardin *et al.* (2022) reported that the knockdown of Flangin, *Gl*Actin, and *Gl*Rac resulted in a defect of the flange formation. These defects impaired the parasite's ability to attach to the intestinal wall, limiting its ability to establish infection and evade host immune responses.

In addition, other studies have reported the different localization of the Rho family GTPases, which were found to be associated with the endoplasmic reticulum (ER) and Golgi apparatus-like encystation-specific vesicles (ESVs). Their study reported that the Rho family GTPases play a role in regulating the encystation process (Krtková *et al*., 2016). Encystation is a process of production of the cyst, an infectious form of *Giardia* that is crucial for the transmission of giardiasis, in which swallowing as low as 10 cysts can cause a person to become ill (Logue *et al.*, 2017). It regulates the encystation process in many ways. For example, it signalling help regulate the production of cyst wall protein (CWP), the maturation of encystation-specific vesicles (ESV), and the secretion of CWP. These processes are crucial in forming a viable cyst.

One of the Rho family GTPases was found to be increased in abundance in the virulence variant of *E. histolytica* when a comparison was performed to the avirulent strain, where it was reported to be increased in abundance among 37 proteins that were differentially regulated (Ng *et al.*, 2018). Additionally, through Protein Analysis Through Evolutionary Relationships database (PANTHER DB) analysis, it was revealed that the protein participates in catalytic activity and the metabolic processes of the parasite. The involvement of the protein in the parasite's catalytic activity and metabolic process showed that it was important for its survival; thus, targeting it would lead to the killing of the parasite (Ng *et al.*, 2018).

Similarly, humans also possess Rho GTPase, and they have been found to be involved in cellular functions such as cytoskeletal regulation and cell signaling (Estrach *et al*., 2002; Mosaddeghzadeh & Ahmadian, 2021). Despite the presence of similar proteins in both humans and parasites, the *Giardia*, GlRac shares only 50% identity with human Rac1, indicating significant divergence between human and parasite Rho GTPases (Krtková *et al.*, 2016). Similarly, *Giardia*'s RhoGAPs also have a low sequence similarity and different structures compared to those in humans.

Importantly, while human Rho GTPases are essential for normal cellular homeostasis, they are not considered virulence factors. In contrast, *Giardia* Rho GTPases are engaged in parasite-specific processes like encystation, cytoskeletal rearrangement, and pathogenesis functions that directly contribute to the organism's ability to survive in the host and cause disease. Therefore, the virulence is not due to the GTPases alone, but rather how *Giardia* uses them in its life cycle to support infection and survival in the host. Thus, inhibiting the parasite's Rho family GTPase and RhoGAP offers a promising therapeutic strategy to combat giardiasis, with a lower risk of disrupting human cellular function.