IDENTIFICATION OF IMMUNOGENIC PROTEINS FROM CYSTS AND TROPHOZOITES OF GIARDIA LAMBLIA BY IMMUNOASSAY

NORHAMIZAH BINTI ROSHIDI

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by

NORHAMIZAH BINTI ROSHIDI

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LIST OF SYMBOLS

=	Equal to
~	About
%	Percentage
>	More than
°C	Degree Celsius
µg	Microgram
µL	Microliter
cm	Centimeter
dH_2O	Distilled water
et al.	*et alii* – ‘and others’
× *g*	Gravity
g	Gram
kDa	Kilodalton
L	Litre
mA	Miliampere
mL	Milliliter
mM	Milimolar
OD_{450nm}	Optical density at wavelength of 450 nm
s	Second
kV	Kilovolt

LIST OF ABBREVIATIONS

EIA	Enzyme immunoassay
ELISA	Enzyme- linked immunosorbent assay
HRP	Horseradish peroxidase
HCl	Hydrochloric acid
IBS	Irritable bowel syndrome
IFA	Immunofluorescent assay
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
NaCl	Sodium chloride
NaOH	Sodium hydroxide
TEMED	Tetramethylethylenediamine
TBS	Tris-buffered saline
TBST	TBS-Tween 20
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
NDI	Neglected Diseases Initiative
WHO	World Health Organization
CDC	Centres for Disease Control and Prevention
NIADH	National Institute of Allergy and Infectious Diseases

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PENGENALPASTIAN PROTEIN IMUNOGENIK DARIPADA SISTA DAN TROFOZOIT GIARDIA LAMBLIA SECARA IMUNOASAI

ABSTRAK

Giardia lamblia wujud dalam dua peringkat dalam kitaran hayat; trofozoit yang motil dan sista yang mampu beradaptasi dalam persekitaran yang lasak, yang menjangkiti manusia dan haiwan dengan penyakit yang dikenali sebagai giardiasis. Giardiasis merupakan masalah kesihatan awam yang penting, dengan kira-kira 280 juta jangkitan simptomatik dalam manusia berlaku setiap tahun dan menyumbang kepada 1.6 juta kes kematian cirit-birit pada 2016. Di Malaysia, kes giardiasis dilaporkan berkisar antara 2.6% kepada 25% dari 1970-2000 dan meningkat kepada 10.4% kepada 28.3% dari 2002 hingga 2019. Walaubagaimanapun, kajian mengenai giardiasis amat terhad dan kebanyakannya bertumpu kepada kajian mengenai ekspresi variasi gen dan pembangunan vaksin. Oleh itu, kajian ini dijalankan untuk mencirikan protein *Giardia* yang boleh membawa kepada pemahaman yang lebih baik mengenai potensi protein *Giardia*-imunogenik untuk aplikasi diagnostik dan terapeutik. Pendekatan kajian ini melibatkan pengkulturan trofozoit dan sista *G. lamblia* secara in-vitro dalam medium kultur yang berbeza, pengekstrakan protein dan pemisahan menggunakan OFFGEL^{TM} sebagai langkah pra-pemeringkatan yang lebih baik sebelum imunoasai dan analisis spektrometri jisim untuk mengenal pasti identiti protein imunogenik yang berpotensi. Bilangan maksimum trofozoit sebanyak 9.6×10^6 sel/ml berjaya dihasilkan dalam medium TYI-S-33 pada pH 7 selama 72 jam pada 37°C manakala bilangan sista tertinggi sebanyak 7.45×10^5 sel/ml diperoleh menggunakan medium TYI-S-33 yang dimodifikasi sepenuhnya mengandungi 0.25 mg/ml hempedu khinzir pada pH 7.8 selama 72 jam pada 37°C.

Pewarnaan gel dengan pewarna Coomassie dan perak menunjukkan penyebaran protein yang tidak seragam, di mana kebanyakkan protein trofozoit bertumpu antara saiz 50 kDa hingga 100 kDa manakala protein sista yang paling menonjol muncul di antara saiz 35 hingga 50 kDa. Analisis protein trofozoit melalui pemendapan Western IgA dan pemendapan Western IgG mengesan sejumlah 21 jalur protein imunogenik yang berpotensi, di mana dua daripada protein tersebut dikenali sebagai protein dengan domain SH2_2 dan rantaian berat dinein. Analisis pemendapan Western IgA dan pemendapan Western IgG bagi protein sista pula mengesan sejumlah 11 jalur protein imunogenik yang berpotensi, di mana tiga daripada protein tersebut diidentifikasi sebagai permodelan semula kromatin ATPase INO80, protein kinase non-spesifik serina/treonina dan rantaian berat dinein IAD-4 melalui kaedah spektrometri jisim, LCMS/MS. Hasil kajian ini menjadi asas dan berpotensi untuk dikaji selanjutnya dalam usaha meneroka potensi dan aplikasi protein ini dalam bidang diagnostik dan terapeutik bagi giardiasis.

IDENTIFICATION OF IMMUNOGENIC PROTEINS FROM CYSTS AND TROPHOZOITES OF GIARDIA LAMBLIA BY IMMUNOASSAY

ABSTRACT

Giardia lamblia exists in two stages in the life cycle; the motile trophozoite and the resistant cyst that is highly adapted to harsh environments, infecting humans and animals with a disease known as giardiasis. Giardiasis is a significant public health concern, with an estimated 280 million symptomatic human infections occurring each year and contributing to 1.6 million cases of diarrheal death in 2016. In Malaysia, cases of giardiasis reportedly ranged from 2.6% to 25% from 1970 to 2000 and rise to 10.4% to 28.3% from 2002 to 2019. However, studies on giardiasis are very limited and mostly focused on the variation of gene expression and vaccine development. Therefore, this study was conducted to characterize *Giardia* proteins that may lead to a better understanding of *Giardia*-immunogenic proteins potential for diagnostics and therapeutic applications. The approach of this study involves in-vitro cultivation of *G. lamblia* trophozoites and cyst in different culture medium, protein extraction and separation using OFFGEL™ as an improved pre-fractionation step prior to immunoassays and mass-spectrometry analysis to identify the identity of potential immunogenic proteins. The maximum number of trophozoites of 9.6 x 10^6 cell/ml was successfully obtained in the TYI-S-33 medium at pH 7 for 72 hours at 37°C, while the maximum number of cysts of 7.45 x 10^5 cell/ml was produced in complete modified TYI-S-33 medium containing 0.25 mg/ml of porcine bile at pH 7.8 for 72 hours at 37°C. Gels stained with Coomassie blue and silver-staining revealed a non-uniform distribution, where the most prominent proteins of trophozoites appeared between size 50 to 100 kDa while the prominent proteins of

cysts appeared between size 35 to 50 kDa. The analysis of IgA-western blot and IgG-western blot of trophozoite proteins detected a total of 21 potential immunogenic bands, of which two of the potential proteins were identified as SH2_2 domain-containing protein and dynein heavy chain. The analysis of IgA-western blot and IgG-western blot of cysts revealed a total of 11 potential immunogenic bands, of which three of the potential proteins were identified as chromatin-remodeling ATPase INO80, non-specific serine/threonine protein kinase and IAD-4 dynein heavy chain via LCMS/MS mass-spectrometry analysis. The results of this study form the basis and potential for further study in an effort to explore the potential and application of these proteins in the diagnostics and the therapeutics of giardiasis.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Giardia lamblia (*G. lamblia*) also known as *G. duodenalis* or *G. intestinalis* is a parasitic protozoan that predominantly infects the small intestine of humans and other animals through the ingestion of contaminated food or water with cysts and direct person-to-person contact (Adam, 2021). It is the causative agent of giardiasis, a diarrheal disease that affects millions worldwide and is recognized as an opportunistic parasitic infection affecting immunocompromised people. Giardiasis is a significant public health concern, with an estimated 280 million symptomatic human infections occurring each year, contributing to 1.6 million cases of diarrheal death in 2016 (Troeger *et al*., 2018). *Giardia* had also been reported as waterborne pathogen causing a mass outbreak worldwide. From 2011 to 2016, 37% (142/381) of protozoan-waterborne outbreaks were reportedly due to giardiasis (Efstratiou *et al*., 2017).

Giardiasis is particularly prevalent in developing countries, with reported infection rates ranging from 20 to 30%, while developed countries typically exhibit rates between 3 to 7% (Halliez and Buret, 2013). The higher rate in developing countries was due to lack of hygiene practices and clean water supply. However, most cases in developed countries involve travellers visiting the high risk countries with giardiasis. Recognizing the importance of addressing this neglected tropical disease, the World Health Organization (WHO) included giardiasis as part of its Neglected Disease Initiative (NDI) in 2004 (Ankarklev *et al*., 2010). Besides, *G. lamblia* is also classified as a Category B pathogen, the

second highest priority organism or biological agent that poses a significant risk to public health and national security (NIAID, 2008).

Giardiasis can affect individuals of any age, presenting symptoms ranging from diarrhea, nausea, constipation, abdominal pains/cramps, weight loss, and malabsorption. However, most patients usually remain asymptomatic. Acute disease in symptomatic patients is established when general signs and symptoms occur, and if left untreated, it could progress into a chronic stage characterized by irritable bowel syndrome (IBS), food allergies, arthritis, ulcers, or chronic fatigue syndrome (Einarsson *et al*., 2016a). Children especially those living in developing countries are particularly vulnerable to giardiasis. The infection in children could lead to serious consequences, including protein-energy malnutrition, iron deficiency anemia, and zinc deficiency. These nutritional deficiencies can lead to impaired growth, stunting, and poor cognitive and educational performance (Roshidi *et al*., 2021). Once a patient is diagnosed with giardiasis, treatment usually involves the use of antiparasitic medications such as metronidazole or nitaxazonide.

Microscopic examination is considered to be the gold standard for diagnosing giardiasis from stool samples. Using a microscope, stool sample is examined to detect presence of *Giardia* cysts or, less frequently, trophozoites as trophozoites may not survive well in the environment. However, there are some limitations to this approach. It requires skilled and experienced microscopists to accurately identify the parasites, and analysis of samples can be time-consuming, leading to delays in diagnosis (Soares and Tasca, 2016). To overcome these limitations, alternative diagnostic techniques such as immunoassays and molecular technique have been developed. Immunoassays including techniques

such as immunofluorescence assays (IFAs), enzyme-linked immunosorbent assays (ELISAs), rapid solid-phase qualitative immunochromatography assays and enzyme immunoassays (EIA) have been developed to detect specific antigens or antibodies associated with *Giardia* infection and have proven to give higher sensitivity compared to microscopy (Geurden *et al.*, 2010). Similarly, molecular techniques such as real-time PCR and LAMP also exhibit high sensitivity but are typically employed in advanced settings.

1.2 Problem statement and Rationale of study

Giardiasis is a common yet neglected cause of diarrheal illness worldwide, and its impact on children's health is often underestimated. With a mean rate of 13.7% in the last 10 years, giardiasis is becoming increasingly important in Malaysia. Despite this seemingly optimistic rate, tackling giardiasis is fundamental to the attainability of the Malaysian Global Nutrition Targets 2025. The targets aim for a 40% reduction in stunting among under-five-year-olds, reflecting the broader objectives embedded within the National Plan of Action for Nutrition of Malaysia III (2016-2025) (Ministry of Health Malaysia, 2016). Given the tie between giardiasis and developmental issues in children, including stunted growth, addressing knowledge gaps about the disease is of paramount importance.

Meanwhile, the presence of *Giardia* in animals, environmental samples, and water sources indicates the potential for zoonotic transmission and the importance of a holistic One Health approach to address the disease. However, the challenge lies in the current diagnostic for giardiasis which heavily relies on

microscopy techniques (with or without staining) with recognizably low sensitivity, emphasizing the need for a more accurate diagnostic alternative. Complicating the issue further is the scarcity of comprehensive surveillance studies and the growing resistance to giardiasis treatment.

While there are numerous studies on the gene expression of different variants and life stages (cysts versus trophozoites) of *G. lamblia*, little is known regarding the immunogenic proteins identified in the parasite. This study aims to identify immunogenic proteins from both cysts and trophozoites of *G. lamblia* in search of diagnostically potential proteins that exist during both life stages. This would be the first step towards identifying potential disease-related biomarkers that can be further studied in future research.

1.3 General objective

To identify immunogenic protein from cysts and trophozoites of *Giardia lamblia* by immunoassays and mass spectrometry analysis.

1.3.1 Specific objectives

1. To establish the culture of *G. lamblia* cysts and trophozoites using different culture media.
2. To extract and compare the protein profiles of *G. lamblia* cysts and trophozoites.
3. To assess the immunoreactivity of the fractionated parasite proteins by IgA-, IgG-, and IgM-western blots.
4. To reveal the identity of immunogenic proteins identified in this study by mass spectrometry analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 *Giardia lamblia*

2.1.1 Morphology and life cycle of *G. lamblia*

G. lamblia has two morphologically distinct stages in its life cycle. The motile form and pear-shaped cell is called trophozoites, which can only survive in the small intestine of the host. The size of trophozoites is about 12 to 15 µm long, 5 to 7 µm wide, and 1 to 2 µm thick. Meanwhile the other stage is the resistant cyst which plays a crucial role in the transmission of giardiasis. *Giardia* cysts are morphologically more simple with an oval shape and range in size from 6 to 10 µm while the size of the cyst wall varies from 0.3 to 0.5 µm in thickness (Adam, 2001).

The trophozoite of *Giardia* has a complex cytoskeleton that maintains the shape of the parasite. This cytoskeleton comprises an adhesive disc, a median body and four pairs of flagella which develop ventrally, anteriolaterally, posteriolaterally, and caudally from the cell body, the median body, and the ventral disk. The convex side represents the dorsal surface while the cut side of the pear represents the ventral surface of the cell. The flagella are important for parasite motility and attachment to the epithelial surface by creating negative pressure under the ventral disc that functions as a sucking disc (Carranza and Lujan, 2010). Besides, trophozoites possess two diploid, oval shaped nuclei surrounded by nuclear envelopes that remain practically intact throughout the cell cycle (Carranza and Lujan, 2010). These nuclei are located in the anterior half of the cell, on the left and right sides of the longitudinal axis and divide by binary fission (Solari *et al.*, 2003). Apparently, both nuclei that contain the same genetic information are transcriptionally active, divide at

the same time and are equally partitioned at cytokinesis. Moreover, the outer nuclear membrane of a trophozoite has sparsely scattered ribosomes, whereas encysting cells have a nuclear envelope studded with ribosomes as well as a closely associated structure resembling the endoplasmic reticulum (Andersson *et al.*, 2010).

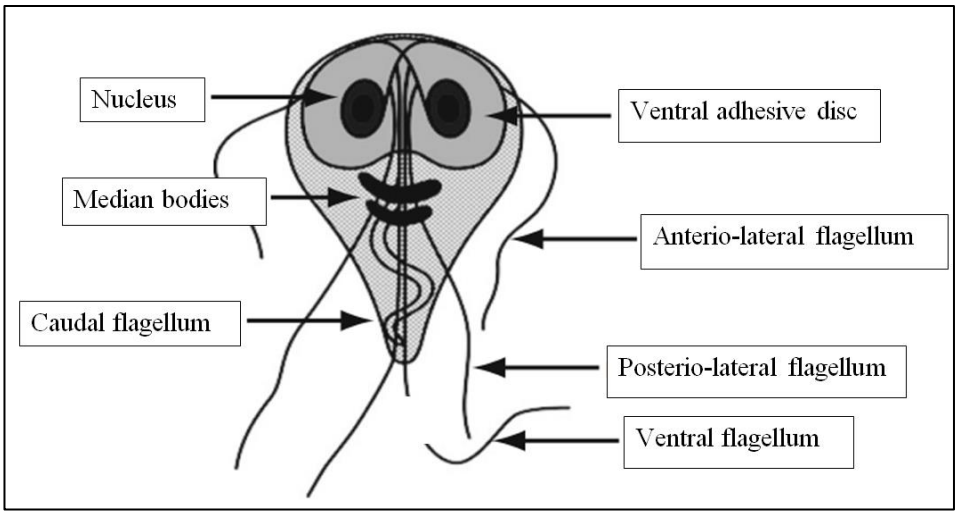
In contrast, the cyst of *Giardia* has a cyst wall composed of an inner membranous layer including two membranes that enclose the periplasmic space and an outer filamentous layer (Solari *et al.,* 2003). The cytoplasm of a mature cyst contains four nuclei due to a nuclear division without cytokinesis, contracted flagella, and fragmented portions of the ventral disc (Carranza and Lujan, 2010). These nuclei can move and be located in different parts of the cysts due to the disassembly of the cytoskeleton during encystation (Andersson *et al*., 2010). Besides, cyst could be present in two types. Type 1 cysts were oval and phase bright, the cell body was evenly distributed within the cyst wall and the cyst wall was readily visible. Cytoskeletal structures such as the fragmented disc or contracted flagella can be visualized inside the cyst. These cysts are the best quality for excystation. Meanwhile, Type 2 cysts had a cell body or cytoplasm appears distorted or shrunken away from the cyst wall (David and Gillin, 2011). Figure 2.1 shows the differences in morphology of *Giardia* cyst and trophozoites.

The unique morphologies of the trophozoites and cysts remarkably enhance their adaptability, allowing them to survive in substantially diverse and challenging environments. For instance, the trophozoite with an adhesive disc and flagella helps with motility and attachment in the human small intestine. Meanwhile, the thick outer wall helps the cysts to survive in cold water and harsh conditions for months, making it more likely to cause infection in humans and responsible for the transmission of giardiasis.

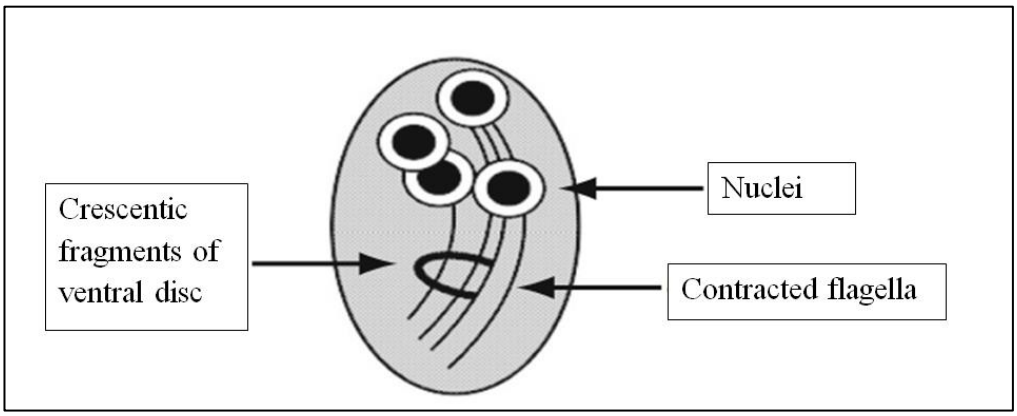
Giardia infection in humans is initiated by the ingestion of infectious cysts through contaminated food or water. During excystation, each cyst is stimulated to excyst by the gastric acid in the stomach and the presence of bile and trypsin in the duodenum to develop into an excyzoite, an intermediate form that undergoes two rounds of cytoplasmic divisions and one round of nuclear division to produce four trophozoites (Einarsson *et al.*, 2016a). These trophozoites then utilize flagellar motility to search for suitable sites for intestinal attachment and colonization. The trophozoite attaches to the intestinal epithelial cells using the adhesive disc to proliferate in the small intestine and resist peristaltic flow (Nosala and Dawson, 2015).

As the trophozoite senses a change in the environment while being transported further down the small intestine, it initiates encystation to survive the outside environment. Due to changes in pH which is slightly alkaline and a high bile concentration in the small intestinal lumen, the trophozoite prompts to encyst forming a cyst with four nuclei and a thick outer wall (Birkeland *et al*., 2010). Figure 2.2 illustrates the life cycle of *G. lamblia* in the human host, demonstrating the transformation of cysts into trophozoites and vice versa through the excystation and encystation processes.

The formation of the cyst wall involves the regulated secretion of cyst wall protein components. The cyst is water resistant and can persist for weeks in the environment until it is ingested by a new host (Carpenter *et al.*, 2012). Since a patient can shed 10^8 viable cysts per gram of faecal material, this amount of cell numbers of cysts enhances the probability of infecting a new host as the infectious dose has been reported to be as low as ten to a hundred cysts (Andersson *et al.*, 2010).



A



B

Figure 2.1 Morphology of *G. lamblia* A: *Giardia* trophozoites; B: *Giardia* cyst (images adopted from Smith & Mank, 2011).

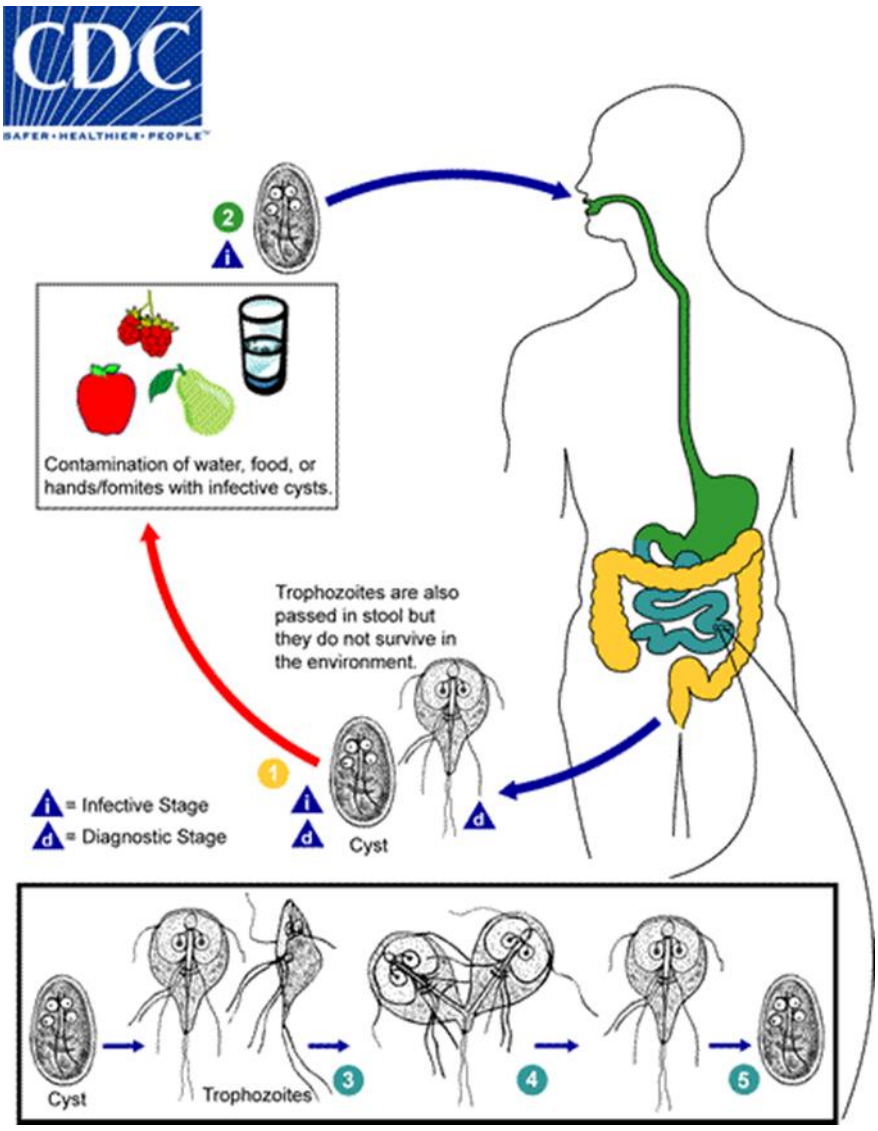


Figure 2.2 The life cycle of *G. lamblia*.1: The cyst and trophozoite were excreted through stool. 2: The ingestion of cysts through contaminated food or water. 3: The transformation of cysts into trophozoite through excystation. 4: Trophozoites were proliferated and attached to the small intestine. 5: Encystation of trophozoite to cyst to be excreted through stool to the environment (CDC, 2017).

2.1.2 Cultivation techniques for *Giardia* trophozoite and cyst

In humans, *G. lamblia* trophozoites colonize and proliferate in the upper small intestine before entering colon and transform into cysts through a process termed encystation. Therefore, a similar condition to the intestine is needed to culture *G. lamblia*. Axenic cultures of *Giardia* were initially grown by Meyer using a medium that required human serum and chick embryo extracts (Meyer, 1970). Later, Diamond discovered that TYI-S-33 medium which was formulated to culture *Entamoeba* and *Trichomonad* species could also support the axenic growth of *Giardia* trophozoites. In 1983, Keister modified Diamond's medium into a complete modified TYI-S-33 that specifically enhanced giardial growth and was used in this study to culture the trophozoites. Keister's modifications of TYI-S-33 medium involved the addition of bovine bile, higher concentrations of cysteine, a pH adjustment to 7–7.2, and sterilization by filtration, rather than autoclaving (Davids and Gillin, 2011).

To induce encystation of *Giardia,* Gillin *et al.* (1987) evaluated that small intestine contained factors such as bile salts, calcium and iron that could stimulate encystation, especially bile which had been suggested to stimulate encystation by sequestering cholesterol. Later, a higher number and quality of biologically active cysts were obtained by the addition of fatty acids, adjusting medium pH to slightly alkaline, 7.8 and addition of lactic acid hemicalcium salt in the growth medium. Hence, the findings of these studies had provided one of the most widely used method for giardial encystation, which includes a growth medium of TYI-S-33 without the use of bile (pre-encystation) followed by encystation in a medium with porcine bile with addition of lactic acids at pH 7.8 (Method 1)(Gillin *et al*., 1987). Another common method (Method 2) for encysting *Giardia* does not require a pre-

encystation period, but requires excess bovine bile in the growth medium (Sun *et al*., 2003). Method 2 used TYI-S-33 medium containing 12.5 mg/ml of bovine bile at pH 7.8 with addition of lactic acid hemicalcium. Moreover, Method 2 is generally favored due to its advantage of taking less time to produce abundant cysts in vitro than Method 1.

2.2 Giardiasis

2.2.1 Disease epidemiology: Global and Malaysia

The worldwide prevalence of giardiasis is reportedly higher in low-income countries ranging between 20 to 40% such as Bangladesh, Nepal, Mexico and Kenya. The high prevalence of giardiasis is largely attributed to factors such as poverty, lack of clean water supplies and poor hygienic conditions that are pronounced in these vulnerable areas for the transmission of giardiasis (Pickering *et al*., 2019; Roshidi *et al*., 2021). Notably, a significant portion of these cases are among children, with an estimate of 15 to 20% occurring in young children under the age of 10 years. This is supported by a recent study in Algeria which reported that 62 of the 97 individuals infected with giardiasis were children aged between 1 to 10 years (Belkessa *et al*., 2021). Likewise, studies conducted among children in rural Bangladesh and Kenya reported that 88.4% of children with a median age of 2.7 years and 91·8% of children with a median age of 2.2 years were infected with *Giardia* as determined by the detection of stool specimens respectively (Benjamin-Chung *et al*., 2021).

Meanwhile, the prevalence of giardiasis in high-income countries is reported to be 2 to 7%, where almost 40% of cases occurring in travellers returning from highly endemic areas. Thus, giardiasis is perceived as a re-emerging infection

commonly associated with traveling and presumably contaminated water supplies (Lalle and Hanevik, 2018). A study conducted by Takaoka *et al.* (2016) reported that 92 of 3306 returning UK travellers (2.8%) were diagnosed with giardiasis, and the incidence rate per 1000 person-months was 12.5 (Takaoka *et al*., 2016). In 2012 to 2017, 111 giardiasis outbreaks (760 cases) were reported in 26 states of the United States with three main modes of transmission identified: water exposure in 29 outbreaks, person-to-person contact in 28 outbreaks, and contaminated food in 6 outbreaks while the other 48 outbreaks could not be determined (Conners *et al*., 2021). Furthermore, Southwest England has been reported to have the highest rate of *Giardia* infections with 20.1 cases per 100,000 populations while Canada reported an infection rate of 19.6 per 100,000 population per year (GOV-UK, 2019; Laupland and Church, 2005).

The significantly higher prevalence of giardiasis in lower-income countries suggests the necessity to focus on improving water sanitation systems, enhancing public hygiene practices, and prioritizing health education. Meanwhile, the prevalence in high-income countries, linked to travel and potentially contaminated water supplies, signifies the importance of persistent global health vigilance and collaboration in the face of infectious diseases.

In Malaysia, most of the studies regarding giardiasis were conducted among indigenous peoples and schoolchildren while limited studies were performed on communities such as migrants and immunocompromised individuals. Over the recent decade (2011 to 2020), the prevalence of giardiasis among indigenous populations, HIV/AIDS patients, migrants, and inmates ranged from 0.3 to 34.6% in Malaysia (Roshidi *et al.*, 2021). The overall prevalence of giardiasis among indigenous people in Malaysia was 11.6%. At the state level, the highest prevalence of giardiasis was

15.9% in Pahang followed by 14.9% in Negeri Sembilan and 13.4% in Kedah while the lowest was 4.6% in Malacca (Choy *et al*., 2014). Meanwhile, the highest prevalence of giardiasis among the indigenous schoolchildren was observed at 34.6% for ages 7 to 12 years in Pahang, 21.6% for ages 6 to 12 years in Perak, and 14.5% in Terengganu (Elyana *et al*., 2016; Adli and Ghani., 2020; Gee Hong Tang, 2020). In another study performed by Al-Mekhlafi *et al*. (2013), the prevalence of severe underweight, stunting and wasting among children infected with giardiasis was found to be 28.3%, 23.8%, and 21.0%, respectively (Al-Mekhlafi *et al*., 2013). Lastly, the prevalence of giardiasis in HIV/AIDS patients and inmates was reported to be 5.7% among 122 HIV/AIDS patients and 0.3% among 294 inmates respectively (Lim *et al*., 2011; Angal *et al*., 2015).

Besides humans, *Giardia* was also found in animals and the environment. In cattle farms located near Sungai Langat Basin, *Giardia* cysts were detected in 14.6% of bovine fecal samples, and 6.7% in cattle wastewater samples (Farizawati *et al*., 2005). Additionally, *Giardia* has been found in both treated and untreated water samples in Malaysia. In a study conducted in Selangor, 90% of raw water samples from two drinking water plants were found to contain *Giardia*. Similarly, in Negeri Sembilan, 51.6% of raw water samples from three water treatment plants tested positive for *Giardia* (Ahmad *et al*., 1997).

The presence of *Giardia* in humans, animals and the environment, including treated and untreated water samples, underscores the significance of giardiasis as a public health concern that requires a comprehensive and multidisciplinary approach to mitigate and address its impacts effectively.

2.2.2 Clinical manisfestation of giardiasis

Giardia infections are often self-limiting in individuals with competent immune systems, leading to both asymptomatic and symptomatic infections. While, it is common for immunocompetent individuals to develop acute and chronic giardiasis, those with compromised immune systems are at an increased risk of developing chronic giardiasis (Einarsson *et al*., 2016a). Clinical manifestations such as diarrhea with or without malabsorption syndrome, nausea, vomiting and weight loss typically appear approximately 7 to 12 days post-infection. While some patients may experience mild and acute illness that resolves spontaneously, others could suffer from prolonged, severe diseases that do not respond to standard treatment. Without proper treatment, chronic *Giardia* infections can occur and lead to complications such as irritable bowel syndrome (IBS), food allergies, arthritis, or chronic fatigue syndrome (Einarsson *et al*., 2016a; Halliez, and Buret, 2013).

Among these, IBS is one of the most common human gastrointestinal disorders in the developing world, frequently giving symptoms such as bloating, abdominal pain, nausea, flatulence and diarrhea or constipation (Spiller and Garsed, 2009). The severity of symptoms during the acute phase of giardiasis appears to represent a risk factor for the development of post-infectious fatigue and abdominal symptoms (Cotton *et al.*, 2011). Several cases of myopathy and hypokalaemia induced by giardiasis have been reported in both immunocompromised and immunocompetent patients. This suggests that *Giardia* can trigger muscular manifestations independently due to the host's immune status (Kanokwanvimol, 2017). During the chronic stage, lethargy, headache and muscle pain with progressive weight loss, loss of appetite and malabsorption could also be present.

In children, the chronic infection could manifest with slowed growth, with malabsorption of essential nutrients such as fat, glucose, lactose, xylose, vitamin A and B12 observed in some patients. This malnutrition, specifically the lack of adequate protein, energy and micronutrients – particularly vitamin A and iron, often leads to stunting and wasting, which are unfortunately common during infancy when the infection strikes. These physical health impacts have further implications on cognitive functionality and lead to an increased susceptibility to additional infections, impair cognitive function, and adversely affect educational performance. Furthermore, diarrheal disease during early childhood was found to impair visual-motor coordination, auditory, short-term memory, and cortical cognitive functions (Ajjampur *et al.*, 2011; Halliez, & Buret, 2013). In some cases, urticaria, cholecystitis and pancreatitis have also been reported in giardiasis cases (Ivanov, 2010).

There are studies that associate low serum ferritin and iron with affecting psychomotor development and low serum retinol as indicators of vitamin A deficiency that can cause xerophthalmia, a condition of childhood blindness which has been detected in patients with giardiasis (Simsek *et al.*, 2004; Al-Mekhlafi *et al*., 2010). Undoubtedly, younger children are more vulnerable to giardiasis due to their low immunity level, in addition to having low hygiene standards and lifestyles or the presence of infected family members where person-to-person contact within the family members is a possible mode of transmission.

2.2.3 Pathogenesis of giardiasis

Giardia is a non-invasive parasite which infects surrounding tissues of small intestines and entering the bloodstream without passing the epithelium and cause illness. The attachment of trophozoites to the epithelial cells lining of the small intestine can damage enterocytes and loss of the brush border of epithelial, resulting in microvilli shortening and targets specific signalling networks that can activate apoptosis, leading to the loss of intercellular junctions, cytoskeletal rearrangement, and impaired epithelial barrier, which contribute to diarrhea (Einarsson *et al*., 2016a; Certad *et al.*, 2017; Allain and Buret, 2020).

In general giardiasis is multifactorial disease, reflecting the complex interplay between the host and parasite. The pathophysiological stages are believed to involve diffusion shortening of microvilli and inhibition of brush border enzymes. This involves attachment of the parasites and CD8+ T cells. Next, induction of chemokines in intestinal epithelial cells (IECs), resulting in attraction of immune cells like mast cells and dendritic cells (DC) followed by disruption of tight junctions, induction of apoptosis and starvation for arginine in IECs, resulting in less nitric oxide (NO), cell cycle arrest and apoptosis. Other pathophysiological event were, increased intestinal permeability and chloride ion secretion, intestinal hypermotility, crypt hyperplasia and increased mucus secretion. Last but not least, changed composition of bacterial normal flora causing *Giardi*a and host cells secrete specific proteins during the interaction (ESPs) (Tilahun *et al*., 2022)

The loss of the brush border cells is one of the most common gastrointestinal pathologies. Troeger *et al.* (2007) clearly demonstrated that total duodenal surface area is decreased in patients with chronic giardiasis (Troeger *et al*., 2007). This process, as well as microvillar disaccharidase deficiencies, is mediated by activated

CD8+ lymphocytes, consistent with the hypothesis that *Giardia*-mediated immunopathophysiology occurs secondary to intestinal barrier disruption, which indeed is observed at earlier time-points (Scott *et al*., 2004). The data suggest that *Giardia*-induced diffuse shortening of host brush border microvilli might cause diarrhoeal disease via malabsorption and maldigestion (Faubert, 2000; Buret, 2008). The nutrient-coupled absorption of electrolytes occurs in brush border microvilli. Hence, diffuse shortening of brush border microvilli causes small intestinal malabsorption due to impaired absorption of water, glucose and electrolytes (Buret *et al*., 1990; Cevallos *et al*., 1995; Scott *et al*., 2004). In addition, diarrhoeal disease in *Giardia*-infected individuals also results from increased intestinal transit rates due to mast cell degranulation and adaptive immune responses (Deselliers *et al*., 1997; Li *et al*., 2006).

Moreover, some molecules like IgA, IL-6, IL-7, NO (nitric oxide), and mast cells have a clear influence in parasite clearance (Vivancos *et al*., 2018). TH17 cells produce IL-17A as well as other cytokines and, in addition to protective immunity, play a role in autoimmune diseases, including inflammatory bowel disease, meaning that precise regulation is required (Plichta *et al*., 2019). IL-17A has been recognized as a key component of the immune response to *Giardia* infection and has been shown in mice infected with *G. duodenalis* and with *Giardia muris* and facilitates the IgA response (Solaymani-Mohammadi & Singer, 2011; Dreesen *et al*., 2014). Antibodies are required for clearance late in infection in mice, with secretory immunoglobulin A being the most critical antibody isotype. Some studies had shown that lack of arginine inhibits lymphocyte function and affects target of rapamycin in mammalian cells (mTOR), which regulates the formation of dendritic cells. Besides, arginine deficiency also limiting intestinal epithelial proliferation and nitric oxide

generation while the degradation of chemokine ligand 8 (CXCL8) by cathepsin B-like proteases results in reduced neutrophil chemotaxis. In a myosin light chain kinase-dependent way, *Giardia* also causes enterocyte apoptosis and destroys the epithelial barrier (Stadelmann *et al.*, 2013; Banik *et al*., 2013).

Increased intestinal permeability has also been reported in individuals with chronic giardiasis (Troeger *et al*., 2007). Furthermore, the contact between the trophozoite and intestinal cells could cause release of metabolic enzymes and proteases that help parasite colonization (Rodríguez-Fuentes *et al*., 2006; Ringqvist *et al*., 2008). This shows that *Giardia* induces changes in cytoskeleton by rearrangement of F-actin and α-actinin and this damage is associated to parasite adhesion (Humen *et al.*, 2011; Teoh *et al*., 2000). A findings show the existence of lipid rafts in *G. lamblia*, which may be involved in parasite binding to the epithelium (Humen *et al.*, 2011; Weiland *et al*., 2005). It is believed that most of the functional disorders resulting in *G. lamblia* infection are produced by the interaction between the ventral disk of the parasite and the brush border of enterocytes (Smith, 1985). A study indicates that the pathogenic effects of *Giardia* may be further compounded by degradation of local mucins by the parasite which may in turn contribute to the translocation of commensal bacteria through the epithelium. It also indicates that *G. lamblia* may cause mucin depletion in goblet cells of the small intestine, as well as in the colon (Peattie *et al*., 1989). Finally, increased chloride secretion further contributes to diarrhoea in giardiasis (Cevallos *et al*., 1995; Troeger *et al.*, 2007; Baldi *et al.*, 2009).

2.2.4 Diagnosis of giardiasis

2.2.4(a) Microscopic examination

The microscopic analysis of faecal samples is considered as the gold standard for the diagnosis of giardiasis (Adam, 2021). Microscopic techniques utilized in the clinical laboratory include the screening of direct wet mounts, concentrated samples, and permanently stained smears. Both the trophozoites and cysts of *Giardia* can be detected by microscopy either directly or after concentration with sucrose, zinc sulphate or formalin. Trophozoites can sometimes be detected in faecal samples with diarrhea due to increased peristalsis. Given the characteristic movement of the trophozoites, they are preferably visualized in a native smear using recently obtained faeces. However, the detection of cysts in the faeces is preferred for diagnosis. These cysts can be stained prior to examination, with iodine and trichrome being the commonly used stains. (Geurden *et al.*, 2010).

The main issue with the microscopy method lies in its inconsistent sensitivity, while its specificity remains consistent across various studies. El-Nahas *et al.* (2013) and co-workers compared the efficacy of microscopy, direct immunofluorescence assay (DIF), and flow cytometry (FC) methods for the detection of *Giardia* cysts in human stool samples. They found a 76.9% sensitivity and 100% specificity for microscopy, concluding that the direct microscopic method serves as a reliable for first-choice test for *Giardia* diagnosis, while the DIF and FC methods are deemed complementary alternative tests for *Giardia* cysts (El-Nahas *et al*., 2013). Van den Bossche *et al.* (2015) also reported high sensitivity (90%) and specificity (100%) for the microscopy method (Van den Bossche *et al*., 2015). However, when compared to ELISA and PCR methods, some studies indicated lower sensitivity (50 to 55%) for microscopy and maintained 100% specificity when analyzing a single sample. These

findings highlight the need for coproantigen detection-based methods to identify *Giardia* more effectively (Beyhan and Cengiz, 2017; Silva *et al*., 2016).

The major advantage of microscopic examination is the limited cost of consumables. Although laborious, microscopy is considered economical and efficient for diagnosing *G. lamblia*, as it also allows the detection of other parasites in the same specimen (El-Nahas *et al.,* 2013). However, the major disadvantage of this technique is the need for a skilled and experienced microscopist, and the lower sensitivity compared to immunological assays when a single stools are examined, not to mention it is time-consuming as the sensitivity of this technique can be significantly enhanced through repeated fecal examinations, at least three stool samples were needed (Geurden *et al*., 2010; Escobedo *et al.*, 2014). The efficacy of microscopy depends on the number of faecal samples examined, the use of concentration techniques, and the professional skill of the microscopist (Dixon, 2021).

2.2.4(b) Serology

The detection of parasite antigens primarily involve techniques such as immunofluorescence assays (IFAs), enzyme-linked immunosorbent assays (ELISAs), rapid solid-phase qualitative immunochromatography assays and enzyme immunoassay (EIA). Most of these tests have been commercialized and evaluated for human stool samples using monoclonal antibodies against cyst wall proteins (Geurden *et al*., 2010). Study by Gotfred-Rasmussen *et al.* (2016) reported IFAs compared to microscopy with two concentration methods, formol-ethylacetate (FEA) and salt-sugar flotation (SSF) concentration had significantly better sensitivity for

detecting *Giardia* spp. (Gotfred-Rasmussen *et al.*, 2016). The advantage of IFAs compared to microscopy was that IFAs could be used to stain fecal smears without an initial concentration and had a detection limit estimated around 1000 cysts per gram of faeces (Geurden *et al*., 2010).

Meanwhile, a prospective study that evaluated the performance of the ELISA method for *Giardia* detection based on the presence of a specific glycoprotein GSA 65 in cysts and trophozoites and microscopy showed that ELISA had a higher sensitivity and specificity for diagnosis in a single faecal sample (Soares and Tasca, 2016). The advantage of this method is the possibility to analyse a single faecal sample as well as preserved or frozen samples (Soares *et al*., 2016). Similarly, a rapid qualitative EIA also demonstrated high sensitivity (95.9%) and specificity (97.4%) detecting *Giardia* in fecal specimens, without cross-reactions with other parasites, including eight different protozoa and three helminthes (Escobedo *et al.*, 2014).

The major advantage of the EIA and ELISA lies in their capacity to process a large number of samples in batches and yield results on the same day (Soares and Tasca, 2016; Church *et al*., 2005). However, the major drawback of IFAs, ELISA and EIA is their time-consuming nature that requires more than an hour to generate results. To overcome this, rapid solid-phase qualitative immunochromatography assays (IC) or rapid diagnostic tests (RDTs) have been developed to shorten the testing process. This assay allows the detection of antigens of one or more protozoan parasites in a single test format, is easy to perform and interpret and beneficial to be used in resource-limited settings or during outbreaks as it does not require specialised equipment experienced microscopists. A retrospective study conducted by Van den Bossche *et al.* (2015) compared the performance of four commercial

RDTs (ImmunoCardSTAT!®CGE, *Crypto/Giardia* Duo-Strip, RIDA®QUICK *Cryptosporidium/Giardia/Entamoeba* Combi and *Giardia/Cryptosporidium* Quik Chek) for the detection of *G. lamblia*, *Cryptosporidium* spp. and *E. histolytica*. Their findings reported a 100.0% specificity and 58.3% to 100.0% sensitivities for *G. lamblia* (Van den Bossche *et al*., 2015).

2.2.4(c) Molecular diagnosis

PCR-based methods have proven to provide excellent sensitivity and specificity compared to microscopy and antigen detection methods. These techniques have been extremely useful in recent years, particularly in enhancing knowledge and understanding of the systematics, biology, epidemiology, ecology and population genetics of *G. lamblia* (Escobedo *et al.,* 2014). In some industrialized settings, the use of real-time PCR as a tool for the detection of giardiasis is increasing. These procedures, apart from being sensitive and specific, are also fast and easy to perform on a large set of samples.

A study performed by Schuurman *et al.* (2007) compared the performance of microscopy, rapid chromatographic test, and a real time PCR demonstrated a sensitivity of 99%, 98% and 100% respectively for *Giardia* (Schuurman *et al*., 2007). However, Schuurman *et al.* (2007) suggest that, although real-time PCR may be the most sensitive method, the risk of cross- contamination of the sample is high, suggesting this method as a complementary test to microscopy. Another disadvantage of PCR methods is the cost, the requirement of relatively well-equipped laboratories and skilled technicians. These limitations restrict their use in resource-poor settings where *Giardia* infections are endemic (Escobedo *et al*., 2014).

In order to improve PCR amplification and reduce the challenges associated with residual co-amplification of non-specific sequences, Notomi *et al.* (2000) developed a new PCR method known as Loop-mediated isothermal amplification (LAMP) (Notomi *et al*., 2000). LAMP is a gene amplification method that employs six oligonucleotide primers designed based on the *G. lamblia* elongation factor 1 alpha (EF1α) gene sequence, capable of amplifying large amounts of DNA with high specificity and sensitivity under isothermal conditions (Li *et al*., 2013; Adeyemo *et al*., 2018). LAMP has been evaluated for detecting *Giardia* cysts in surface water samples and leafy greens (Ongerth and Saaed, 2020; Lalonde *et al*., 2021). The LAMP is considered to be field applicable because of its simple read-out method, which is observation through the naked eye. The LAMP reaction is also reproducible, rapid, and specific for the detection of *G. lamblia* and has lower costs compared to the other molecular assays (Plutzer and Karanis, 2009).

2.2.5 Biomarker of giardiasis

Biomarkers are biological characteristics that can be objectively measured in biofluid and tissue samples and serve as indicators of physiological as well as pathological processes or pharmacological responses to therapeutic intervention (Ahsan, 2019). Biomarkers can be used to determine disease onset, progression, and patient susceptibility to a certain disease or predict the efficacy of treatment at a particular disease stage (Horvatovich and Bischoff, 2012). Several biomarkers have been identified to aid in the early diagnosis and administration of effective treatments for giardiasis enhancing patient survival and quality of life. For instance, many studies have described the identification of *Giardia* major native proteins as target antigens with various functions and localizations, namely variant surface proteins

(VSPs), giardins, tubulins (cytoskeletal proteins), heat-shock proteins (HSPs), cyst wall proteins (CWPs) and metabolic-proteins such as enolase-a, fructose-1,6-biphosphate aldose (FAB), arginine deaminase (ADI), and ornithine carbamoyl transferase (OCT) (Roshidi *et al*., 2022).

Among these proteins, VSPs have garnered significant attention as they are reported as highly immunogenic proteins that activate the humoral immune response, with VSPH7 and VSP 5G8 serving as notable examples (Lopez-Romero *et al*., 2015; Garzon *et al*., 2020). Other than this, Palm *et al*. (2003) identified a 32 kDa highly immunoreactive protein, α-1-giardin that stimulates the production of anti-*Giardia* antibodies (IgA and IgG2a) and is used as a diagnostic biomarker in several commercial RDTs, such as the Triage Parasite Panel (BioSite Diagnostics, USA) (Palm *et al*., 2003; Priest *et al*., 2010). Meanwhile, HSPs, specifically Hsp 70 and Hsp 90 inhibitors have been shown valuable as therapeutic agents for giardiasis and has an important role in the differentiation process of *Giardia* (Al-Madani and Al-Khuzaie, 2021; Debnath *et al*., 2014). Also noteworthy are the most interesting cyst wall proteins, particularly recombinant CWP2. The rCWP2 immunization was reported to induce immune responses similar to those from live infection with *G. muris* cysts, in which the anti-rCWP2 immunoglobulin A (IgA) antibodies were detected in both feces and serum of the immunized mice, while anti-rCWP2 IgG1 and IgG2a antibodies were detected only in the serum (Larocque *et al*., 2003).

Besides, host- specific proteins such as P-selectin, calprotectin, and Trefoil factor 3 (TFF3) were also found to be significantly increase in the serum of patients infected with *G. lamblia* compared to the control group in response to the *Giardia* infection (Al-Hadraawy *et al*., 2016a; Toma and Al-Hadraawy, 2018). For serological biomarkers, secretory IgA (sIgA) antibodies induced by *Giardia* play an