COMPARATIVE ANALYSIS OF CYSTS AND TROPHOZOITES OF *Giardia duodenalis* USING PROTEOMIC AND BIOINFORMATIC ANALYSIS

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by

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LIST OF SYMBOLS

nm	Nanometer
µm	Micrometer
mm	Millimeter
psi	Pounds per square inch
sec	Second
min	Minute
h	Hour
°C	Degree celcius
nL	Nanoliter
µl	Microliter
ml	Milliliter
L	Liter
g	Gram
mg	Milligram
mM	Millimolar
M	Molar
x g	Relative centrifugal force (RCF)
A°	Angstrom
ppm	Parts per million
kDa	Kilodalton

LIST OF ABBREVIATIONS

ESV	Encystation specific vesicle
CWP	Cyst wall protein
SNP	Single nucleotide polymorphism
PTM	Post-translational modification
Mtz	Metronidazole
PV	Peripheral vesicle
MT	Microtubule
HCMP	High-cysteine membrane protein
CWP	Cyst wall protein
LRR	Leucine rich repeat
NEK	NIMA-related serine/threonine kinase
PLK	Polo-like kinase
CDC	Centers for Disease Control and Prevention
CDK	Cyclin-dependent kinase
PKA	Protein kinase A
cAMP	Cyclic adenosine monophosphate
WHO	World Health Organization
HIV	Human immunodeficiency virus
CDC	Centers of Disease Control and Prevention
MOH	Ministry of Health Malaysia
NIH	National Institute of Health
O&P	Ova and parasite
ELISA	Enzyme-linked immunosorbent assay
IFA	Indirect fluorescent antibody
WB	Western blot
PCR	Polymerase chain reaction
DAP	Disc-associated protein
LAMP	Loop-mediated isothermal amplification
qPCR	Quantitative polymerase chain reaction
nPCR	Nested polymerase chain reaction
DFA	Direct fluorescent antibody

LFA	Lateral flow assay
RDT	Rapid diagnostic test
tpi	Triphosphate isomerase
bg	B-giardin
IEC	Intestinal epithelial cells
USM	Universiti Sains Malaysia
ddH2O	Deionized water
NH_4HCO_3	Ammonium bicarbonate
K_2HPO_4	Potassium phosphate dibasic
KH_2PO_4	Potassium phosphate monobasic
NaCl	Sodium chloride
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
ADI	Arginine deaminase
OCT	Ornithine carbamoyltransferase
FBA	Fructose bisphosphate aldolase
UPL	Uridine phosphorylase
VSP	Variant surface protein
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LB	Low-bile
ORF	Open reading frame
CRISPRi	CRISPR interference
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetraacetic acid
SB	Sample buffer
PBS	Phosphate-buffered saline
SDC	Sodium deoxycholate
UHPLC	Ultra-high-performance liquid chromatography
HCD	Higher-energy collisional dissociation
FDR	False discovery rate
PSMs	Peptide-spectrum match
CC	Cellular component
MF	Molecular function
BP	Biological process

GO	Gene ontology
hpi	hours post-induction of encystation
GFP	Green fluorescent Protein
HCP	High-cysteine protein
DB	Database
ID	Identifier
OST	Oligosaccharyltransferases
AcPh	Acid phosphatases
PPP	Pentose phosphate pathway
PGM	Phosphoglucomutase
PGAM	Phosphoglycerate mutase
ER	Endoplasmic reticulum
HSP	Heat shock protein
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
EGF	Epidermal growth factor
PDI	Protein disulfide isomerase
TCP-1	Chaperonin tailless complex polypeptide 1
DALY	Disability-adjusted life year
FAO	United Nations Food and Agriculture Organization
NO	Nitric oxide

LIST OF APPENDICES

ANALISIS PERBANDINGAN SISTA DAN TROFOZOIT *Giardia duodenalis* MENGGUNAKAN ANALISIS PROTEOMIK DAN BIOINFORMATIK

ABSTRAK

Giardia duodenalis merupakan parasit protozoa yang bertanggungjawab terhadap jangkitan giardiasis, iaitu penyakit gastro-usus yang meluas dan memberikan kesan ketara terhadap kesihatan awam, terutamanya di negara-negara membangun. Parasit ini mempunyai kitar hayat dua fasa, iaitu trofozoit yang bermotil dan sista yang tidak bermotil, dengan penularan utama bagi parasit ini melalui air yang tercemar. Giardiasis menyebabkan simptom seperti cirit-birit, malabsorpsi, dan penurunan berat badan, yang memberi impak serius terhadap kesihatan kanak-kanak. Kaedah diagnostik sedia ada sering kekurangan dari segi sensitiviti dan spesifisiti, manakala pilihan rawatan pula semakin rumit disebabkan oleh kemunculan strain rintangan ubat. Kajian ini bertujuan untuk menjalankan analisis proteomik yang komprehensif terhadap sel trofozoit dan sista *G. duodenalis*, dengan tumpuan kepada pengenalpastian protein utama dan laluan biologi yang terlibat secara siginifikan dalam kitar hayat parasit ini. Analisis ini dijalankan dengan pendekatan proteomik dan bioinformatik, bertujuan menambahbaikkan kaedah diagnostik terutamanya untuk ujian-ujian antigen dengan mengenalpasti protein-protein yang berperanan penting dalam kitar hayat parasit. Kaedah pengekstrakan protein telah dioptimumkan menggunakan pelbagai teknik termasuk lisis SDS, lisis urea, sonikasi, dan sonikasi dibantu RapiGest. Antara kaedah-kaedah ini, lisis urea menunjukkan kepekatan protein tertinggi dan pemisahan protein yang lebih baik. Spektrometri jisim berjaya mengenal pasti sebanyak 712 protein yang tidak bertindih, dengan 145 protein unik kepada trofozoit, 156 protein unik kepada sista, dan 57.7% protein dikongsi antara

kedua-dua peringkat kitar sel. Analisis fungsian menunjukkan bahawa trofozoit menunjukkan peningkatan protein yang berkaitan dengan biosintesis dan pengawalan kitar sel, manakala sista menunjukkan tahap protein yang lebih tinggi bagi penanda pensistaan seperti protein varian-spesifik permukaan (VSP) dan protein membran cysteine tinggi (HCMP). Rangkaian interaksi protein-protein menunjukkan bahawa protein ribosom, enzim glikolitik, dan protein permukaan memainkan peranan pusat dalam kitar hayat parasit. Penemuan ini memberikan pemahaman baharu terhadap biologi *G. duodenalis* serta mencadangkan potensi biopenanda untuk tujuan diagnostik, termasuk protein ribosom, enzim glikolitik, dan giardin. Kajian lanjutan perlu memberi tumpuan kepada pengesahan biopenanda ini serta penambahbaikkan kaedah diagnostik yang lebih sensitif bagi menangani giardiasis secara lebih berkesan.

COMPARATIVE ANALYSIS OF CYSTS AND TROPHOZOITES OF *Giardia duodenalis* USING PROTEOMIC AND BIOINFORMATIC ANALYSIS

ABSTRACT

Giardia duodenalis is a protozoan parasite responsible for giardiasis, a widespread gastrointestinal disease that significantly affects public health, particularly in developing regions. The parasite alternates between the motile trophozoite and the cyst stage, with transmission primarily occurring through contaminated water. Giardiasis causes symptoms such as diarrhea, malabsorption, and weight loss, severely impacting children's health. Current diagnostic methods lack sensitivity and specificity, and treatment options are complicated by emerging drug resistance. This study aims to provide a comprehensive proteomic analysis of *G. duodenalis* trophozoites and cysts, focusing on identifying key proteins and pathways for improving diagnostic approaches particulary for antigen tests by narrowing notable proteins that highly involved in the life cycle of *G. duodenalis* through proteomic and bioinformatics analyses. Protein extraction methods (SDS-lysis, urea-lysis, sonication, and RapiGest-aided sonication) were optimized, with urea-lysis yielding the high protein concentrations and better protein separation. Mass spectrometry identified 712 non-redundant proteins, with 145 unique to trophozoites, 156 unique to cysts, and 57.7% common to both stages. Functional analysis revealed that trophozoites are enriched in proteins related to biosynthesis and cell cycle regulation, while cysts show higher levels of encystation markers such as VSPs and HCMPs. Key protein-protein interactions highlighted ribosomal proteins, glycolytic enzymes, and surface proteins as central to the parasite's lifecycle. These findings offer insights into *G. duodenalis* biology and suggest potential diagnostic markers and therapeutic targets, including

ribosomal proteins, glycolytic enzymes, and giardins. Future research should focus on validating these biomarkers and developing more sensitive diagnostic tools to combat giardiasis.

CHAPTER 1

INTRODUCTION

1.1 Background

20 infectious diseases that have been classified as Neglected Tropical Diseases (NTDs) by the World Health Organization (WHO, n.d.). According to Molyneux and Fenwick (2005), these diseases predominantly affect rural populations in developing countries, where access to sanitation and healthcare is limited. Giardiasis is one of the NTDs, that caused by the protozoan parasite *Giardia duodenalis*, is the most widespread intestinal parasitic infection globally (Escobedo et al., 2018), with prevalence rates reaching up to 15–20 % among children in endemic regions (Cacciò & Ryan, 2008). In Europe, *Giardia* is a leading cause of intestinal parasitic infections in the United Kingdom, with especially high incidence in Eastern Europe (Dunn & Juergens, 2024). In Malaysia, overall prevalence rates 0.44% to 34.6% has been reported up to between 2011 and 2021, particularly among indigenous children (Roshidi et al., 2021; Chin et al., 2016).

Transmission to humans typically occurs via ingestion of *Giardia* cysts through contaminated water or direct contact with infected individuals or animals. Poor hygiene and sanitation are key drivers of transmission. Primary groups at higher risk include international travelers, immigrants, and daycare workers (Dunn & Juergens, 2024; Bédard et al., 2016). While many infections are asymptomatic, others may present with gastrointestinal symptoms such as watery diarrhea, abdominal cramps, bloating, and nausea (Mayo Clinic, 2022), which can lead to malabsorption and weight loss (Coronato-Nunes et al., 2017). These clinical manifestations result from the host immune response to the colonization of intestinal epithelial cells (IECs)

by *Giardia* trophozoites, which compromise barrier function and nutrient absorption, particularly of arginine (Schofield et al., 1990; Stadelmann et al., 2012).

Both life stages of *Giardia* play a crucial role in the infection process, where cysts serve as the transmissible stage between hosts, while trophozoites represent the proliferative stage responsible for symptomatic disease and host tissue disruption. Accurate and sensitive detection of giardiasis is critical for effective disease management, particularly in controlling transmission and ensuring timely treatment. The current diagnostic gold standard, which is stool microscopy, suffers from low sensitivity, is labour-intensive, and requires skilled technicians to distinguish *Giardia* cysts from other protozoa. While polymerase chain reaction (PCR) offers superior sensitivity and specificity, its high cost and technical demands limit its use in low-resource settings (Paulos et al., 2019).

To overcome these barriers, antigen-based detection methods have emerged as a highly promising alternative, offering excellent specificity, faster results, and cost-effectiveness. These characteristics make antigen detection particularly valuable for identifying active infections in endemic areas (García et al., 2003; Onosakponome et al., 2020; Aziz et al., 2024). Recent advancements in antigen-based diagnostics are driven by the identification of highly specific and immunogenic proteins unique to *G. duodenalis*. High-quality genome sequencing of representative isolates from Assemblages A (WB strain) and B (GS strain) has enabled the application of 'omics' technologies to study host-parasite interactions in depth (Emery-Corbin et al., 2020). Among these, proteomics has proven especially useful, allowing comprehensive and quantitative analysis of protein expression during the interaction between *Giardia* and

host IECs, as well as during the parasite's life cycle transition from trophozoites to cysts (Balan et al., 2021; Emery-Corbin et al., 2021).

1.2 Problem statements and rationale of the study

The standard diagnostic method for detecting *Giardia* infection often suffer from low sensitivity and specificity due to intermittent cyst shedding and reliance on the expertise of laboratory personnel (Vicente et al., 2024; Aziz et al., 2024), resulting in frequent inaccuracies and making reliable identification of giardiasis challenging. Additionally, the rise of drug-resistant *Giardia* strains (Müller et al., 2019) underscores the urgent need for a deeper understanding of the parasite's biological mechanisms to inform the development of more effective treatment strategies. While considerable research has explored the transcriptional regulation of the encystation process, there is a lack of proteomic insights into this regulation, and the specific roles of proteins across *Giardia*'s various life stages remain largely unclear. A thorough proteomic analysis of *Giardia* trophozoites and cysts is therefore essential to address these knowledge gaps, paving the way for the development of more precise diagnostic and therapeutic approaches for giardiasis. Proteomic studies could provide a more holistic understanding of key processes and pathways involved in *Giardia* infection, supporting the discovery of potential biomarkers for diagnostic and therapeutic applications in giardiasis (Einarsson et al., 2015)

Between 2012 and 2022, only 31 articles related to the proteomics of *G. duodenalis* were published (Aziz et al., 2022), with limited focus on the encystation process, leaving significant gaps in understanding the functional activities involved during this phase. Therefore, comprehensive proteomic profiling of *Giardia*

trophozoites and cysts is essential to bridge these gaps, ultimately enabling the development of better detection tests to manage giardiasis effectively.

1.3 Objective(s) of study

This study aims to conduct a comprehensive comparative proteomic analysis of *G. duodenalis* trophozoites and cysts, both derived from *in vitro* axenic cultures. By analyzing protein profiles from each life stage, the research seeks to identify key proteins and pathways involved in the parasite's life cycle, with a focus on the encystation process. Using proteomic and bioinformatics approaches, this project also aims to discover potential biomarkers for improved and more accurate antigen-based diagnostics for giardiasis.

1.3.1 Specific Objectives

1. To evaluate the efficiency and reproducibility of four different protein extraction methods for *G. duodenalis* trophozoites and cysts based on protein yield and quality of separation.

2. To identify and quantify proteins from trophozoites and cysts using mass spectrometry-based proteomic analysis.

3. To perform a comparative analysis of the total proteomes to identify differentially expressed proteins between the two life stages.

4. To construct and analyze protein-protein interaction (PPI) networks of the identified proteins using bioinformatics tools.

CHAPTER 2

LITERATURE REVIEW

2.1.1 Life cycle

G. duodenalis undergoes two main stages in its life cycle; the motile trophozoite and the resistant cyst. The cyst stage is crucial in its life cycle as it serves as the bridge for transmission from the faeces of one host into the environment, and then to a new host. It takes a minimum of ten cysts to initiate the infection (Thompson and Monis, 2012). These cysts travel through the gut to the duodenum, where elevated bile and pH levels higher than those in the acidic stomach, trigger excystation. Excystation refers to the transformation of the ingested cyst into motile trophozoites within the duodenum. During this process, flagella and the cell body of the excyzoite develop through an opening at one pole of the cyst, resulting in the formation of four trophozoites through two rounds of division (Bertrand and Schwartzbrod, 2007).

The trophozoites reproduce by binary fission in the small intestine, with some reverting back to cyst form. These trophozoites aggregate in the proximal small intestine, reaching maximum colonization typically within a week (Barash et al., 2017). During infection, encystation is triggered, where trophozoites transform into cysts. At the onset of encystation, encystation-specific vesicles (ESVs) develop, and the trophozoites round up, disassembling certain cytoskeletal components for storage inside the cysts (Balan et al., 2021; Palm et al., 2005). The cells continue to divide until mature cysts with four nuclei are produced. These cysts along with some trophozoites are then excreted into the environment through host's faeces contaminating soils and waters, and continuing the life cycle of *G. duodenalis*. The life cycle of *G. duodenalis* is shown in Figure 2.1.

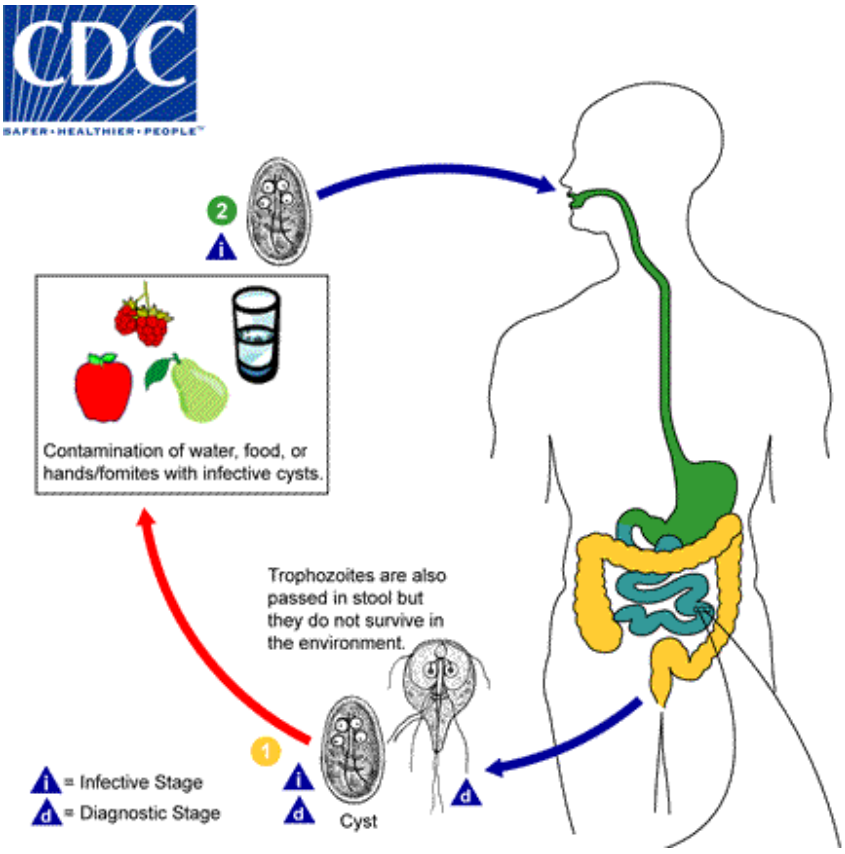

Figure 2.1 Life cycle of *G. duodenalis*. Retrieved from Centers for Disease Control and Prevention (CDC) website. Available at (https://www.cdc.gov/dpdx/giardiasis/index.html)

2.2 Historical background of *Giardia duodenalis*

Giardia duodenalis, a common zoonotic intestinal parasite, is among the ten most significant parasites affecting humans (Dawson and House, 2010). This microorganism was first discovered by van Leeuwenhoek in 1681 when he examined his own stool with a microscope (Dobell, 1920; Adam, 2001). However, this protozoan was not generally recognized until 1859, when Lambl provided a more detailed description of the organism initially classifying it as part of the genus *Cercomonas* and naming it *Cercomonas intestinalis*. Over time, some named the genus in his honor (for example, *Lamblia intestinalis* suggested by Blanchard in 1888), while others named the human-infecting species after him, calling it *G. lamblia* by Kofoid and Christiansen

in 1915 (Adam, 2001). From 1859, this parasite was initially introduced as *Cercomonas* until 1882 and 1883, when Kunstler described an organism in tadpoles, matching the description documented by Lambl in 1859, and introduced *Giardia* as the genus name. Initially, Blanchard then suggested the name *Lamblia intestinalis* in 1888 to honor Lambl's work, which Stiles revised to *G. duodenalis* in 1902. Following this, Kofoid and Christiansen proposed the names *G. lamblia* in 1915 and *G. enterica* in 1920. Later, Simon classified the species based on morphology, distinguishing *G. lamblia* for humans and *G. muris* for rodents (Simon, 1922; Adam, 2001). However, controversy arose over naming species according to their host of origin rather than relying solely on morphology, which led Kulda and Nohynkova (Adam, 2001) to propose 40 species names based on the host of origin. Currently, three names for the human-associated form are widely accepted: *G. lamblia* (since the 1970s), *G. duodenalis* (since the 1980s), and *G. intestinalis* (since the 1990s).

Seven species names have been broadly recognized: *G. duodenalis*, *G. muris*, *G. agilis*, *G. psittaci*, *G. microti*, *G. ardea*, *G. peramelis*, and *G. cricetidarum* (Adam, 2021). *G. duodenalis* is the most studied species within the genus of *Giardia,* primarily due to its significant pathogenicity and role as the causative agent of giardiasis in humans. Its adaptability to the different conditions within the gastrointestinal tracts of various hosts has made it a focal point for understanding its biological mechanisms. Furthermore, accurate methods have been developed to sub-classify the heterogeneity within *G. duodenalis*, leading to the identification of eight genetic assemblages (designated A—H) (Heyworth, 2016). In fact, El Basha et al. (2016) found that *Giardia* assemblages A and B are more prevalent in children compared to adults, based on investigations using light microscopy and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Understanding these different

assemblages, which are responsible for infection across various hosts, especially humans, enables researchers with the insight to develop more effective diagnostic tools and treatment strategies crucial for the enhanced management and control of giardiasis

2.3 Biology of *G. duodenalis*

2.3.1 Morphology

2.3.1(a) Trophozoites

The morphology of trophozoite is described as a pear-shaped, binucleate cell with eight flagella and an adhesive ventral disc (Al-Saad and Al-Emarah, 2014). The length of *G. duodenalis* trophozoites ranges from 12 to 15 µm, and their width ranges from 6 to 8 µm (Monis et al., 2009). Flagella allow the survival of *Giardia* within hosts, by facilitating movement to appropriate sites on the intestinal villi for colonization, while the ventral discs allows attachment, which is crucial for *Giardia* pathogenesis and replication (Brown et al., 2016). Behind the ventral disc lies a large, curved median body, unique to *Giardia*. This body lacks a Golgi complex, lysosomes, and smooth endoplasmic reticulum, consisting only of a parallel set of microtubules shaped like a claw (Monis et al., 2009). However, the precise function of the median body remains unknown (Hagen et al., 2020).

2.3.1(b) Cysts

The cyst is an ellipsoid-shaped, tetranucleate cell with fewer organelles than trophozoites, measuring 9–14 µm in length and 6–11 µm in width (Al-Saad and Al-Emarah, 2014). Cyst contains eight axonemes along its long axis, which are associated to the flagella development during encystation. The cyst wall, approximately 0.25 µm thick, consists of a filamentous outer later that protects *G. duodenalis* from hypotonic lysis in the environment (Ankarklev et al., 2010; Chavez-Munguia et al., 2004). The

cyst wall is composed of four proteins, namely cyst wall proteins CWP1, CWP2, CWP3 (Luján et al., 1995; Sun et al., 2003), and a high-cysteine (non-variant protein) (HCMP) (Davids et al., 2006). Each of these proteins is responsible for the survival and pathogenic mechanisms. The morphological features of *G. duodenalis* trophozoites and cysts are shown in Figure 2.2.

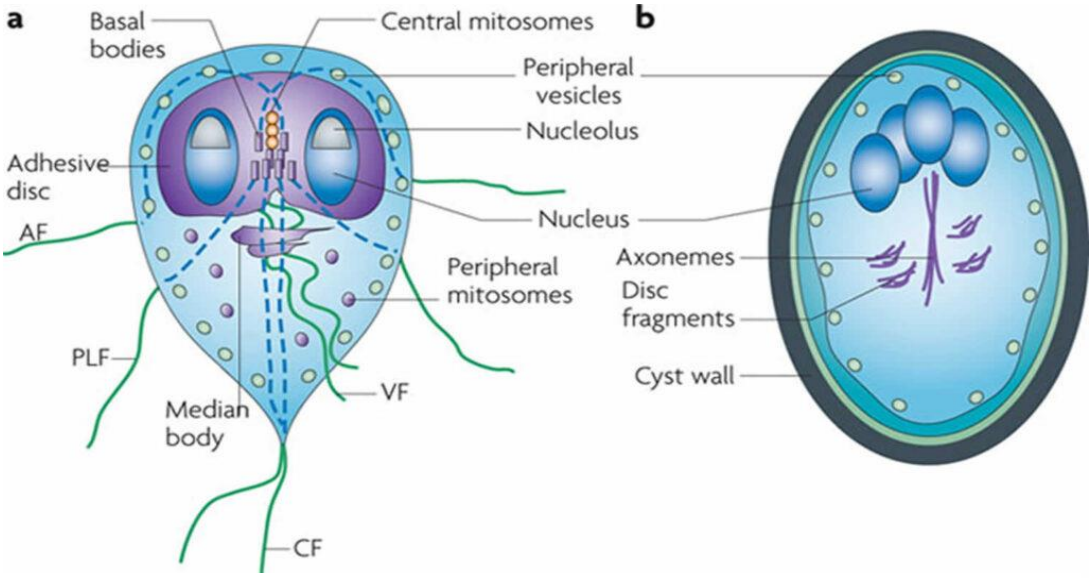

Figure 2.2 The structure of *G. duodenalis* trophozoites and cysts. Adapted from Health Jade (2020) [online]. Available at https://healthjade.net/giardia-duodenalis (Accessed: 16 August 2024)

2.3.2 Cell differentiation

2.3.2(a) Encystation

Encystation involves the gradual transformation of motile trophozoites into cysts, the internalization of flagella and the formation of the cyst wall (as shown in Figure 2.3), rendering the trophozoites unable to attach to surfaces. This process can be mimicked *in vitro* by stimulating external factors like high bile levels and slightly alkaline pH, marked by the synthesis of CWPs and the development of ESVs, which transport CWPs to form the new cyst wall (Thomas et al., 2021). Currently, only four CWPs are well known proteins associated with encystation (Lauwaet et al., 2007).

Three CWPs (CWP 1, CWP 2, and CWP 3) contain leucine-rich repeat (LRR) domain, while the high HCMP resembles trophozoite variant surface proteins and is up-regulated during late encystation (Davids et al., 2006). Once the CWPs reach the cell surface, these CWPs are processed and modified to form a new cyst wall (Thomas et al., 2021).

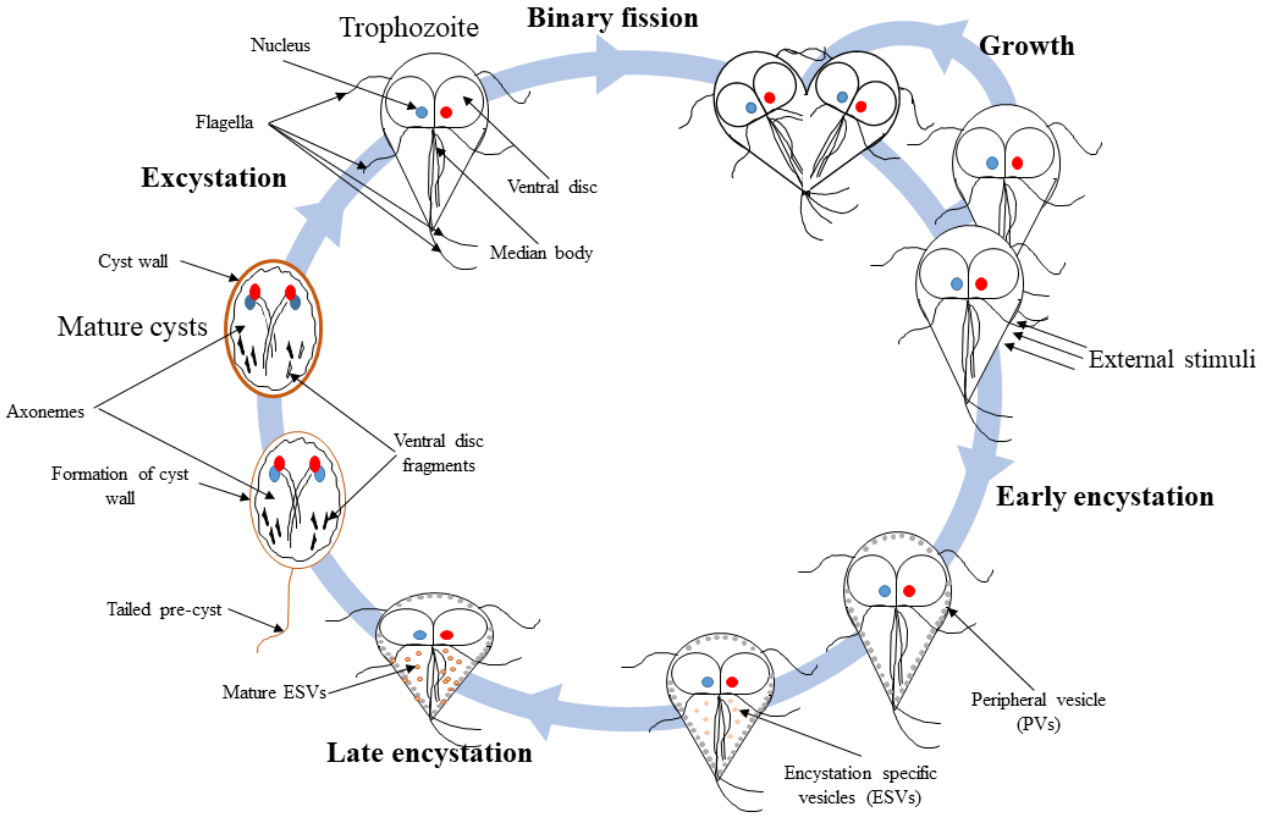

Figure 2.3 Schematic diagram of encystation and excystation in *G. duodenalis*. Adapted and modified from Einarsson & Svärd (2015).

Unlike CWP1–3, which are found in the cyst wall, HCMPs are identified in trophozoites and co-localize with CWPs in the ESV during encystation (Davids et al., 2006). Davids et al. (2006) reported that this protein is up-regulated during late encystation and is a novel *Giardia* protein. It contains a high cysteine content, explaining the parasite's high requirement for cysteine for growth and survival (Gillin and Diamond, 1981; Lujan and Nash, 1994). These findings suggest *Giardia* encystation provides an exclusive opportunity to discover the principles of membrane trafficking and the roles of its proteins during infection, potentially leading to the development of *Giardia*-specific diagnostic target or vaccine candidates.

2.3.2(b) Excystation

Giardia cysts can revert to trophozoites via excystation, enabling the parasite to proliferate and obtain nutrients from the host. Excystation is triggered when cysts encounter low pH and minimal pancreatic secretions. Few studies have reported that this process could be considered as an adaptation mechanism of *G. duodenalis* in humans (Lujan, 2011). Researchers have conducted *in vitro* excystation by mimicking the acidic conditions of the stomach passage, which offers advantages over natural excystation. *In vitro* methods allow for a better understanding of the mechanisms and proteins involved, providing more consistent physiological conditions and contamination-free studies of *G. duodenalis*'s biological processes. Bingham and his co-workers (1979) first successfully conducted *in vitro* excystation by incubating the cysts in an acidic aqueous solution (pH 2.0), followed by rinsing in distilled water and incubation in growth medium. Boucher and Gillin (1990) later established a method to determine the excystation efficiency of *Giardia* cysts in low pH conditions and in the presence of proteases. However, *in vitro* excystation from faecal sample is less common due to the low success rate and difficulty in maintaining cysts, as *Giardia*

trophozoites are the only life stage that proliferates. Nevertheless, some studies have reported successful *in vitro* excystation from trophozoites *in vitro* culture (Davids and Gillin, 2011; Fink et al., 2020).

Only a few proteins have been identified as playing vital roles during *Giardia* excystation. One such group is protein kinases, which mediate the cell-signalling pathways crucial for giardial colonisation in hosts and excystation in response to environmental stimuli. Four main kinase families regulate chromosomal segregation during the eukaryote cell cycle: NIMA-related serine/threonine kinases (NEKs), Aurora kinases, Polo-like kinases (PLKs), and cyclin-dependent kinases (CDKs) (Lagunas-Rangel et al., 2021; Malumbres and Barbacid, 2007), for which only a few number of proteins from these families have been identified thus far. For example, Abel et al. (2001) reported on protein kinase A (PKA), a serine/threonine kinase family dependent on cellular levels of cyclic AMP (cAMP). PKAs phosphorylates numerous proteins, including enzymes, cytoskeletal proteins, ion channels, and transcription factors (Abel et al., 1997). In another study, Smith et al. (2013) explored the roles of *Giardia* NEK1 and NEK2, finding that *NEK*1 regulates the microtubule dynamics of the ventral disc, while NEK2 controls the mitotic commitment and chromosome distribution to daughter parasites. This was supported study by Smith et al. (2012), who found that overexpressing NEK1 and NEK2 during *in vitro* excystation inhibited *Giardia* excystation. Furthermore, as noted by Lagunas-Rangel et al. (2021) Langunas-Rangel et al. (2021), *Giardia* possesses a surprisingly large number of NEKs, indicating a complex regulatory mechanism for its four distinct pairs of flagella. Therefore, these proteins may also be potential biomarkers for diagnosis, given their roles in cyst survival and parasite motility.

2.4 Giardiasis, transmission and disease management

Intestinal protozoan infections are common in humans, especially in high-risk groups such as children, pregnant women, and immunocompromised individuals, resulting in significant morbidity and mortality. Over 60 million cases of protozoan diarrheal diseases are reported annually (Savioli et al., 2006). A significant contributor to these cases is *G. duodenalis*, which causes giardiasis, a gastrointestinal disease. Recognized by the World Health Organization (WHO)'s Neglected Diseases Initiatives in 2004, giardiasis leads to nutritional disorders that severely impact the quality of life, particularly in areas with poor hygiene and sanitation. The spread of giardiasis is also influenced by travel-associated individuals, including refugees, adoptees, and immigrants. Between 25% and 50% of travellers from developing countries experience diarrhea (Ekdahl and Andersson, 2005). Refugees alone account for approximately 18–50 % of local giardiasis cases annually in United States (Bedard et al., 2016). Moreover, over 70% of giardiasis cases among refugees and immigrants involve individuals under the age of 18, highlighting the need for routine screening for *Giardia* during initial health assessments and enhanced giardiasis surveillance. Specialized health education programs are essential to inform travellers about the risks of giardiasis. Additionally, some studies have documented an association between *Giardia* infection and food allergies (Di Prisco et al., 1993) as well as patient intolerance (Litleskare et al., 2015).

G. duodenalis infection is commonly associated with diarrhea, but it can also be asymptomatic or present with a range of clinical symptoms from acute to chronic (Adam, 2021; Nash et al., 1987). Symptoms may include nausea, vomiting, and weight loss. While the infection is typically treatable and resolves within weeks, it poses a significant risk for young children, who may experience persistent diarrhea,

malabsorption, and impaired growth and development for several months (Coronato-Nunes et al., 2017). Diarrheal diseases are responsible for one in nine child deaths, and giardiasis partly contributed to an estimated 1.6 million diarrheal deaths reported in 2016 (Troeger et al., 2007). Malnutrition due to giardiasis is particularly concerning, as it has been linked to micronutrient deficiencies and impaired cognitive development.

Coronato-Nunes et al (2017) reported that children with giardiasis have significantly higher rates of stunting and underweight compared to healthy children, at 21.7 % vs. 5.6 % and 21.7 % vs. 7.3 %, respectively (Coronato-Nunes et al., 2017). Additionally, a recent study using a gerbil model (González Maciel et al., 2024) demonstrated that *Giardia* infection significantly reduced zinc levels in the duodenum and cognition-related tissues, such as the cerebellum and hippocampus. Zinc deficiency is associated with various brain disorders, and *Giardia* infection has been shown to contribute to this alteration. Therefore, effective management of giardiasis is crucial not only to alleviate symptoms but also to improve nutritional status and overall health in affected children.

Giardia is also recognized as a foodborne pathogen. In 2010, the WHO reported that *Giardia* caused 28.2 million cases of foodborne illness and 26,270 disability-adjusted life years (DALYs) (Ryan et al., 2019). In 2014, *Giardia* was ranked 11th among 24 foodborne parasites by a joint assessment from the United Nations Food and Agriculture Organization (FAO) and WHO (FAO/WHO, 2014). This ranking, lower than *Cryptosporidium*, suggests potential underreporting due to limitations in current detection and surveillance methods. The prevalence and impact

of *G. duodenalis* on foodborne infections are likely much higher than currently reported.

Giardiasis cases in Malaysia have primarily been reported among young children, indigenous communities, immunocompromised individuals, and migrants between 2000 and 2020 (Lim et al., 2011; Lim & Ahmad, 2004; Sahimin et al., 2016; Roshidi et al., 2021), with reported prevalence rates ranging from 0.44% to 34.6%. According to prevalence studies reviewed by Roshidi et al. (2021), young children from indigenous communities are the most susceptible group, accounting for 84.6% of the cases. This high prevalence is attributed to poor sanitation and hygiene, unsafe water sources, and low socioeconomic status, which contribute to improper food handling and unhygienic living conditions (Yusuf et al., 2007; Sinniah et al., 2012; Ngui et al., 2011; Roshidi et al., 2021).

2.4.1 Transmission

Direct transmission between individuals is common, especially among infants and young children in residential institutions where personal hygiene may be compromised, leading to prevalence rates of up to 27 % among children under ten years old (Al-Mekhlafi et al., 2013). Transmission during sexual activity, primarily through oral-anal contact, has also been reported (Escobedo et al., 2018), although this aspect is less studied due to the similarity in clinical manifestations of *Giardia* infection among HIV patients and the general population.

Giardia cysts are among the most commonly detected protozoa in untreated water, including domestic water sources (Ahmad et al., 1997; Richard et al., 2016), and recreational lakes (Azman et al., 2009). This widespread presence is associated with factors such as nearby human populations, agricultural runoff, and inadequate

waste disposal (Richard et al., 2016). Livestock, especially in the rainy season, contribute significantly to *Giardia* contamination in water sources through defecation. Runoff from these areas can lead to *Giardia* cysts entering untreated water, and ingestion of as few as ten cysts via the fecal-oral route can cause infection (Ryan et al., 2019), representing a major public health risk, particularly where water treatment infrastructure is limited (CDC, 2019).

Outbreaks of giardiasis can result from drinking contaminated water from lakes, rivers, or poorly maintained municipal supplies (Conners et al., 2021). From 2011 to 2016, 381 documented outbreaks linked to waterborne protozoa transmission were recorded, with *Giardia* species identified in 37% of the 142 cases (Efstratiou et al., 2017). In Malaysia, giardiasis rates in waterborne outbreaks are at 25% (Feng and Xiao, 2011; Roshidi al., 2021). Symptoms of giardiasis can be particularly severe in young children and immunocompromised individuals. The serious impact of giardiasis has prompted its classification as a nationally notifiable disease in the United States by WHO to strengthen public health surveillance (CDC, 2024). However, in Malaysia, this infectious disease is not classified as a notifiable communicable disease by the government (Ministry of Health Malaysia [MOH], 2003; MOH, 2017), which suggests a lack of surveillance and potential underreporting of giardiasis cases among the population.

Given its capacity to cause widespread waterborne outbreaks, *Giardia* is also classified as a potential bioterrorism threat. It is listed as a Category B bioterrorism agent by the CDC and National Instittute of Health (NIH) due to its moderate ease of dissemination and potential for high morbidity (Bashyal et al., 2017). *Giardia*'s resilience in various environmental conditions and low infectious dose make it suitable

for intentional water contamination, posing risks to public health systems (Bashyal et al., 2017). Although *Giardia* has a lower mortality rate than other bioterrorism agents, its ability to cause extensive illness and induce public alarm highlights the need for preparedness and response strategies (CDC, 2018). Preventing these outbreaks involves ensuring clean water treatment, promoting good hygiene, and educating the public on the risks of consuming untreated water.

2.4.2 Epidemiology

Among the eight known genetic assemblages of *G. duodenalis* (as listed in Table 1), only assemblages A and B are found in humans, whereas the remaining six (C–H) are host-specific, with assemblage B has a higher prevalence than assemblage A worldwide (Ryan and Cacciò, 2013). In Malaysia, *G. duodenalis* assemblage B is overwhelmingly dominant (over 97.6%) among 42 indigenous people infected with giardiasis (Feng and Xiao, 2011). This suggests that assemblage B may have an advantage over assemblage A in terms of transmission, adaptation, and survival in different environments due to its higher genetic diversity (Ogbuigwe et al., 2022).

Studies have confirmed that zoonotic transmission can occur between humans and non-human hosts. In England, a case-control study showed an association between giardiasis and contact with livestock and pets (Xiao and Fayer, 2008). Another study found assemblages A and E in both humans and livestock, suggesting that environmental contamination by faeces facilitates the transmission of *Giardia* in Vietnam (Iwashita et al., 2021). These insights deepen our understanding of the association between genotyping, zoonotic potential, and host specificity of *G. duodenalis*.

Table 1 The sub-classification of *G. duodenalis*.

Assemblage	Hosts	References
A	Mammals, including humans	Heyworth (2016)
B	Mammals, including humans	Heyworth (2016)
C	Dog, cattle, pig	Covacin et al. (2011); Minetti et al. (2014)
D	Pig, cattle, cat, deer	Feng and Xiao (2011); Geurden and Olson (2011); Liu et al. (2014)
E	Livestock, cat, deer	Feng and Xiao (2011); Lebbad et al., (2010); Liu et al. (2014)
F	Cat	Lebbad et al. (2010)
G	Rat, mouse	Lebbad et al. (2010); Zhao et al. (2015)
H	Seal, gull	Lasek-Nesselquist et al. (2010)

According to Feng and Xiao (2011), the prevalence of human giardiasis is 22.5 % higher in developing countries (8–30 %) compared to developed countries (0.4–7.5 %). In Malaysia, *G. duodenalis* is more prevalent in the least affluent segments of the population, including indigenous communities and densely populated areas such as prisons and slums (Roshidi et al., 2021). Over 80 % of cases have been reported in these populations, particularly among children. The lack of access to clean water and proper sanitation significantly contributes to the high incidence of *G. duodenalis* cases in these areas. The higher incidence of giardiasis in rural communities compared to urban areas can be attributed to the lack of education and insufficient government efforts in these regions. Studies on *G. duodenalis* in environmental samples and domestic animals also have been conducted. Similarly, Bertrand and Schwartzbrod (2007) detected assemblages A and E were detected in wastewater samples from a slaughterhouse. In contrast, urban wastewater samples had revealed only assemblages A and B, indicating contamination from human sources.

2.4.3 Diagnosis of *G. duodenalis*

2.4.3(a) Traditional method

The most important indicator for suspecting giardiasis in a symptomatic patient is the presence of persistent watery diarrhoea, which may sometimes be foul-smelling and alternate with soft, greasy stools, along with other symptoms such as abdominal pain or signs of malabsorption. (Mayo Clinic, 2022). However, it is possible to distinguish giardiasis from other intestinal issues through a differential diagnosis that includes reviewing the patient's history of exposure to giardiasis risk factors and examining samples for the presence of cyst or trophozoite forms. In areas where giardiasis is common, it is also important to consider the possibility of recurrent infections, especially with the help of molecular epidemiology. Accurate detection of giardiasis is essential for the effective treatment and prevention of the disease.

The gold standard for diagnosing *G. duodenalis* is the ova and parasite (O&P) examination in faecal samples under a light microscope. Trophozoites are mainly observed in diarrhea samples. Meanwhile, in asymptomatic individuals and healthy carriers, cysts are more likely to be observed in non-diarhea samples under the microscope than trophozoites (Sahimin, 2017). Various microscopic techniques are utilized, including direct wet mounts, concentrated samples, and permanently stained smears (Escobedo et al., 2018). The direct wet mount of a fresh faecal sample allows the observation of the motile trophozoites, while stained samples immobilize the trophozoites (Hooshyar et al., 2019).

However, the sensitivity and specificity of these techniques depend on several factors, including the number of fecal samples examined, the use of direct or concentration methods, and the skill of experienced technicians (Hooshyar et al., 2019). *G. duodenalis* exhibits variable patterns of excretion, which can lead to false-

negative results during detection. To achieve higher sensitivity, at least three samples are required. Additionally, fresh samples are necessary because *Giardia* trophozoites in faecal samples tend to degrade without fixatives and preservatives. The current gold standard is cumbersome and lengthy, which can affect its reliability and performance. Several studies have reported that the sensitivity and specificity of microscopic examination are generally low. Consequently, many molecular techniques have been developed over the past decades to improve *Giardia* detection and provide better management of giardiasis.

2.4.3(b) Serological techniques

Immunological assay and other serological methods such as ELISA, ICA, WB test are indeed common options for both diagnostic or research purposes and often offer better specificity and sensitivity compared to microscopy detection. Some studies recommend serological methods as the primary detection tools, especially for large-scale population screening in areas with high *G. duodenalis* prevalence. This is because serological methods provide high throughput as many samples can be tested at once in a single day and require only a single sample to detect the parasite (Hooshyar et al., 2019).

Commercial direct and indirect immunofluorescence assays have been introduced. One commercial kit, Tri-Combo (Techlab, USA), is more cost-effective than other commercial assays, reducing the turnaround time for detection and eliminating the need for expertise to analyze the results (Den Hartog et al., 2013). Additionally, fluorescent-monoclonal-antibody detection, another serological technique, targets and labels the antigens within the cell, increasing the specificity and sensitivity for *G. duodenalis* detection, which is beneficial for epidemiological studies and better giardiasis control (Adeyemo et al., 2018).

Recently, the development of nanoscale analytical instruments, such as biosensors, has gained much attention. These devices combine biological receptors, such as immobilized antibodies, with a transducer to create a signal over the analyte in the sample (Alhindawi et al., 2024). Biosensors have been reported to exhibit high sensitivity and selectivity for *G. duodenalis* detection in water bodies, even at low concentrations (as few as 10 cysts/ml) and within a short period (Xu and Muiharasan, 2010). However, biosensors for *Giardia* detection are still in their infancy. Many aspects should be considered before they can be commercialized as a new diagnostic method for giardiasis.

One critical aspect is the validation of biosensor performance on clinical samples, as there still lacks a sufficient body of studies in this area. Rigorous validation studies should be conducted to demonstrate the accuracy, reliability, and effectiveness of biosensors. Additionally, biosensors should be tested in real-world settings to gather clinical evidence supporting their efficacy and versatility, which is crucial for regulatory approval and gaining the trust of healthcare professionals. Overall, biosensors hold the potential for miniaturization and integration into devices for on-site detection, possibly making them cheaper, faster, and enabling higher sensitivity and accuracy compared to other commercial and molecular tests (Alhindawi et al., 2024).

2.4.3(c) Advanced molecular techniques

The detection of *Giardia* DNA by real-time polymerase chain reaction (PCR) and quantitative PCR offers higher sensitivity and specificity than traditional methods (Schuurman et al., 2007). However, Schuurman et al. (2017) reported a risk of cross-contamination, which can lower specificity. Additionally, PCR is limited to detecting only a single relevant gastrointestinal parasite and requires an additional DNA

extraction step, which is tedious and complex. The expensive machinery needed for PCR can take up to five hours to analyze results, making it unsuitable as a primary detection method for *Giardia*. Instead, it is more appropriately used as a complementary test to microscopy due to its high sensitivity for *G. duodenalis* detection (Alhindawi et al., 2024).

The development of PCR techniques capable of detecting multiple gastrointestinal diseases, such as commercial multiplex PCR assays (Autier et al., 2018; Laude et al., 2016) and in-house multiplex PCR assay (Llewellyn et al., 2016) , has shown promise. The multiplex PCR assay provides superior performance compared to microscopic examination, as it can detect *Giardia* DNA even when present in low quantities (Heyworth, 2014). Furthermore, multiplex PCR can identify multiple pathogens simultaneously, making it particularly valuable in cases of co-infection (Won et al., 2016). This method not only simplifies the diagnostic process but also improves the accuracy and reliability of results (Heyworth, 2014).

Llewellyn et al. (2016) reported that multiplex PCR detects significantly more parasites than microscopy. When coupled with automated nucleic acid extraction, these assays do not require trained technicians, significantly reducing turnaround time (Laude et al., 2016). However, the sensitivity of multiplex PCR assays varies from 41% to 89% compared to microscopy (Autier et al., 2018); partly influenced by the DNA extraction process. Despite the current limitations, multiplex PCR assays are suitable for epidemiologic studies where multiple parasites are present, although performance improvements are needed, such as improving the sensitivity and specificity as well as the ability of multiplex PCR to detect multiple pathogens

simultaneously without cross-reactivity or loss of sensitivity is important, for them to become reliable diagnostic methods for giardiasis.

The real-time loop-mediated isothermal amplification (LAMP)-based assay is used robustly in water resource monitoring to detect wide range of gastrointestinal parasites, including *G. duodenalis*. It is proven to be rapid, effective and easy to perform (Plutzer and Karanis, 2009). LAMP is also more sensitive and specific than conventional PCR and advanced PCR techniques like nested PCR (nPCR) and quantitative PCR (qPCR) (Foo et al., 2020). Unlike PCR, LAMP is not affected by the presence of inhibitors (Plutzer and Karanis, 2009; Sotiriadou and Karanis, 2008). LAMP-based assays can be integrated with field-friendly equipment, such as lateral flow dipsticks, microfluidic methods, and smartphone-based technology (García-Bernalt et al., 2021). However, LAMP requires specific primer designs based on published gene sequences, and only a limited number of organism-specific LAMP primers have been studied (Plutzer and Karanis, 2009). Variations in primer binding regions can affect the ability of *Giardia*-LAMP primers to amplify the target region of *G. duodenalis* DNA. Nonetheless, compared to PCR, LAMP is more suitable for field-testing in developing countries and is an ideal candidate for giardiasis detection, potentially replacing current reference methods. Future studies should focus on miniaturizing LAMP-based methods to make them portable and accessible in developing countries.

Overall, these developments highlight the need for rapid and simple *G. duodenalis* detection in both environmental and clinical samples. Reliable diagnostics allow for better management of giardiasis, preventing transmission and reducing its burden on communities. Cost considerations are crucial, especially for low-income