THE ROLE OF ALPHA-GIARDIN IN RESPONSE TO DRUG TREATMENT IN *GIARDIA INTESTINALIS*

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

×	Multiply
°C	Degree Celsius
µL	Microlitre
µM	Micrometre
ADI	Arginine diaminase
AMP	Antimicrobial peptides
AnnAt1	*Arabidopsis thaliana*
DC	Dendritic cells
DNA	Deoxyribonucleic acid
e.g.	*exempli gratia* - 'for example'
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
etc.	*et cetera*
GDH	Glutamate dehydrogenase
G6PD	Glucose 6 phosphate dehydrogenase
H	Hour
H_2O_2	Hydrogen peroxide
HUSM	Hospital Universiti Sains Malaysia
i.e.	*id est* - 'that is'
min	Minutes
mL	Millilitre
NAOH	Sodium hydroxide
O&P	Ova and Parasite
OCT	Ornithine carbamoyl transferase
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFOR	Pyruvate ferredoxin oxidoreductase

qPCR	Real time polymerase chain
ROS	Reactive oxygen species
RT	Room temperature
TRxR	Thioredoxin reductase
USM	Universiti Sains Malaysia
VSPs	Variant surface proteins
WHO	World Health Organization

PERANAN ALPHA-GIARDIN TERHADAAP UBAT DALAM *GIARDIA INTESTINALIS*

ABSTRAK

Giardiasis yang disebabkan oleh parasit protozoa usus, dikenali sebagai *Giardia intestinalis,* telah memberi kesan negatif kepada 280 juta penduduk dunia terutama kanak-kanak yang berumur lima tahun ke bawah. Pengambilan sekurang-kurangnya sepuluh cyst *G. intestinalis* boleh menyebabkan jangkitan di mana akan mengakibatkan komplikasi seperti dehidrasi dan kekurangan berat badan. Terdapat beberapa ubat yang boleh didapati untuk merawat jangkitan giardiasis namun, kerintangan terhadap ubat, reinfeksi dan pengulangan simptom semakin meningkat. Oleh itu, penghasilan ubat baharu adalah penting untuk mengawal giardiasis. Kajian ini dilakukan untuk mengkaji peranan alpha-giardin disebabkan oleh ubat di dalam *G. intestinalis*. Hasil kajian menunjukkan Mebendazole (MBZ) lebih berkesan terhadap *G. intestinalis* dengan nilai IC_{50} sebanyak 0.06 jika dibandingkan dengan Metronidazole (MTZ) iaitu sebanyak 9.177. Trofozoit pada nilai kepekatan ubat yang tinggi menunjukkan pengurangan pertumbuhan secara signifikan. Radikal bebas tidak dapat dikesan apabila menggunakan MBZ dan MTZ. Selain itu, *G. intestinalis* yang dirawat menggunakan 100 µM MBZ dan MTZ menunjukkan ekspresi alpha-2 giardin yang signifikan ($p \leq 0.05$). Kesimpulannya, alpha-2 giardin berpotensi untuk dijadikan penanda dalam penghasilan ubat atau vaksin baru bagi mengawal jangkitan giardiasis.

THE ROLE OF ALPHA-GIARDIN IN RESPONSE TO DRUG TREATMENT IN *GIARDIA INTESTINALIS*

ABSTRACT

Giardiasis, which is caused by the intestinal protozoan parasite *Giardia intestinalis*, affects approximately 280 million people around the world, particularly in children below five years of age. Oral ingestion of as few as ten cysts of *G. intestinalis* can cause infection in human which lead to complications such as severe dehydration and weight loss. There are several antigiardial drugs available for giardiasis treatment; however, issues such as drug resistance, reinfection and recurrence of symptoms have been highly reported. Thus, the finding for a novel potential drug targets is important to control giardiasis. In this study, we sought to examine the role of alpha-giardins in response to drug treatment in *G. intestinalis*. Our results showed that *G. intestinalis* were more susceptible to the action of Mebendazole (MBZ) as the IC_{50} value for MBZ was 0.06 µM when compared with Metronidazole (MTZ) of 9.177 µM. Trophozoites treated with high concentration of MBZ or MTZ showed significant reduction in viability. The release of reactive oxygen species were not detected in either MBZ or MTZ-treated *G. intestinalis*. We also found that *G. intestinalis* treated with 100 µM MBZ or MTZ showed significant upregulated expression of alpha-2 giardin ($p \leq 0.05$). In conclusion, alpha-2 giardin could serve as a novel potential target for future drug design and vaccine development.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Overview

Giardiasis is a worldwide health problem that requires public attention. The causative agent for human giardiasis, *Giardia intestinalis*, is an anaerobic flagellated protozoan that affected over 200 million people with 500,000 new cases being reported annually (Painter et al., 2015, Ankarklev et al., 2010). It is accounted as one of the most common diarrheal diseases among children aged less than five years old (Ehsan et al., 2017). Giardiasis occurrence is on a decreasing trend due to the effectiveness of current treatment. However, side effects and drug resistance as well as re-occurrence are still a problem. Thus, the search for new drug or drug targets is important to prevent the disease.

Although giardiasis infections are mostly asymptomatic, chronic and persistent diarrhea could be seen in symptomatic patients (Minetti et al., 2016). This can cause life threatening situation especially among younger children living in third world country where access to medicine are limited. Besides, the affected children showed growth disorders although the giardiasis infections are cleared. Most children with *Giardia* infection, show growth retardation and reduced weight when compared to those uninfected children (Fraser et al., 2000).

The current standard antigiardial drug is 5-nitro derivatives of imidazole groups (metronidazole, tinidazole), furan (furazolidone) and thiazole (nitazoxanide). Alternative drugs are acridines (quinacrine, chloroquine), aminoglycosides (paromomycin) and benzimidazoles (albendazole, mebendazole) (Cañete, 2016).

Even though these drugs are effective for the control of giardiasis, issues related to side effects, recurrence and resistance still exist. For example, furazolidone may cause complications in patient of a certain genotype (G6PDH-deficient patient) and quinacrine can lead to yellow discoloration of skin, long term use of albendazole has caused reversible neutropenia (Gardner and Hill, 2001). Recurrences after drug treatment have also become big issue. According to Hanevik and colleagues, the persistence of chronic giardiasis is as high as 32% 7 months after infection. In another study, chronic symptoms such as irritable bowel syndrome and chronic fatigue still persist in infected patients 6 years after *Giardia* infection (Hanevik et al., 2014). The development of resistance towards most antigiardial drugs has also become one of the arising issues. The resistance to metronidazole occurs in 20% of cases (Jarrad et al., 2016). Therefore, the identification of new drug targets is vital to solve this problem.

Cytoskeleton of the *Giardia* is important in pathogenicity as well as in supporting its motility, ability to attach to the mucosa and resistance against degradation by bile salts for *Giardia* survival in host's body (Elmendorf et al., 2003). Cytoskeleton of the *Giardia* consists of tubulin as well as four different giardin families which are the alpha-giardins, beta-giardins, gamma-giardins and delta-giardins. In *Giardia*, 21 alpha-giardins have been identified and they are assigned to the family E annexins (Morrison et al., 2007). According to Weiland and colleagues (Weiland et al., 2005), most alpha-giardins are localized to the plasma membrane and flagella, while only a few are attached to the adhesive disc of the trophozoites. Recent finding by Paz Maldonado and colleagues (Paz-Maldonado et al., 2013), showed that alpha-2-giardin was upregulated in *G.intestinalis* when exposed to

albendazole. However, another contrary report showed that alpha-giardins were down-regulated under metronidazole-induced stress (Ansell et al., 2016).

The plant annexin, *Arabidopsis thaliana*, *AnnAt1* (annexin 1), has been identified to play an important role in protecting cells from oxidative stress (Konopka-Postupolska et al., 2009). Since annexins of *Taenia and Schistosoma spp* have been shown to protect cells from oxidative stress (Hofmann et al., 2010*),* it is postulated that alpha-giardins may also protect *Giardia* from drug-induced oxidative stress. Hence, the understanding of whether alpha-giardins help *Giardia* to survive under drug-induced oxidative stress in the host is important in order to identify new drug targets to combat giardiasis.

1.2 Introduction to *Giardia intestinalis*

Giardia intestinalis, also known as *Giardia lamblia* or *Giardia duodenalis*, is the protozoan parasite causes chronic diarrheal diseases worldwide. Infection is acquired through ingestion of cysts from contaminated water or food, or through fecal-oral route (Einarsson et al., 2016). *G. intestinalis* exists in two distinct forms in the environment, the trophozoites (vegetative form) and the cyst (infective form). During trophozoite stage, these parasites will multiply and reproduce asexually in the host's small intestine, while cysts will be shed to the environment through the host's feces and transmit the disease (Weiland et al., 2005).

G. intestinalis is a member of the family Hexamitidae, order Diplomonadida, class Zoomastigophora and of phylum Sarcomastigophora. Currently, there are 40 *Giardia* species that have been identified, and only six have been morphologically

recognized (Feng and Xiao, 2011). *G. intestinalis* is the only species that causes infection in humans and a variety of animals. *G. muris* infects rodents whereas *G. aredeae* and *G. psittaci* infect birds. *G. agilis* is found to infect amphibians (Lopez‐Romero et al., 2015).

There are eight assemblages (genotypes) exist that infect a wide range of mammals and humans. These assemblages are named as assemblage A-H (Heyworth, 2016). The assemblages A and B have been found to cause infection in humans and other mammals. However, other assemblages including C, D, E, F, G and H do not cause infection in human. Assemblage C and D infect domestic animals such as dogs while assemblage E infect hoofed animals, domestic ruminants and pigs. Assemblage F infects cats, assemblage G infects rodents such as mice, and assemblage H infects seals (Feng and Xiao, 2011, Lopez‐Romero et al., 2015). Figure 1.1 shows the established *Giardia* species and *G. intestinalis* assemblages.

Species	Major host(s)
G. agilis Kunstler, 1882	Amphibians
G. ardeae Noller, 1920	Birds
G. microti Benson, 1908	Muskrats and voles
G. muris Benson, 1908	Rodents
G. psittaci Erlandsen and Bemrick, 1987	Birds
G. varani Lavier, 1923[a]	Lizards
G. duodenalis Davaine, 1875	Mammals
Assemblage A (=*G. duodenalis* sensu stricto?[b])	Humans, nonhuman primates, domestic and wild ruminants, alpacas, pigs, horses, domestic and wild canines, cats, ferrets, rodents, marsupials, other mammals
Assemblage B (=*G. enterica*?[b])	Humans, nonhuman primates, cattle, dogs, horses, rabbits, beavers, muskrats
Assemblage C (=*G. canis*?[b])	Domestic and wild canines
Assemblage D (=*G. canis*?[b])	Domestic and wild canines
Assemblage E (=*G. bovis*?[b])	Domestic ruminants, pigs
Assemblage F (=*G. cati*?[b])	Cats
Assemblage G (=*G. simondi*?[b])	Mice, rats
Assemblage H	Seals

Figure 1.1 ***Giardia species and G. intestinalis assemblages***

Source: Feng & Xiao (2011)

Giardia trophozoite was first discovered by Antony van Leeuwenhoek, a Dutch microscopist in 1681 after he observed his own diarrheal feces and named this protozoan as *G. intestinalis* (Adam, 2001*)*. However, he did not make further observation on the pathology of this parasite. *G. intestinalis* was then further studied and described by Vilem Lambl in 1859. He observed the presence of this parasite in the stools of children with diarrhea. However, he believed that this parasite was a commensal and not responsible to cause infection. The controversy about the number of *Giardia* species continued for many years and more than 40 names have been proposed by few investigators during the first half of the 20th century. The species

was later widely known as *G. lamblia* and *G. intestinalis* in the medical field. This parasite has been recognized as pathogenic in the late 1970s (Ford, 2005). However, these parasites are non-invasive as it remains confined to the lumen of small intestine and do not spread via bloodstream or gastrointestinal tract.

1.2.1 Epidemiology of Giardiasis

The epidemiologic distribution of *Giardia* in human population is due to the exposure and ingestion of infectious cysts through contaminated water and food, or through fecal oral infection. The estimated prevalence of *Giardia* infections range between 2% and 5% in developed countries, and 20% to 30% in developing countries (Roxström-Lindquist et al., 2006). *Giardia* infection showed high prevalence rate in children less than ten years old (Painter et al., 2015). The highest prevalence of giardiasis occurrence has been reported in western Nepal with 73.4%, and this is mostly due to poor sanitation. In the United States, giardiasis has been notified since 2002 with about 2.5 million cases reported annually. The insufficient of clean water supplies appear as the cause of diarrhea among travellers to the regions of Africa, Asia and Latin America (Abbas et al., 2011).

According to a surveillance survey of giardiasis during 2011 and 2012, there are 16, 868 cases in 2011, and 15, 223 cases in 2012 per 100 000 population in 2011 and 5.8 in 2012. Giardiasis has been identified as the main cause of diarrhea among young children aged 1-9 years old. The occurrence in children may be related to the increased recreational water exposure, poor hygiene practices and close contact with infected children in child care center. The occurrence of giardiasis also has been reported to be higher among males especially in adults, which might be due to their

higher exposure to outdoor activities, occupation status and homosexual practices (Painter et al., 2015).

In Malaysia, the prevalence of giardiasis could be underreported. The prevalence of giardiasis was the highest among aboriginal communities (29.2%), with higher cases diagnosed in West Malaysia as compared to East Malaysia (Choy et al., 2014). Giardiasis has been found to be the highest among aboriginal communities in Pahang (15.9%), followed by Negeri Sembilan (14.9%) and Kedah (13.4%). However, the cases were found to be low among communities from Malacca (4.6%). The occurrence of *Giardia* infection was high among aboriginal communities in West Malaysia especially in children aged less than 12 years old. This is caused by the factors such as inadequate sanitary and healthcare facilities, no clean water supply, poor hygiene practices, lack of health education and poor housing conditions (Mohamed et al., 2008). Figure 1.2 shows the distribution of giardiasis in Malaysia.

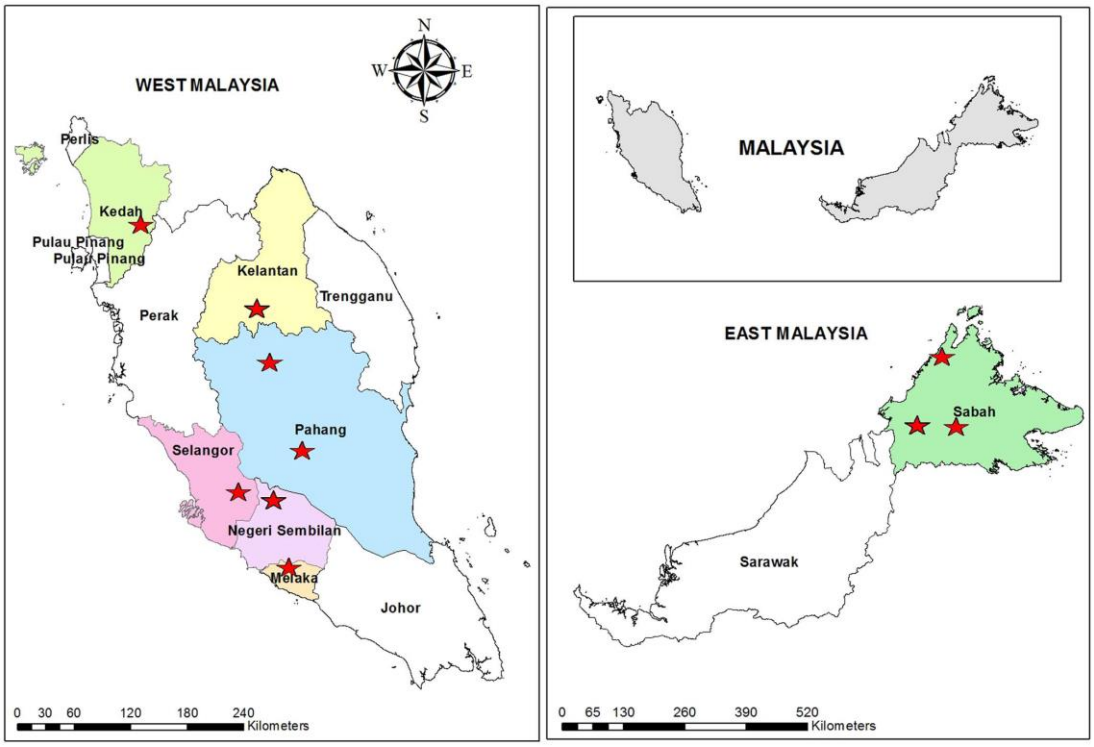

Figure 1.2 Occurence of Giardiasis in Malaysia.

A geographic map showing the location of district (stars) and states (coloured) involved.

Source : Choy et al., (2014)

1.2.2 Morphology and life cycle

G. intestinalis is an unique organism as it possesses two equal sized nuclei that are similar in appearance, DNA content, transcription and time of replication (Morrison et al., 2007). It has two different stages of development, the trophozoites and the cyst. The trophozoites have a pear shaped body with convex dorsal side and an enlarged anterior and narrower posterior. The size ranges from 8 to 20 µm long and 5 to 16 µm wide with an average length range from 10 to 15 µm. *Giardia* trophozoites contain two nuclei and median bodies with four pairs of flagella which are anterior, posterior, ventral and caudal flagella that help in locomotion and attachment to the host's intestines (Adam, 2001). *Giardia* trophozoite is lack of mitochondria,

peroxisome, rough endoplasmic reticulum and Golgi apparatus. However, it has a unique and complex cytoskeleton structure, consists of microtubules and microfilaments which aids in cell motility (Elmendorf et al., 2003).

The cysts which are responsible to cause disease are oval and elliptically shaped. The size ranges from 8 to 19 µm long and 7 to 10 µm wide. Each cyst contains four nuclei, four median bodies and is covered by 0.3 to 0.5 µm thick cyst wall. The cyst wall is composed of β-1, 3-N-acetyl-D-Galactoseamine polymers and cysteine that surrounds the outer filamentous layer and inner membrane layer (Ringqvist, 2009). *Giardia* cysts are very resistant to the environmental stress including chlorination, UV exposure and freezing. Hence, they can survive for several months in cold water and are responsible for the disease transmission (Painter et al., 2015).

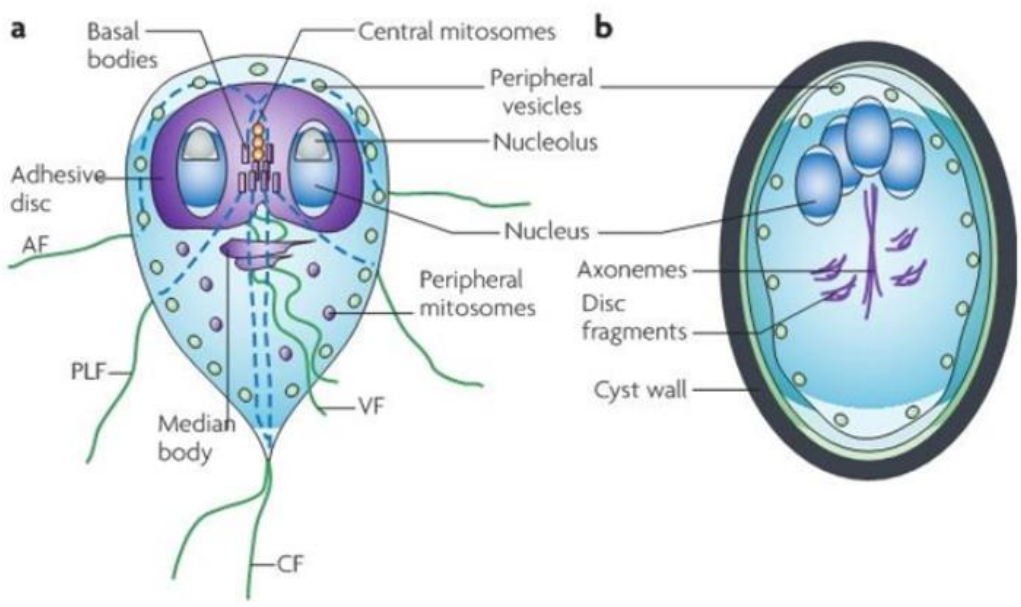

Figure 1.3 Morphological characteristics of the trophozoite (a) and cyst (b) of *Giardia*

The life cycle of *G. intestinalis* is very direct and simple, in which it only involves two stages, the excystation and encystation stages. *Giardia* infection in the host begins through ingestion of cyst through contaminated drinking water or food, or via fecal-oral route (Weiland et al., 2005). The infectious doses are about 25 to 100 cysts; however ten cysts are sufficient enough to cause infection in human as it can reproduce in the small intestine. The excystation stage takes place when the ingested cysts traveled through the host's digestive system and excyst to become trophozoites in the duodenum. The excystation process was stimulated by the acidic environment in the stomach of the host. The low pH of the stomach acid and the influx of bile from the gall bladder lead to the loss of cyst wall's rigidity. Releasing excyzoite then will divide and produce trophozoites in the duodenum and jejunum of the host (Bernander et al., 2001, Adam, 2001, Ringqvist, 2009). Each cyst will divide twice and produce four trophozoites during the excystation stage.

The trophozoites will then attach to the intestinal epithelial cell by using adhesive disk that are resistant to be removed via bulk flow in the intestinal lumen (Einarsson et al., 2016, Lopez‐Romero et al., 2015). During attachment in the small intestine, the trophozoites reproduce asexually by longitudinal binary fission and either remains in the lumen of the proximal small bowel as free organism or being attached to the mucous lumen by a ventral sucking disk. As the trophozoites transported down to the colon, the encystation stage will take place due to exposure to the bile salts and fatty acids. Alkaline pH, the presence of high level of bile and low level of cholesterol will trigger the encystation process of *Giardia* trophozoite (Lauwaet et al., 2007). During the process, the trophozoites disc will disassemble and compartmentalize structural organelles and reform to the shape of the cyst. The encystation stage is complex and involves downregulation of trophozoite-specific

genes and induction of encystation-specific gene (Hill and Nash, 1995). In the early phase, the encystation-specific vesicles will develop and mature as well as other proteins and protease. The late phase of encystation involves changes of the gene expression, transportation of the cyst wall protein to the cell surface, the assembly of trophozoites with N-acetylgalactosamine (GalNAc) into the cyst wall and DNA replication (Einarsson et al., 2016, Hill and Nash, 1995). The cysts will then are passed to the environment and are able to survive for months outside the host. About 300 million of cysts can be shed through the feces of infected individuals (Ford, 2005). As the cysts can survive for a long period of time especially in the damp and cool environment, the life cycle will be repeated again. Figure 1.4 shows the summary of *G. intestinalis* life cycle.

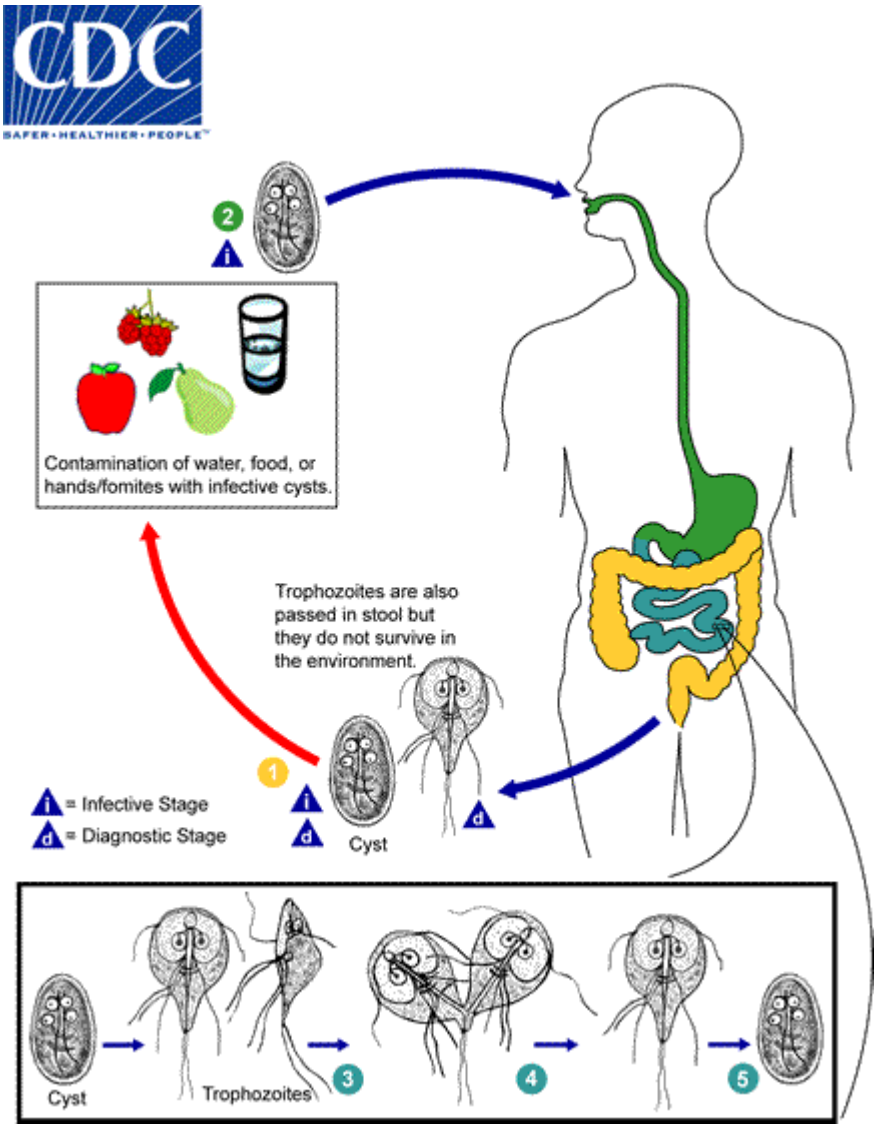

Figure 1.4 Life cycle of *G. intestinalis*

Source : www.cdc.gov

1.2.3 Transmission routes of Giardiasis

Giardia infection can be found worldwide especially in the areas with poor sanitation and contaminated water supply (Einarsson et al., 2016). In humans, giardiasis can be acquired through the consumption of untreated or improperly treated contaminated water, ingestion of contaminated uncooked foods especially vegetables or fruits, or by intimate contact with infected individuals through fecal-oral route (Painter et al.,

2015). Giardia infection can also occur through direct zoonotic transmissions from pets (cats and dogs) to human. Although giardiasis most commonly affects travellers and those camps in the wilderness, anyone is at risk of being infected. The high risk groups of exposure to *Giardia* infection includes children in childcare settings, those having close contacts with infected people, those consume contaminated water and foods, those involved with water activity such as swimming and playing in recreational water especially in lakes, ponds, rivers and streams, those who have contacted with infected animals, and individuals exposed to human feces through sexual contact (Roxström-Lindquist et al., 2006).

According to Yoder and colleagues (Yoder et al., 2012), the occurrence of giardiasis commonly shows seasonal variability in which the number of cases are higher during summer and early autumn in temperate countries. It is probably due to the increased of outdoor activities during warm season which leads to the exposure of contaminated waters. In the United States, the reported cases of giardiasis are higher in northern states as compared to the south of the city. This is most likely due to the increase of cyst viability in colder climates (Yoder et al., 2010).

Giardiasis also shows a bimodal age-related distribution in which most of the cases occurred among children under ten and adults aged between 25 and 44 years of age (Yoder et al., 2012). This is probably caused by the differences in the immune status or the frequency of exposure among different age groups. The higher cases of *Giardia* infection among children under ten are probably caused by the poor sanitary practices in day care centers (Mohamed et al., 2008). Giardiasis is higher in males as compared to females. This is probably due to the sexual transmission among homosexual men practicing anal-oral sexual (Yoder et al., 2012).

1.3 Host-parasite interaction

Giardiasis is one of the major gastrointestinal diseases throughout the world which is caused by the protozoan parasite *Giardia intestinalis*. It is the most common protozoan infection that causes 280 million cases annually. This disease affects human especially children, and seen mostly in developing and industrialized countries (Yoder et al., 2012).

The interaction between *Giardia* trophozoites and the intestinal epithelial cells (IECs) of the host have been characterized *in vitro* cell culture systems and *in vivo* (rodent) models (Dreesen et al., 2014). The mechanism of the disease is still poorly understood, however it is thought to be multifactorial due to the parasite genetic makeup, the host age, gender, nutritional and immune status as well as the presence of other diseases or infections (Cotton et al., 2011). According to Ankarklev and colleagues (Ankarklev et al., 2010), *Giardia* is not invasive compared to other intestinal parasites and it does not secrete any known toxins.

Cysts are one of the virulence factors for *Giardia* infection. Cysts can survive for several weeks in the environment especially in fresh waters, untreated wastewaters and agricultural run-off from manure-fertilized fields (Plutzer et al., 2010). Other virulence factors are the trophozoite adhesive disk, flagella and the variant surface proteins (VSPs). These trophozoites structures are important for their attachment to the host cell, and to keep them from being excreted by the complete clearance of human immune system (Ringqvist, 2009).

Giardia infection was acquired through the ingestion of cysts. The emerging trophozoites of *Giardia* will inhabit and attach to the mucosal surface of host's small intestine and replicate by binary fission. The shortening of microvilli and inhibition