COMPARISON OF THE SENSITIVITIES OF PCR METOHDS FOR GENOTYPING OF *GIARDIA DUODENALIS*

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ABSTRACT

Giardia duodenalis is a unicellular organism that infects the small intestine of humans and a variety of other mammalian hosts. The detection of this organism is usually based on microscopic methods following the application of fecal concentration techniques. The development of immunofluorescence microscopy has generally improved the sensitivity of detecting and quantitating fecal *Giardia* cysts compared to conventional microscopy. However, neither traditional microscopic methods nor immunofluorescence microscopy can discriminate between morphological identical, or similar, organisms that are genetically different. Thus, molecular methods, particularly PCR-based techniques, have been developed as diagnostic methods to solve this problem. To date, several techniques for DNA extraction and PCR amplification have been published; however, comparisons of their sensitivities have not been conducted.

An evaluation of the sensitivities of three DNA extraction methods (i.e. FTA filter paper, QIAamp stool mini kit, and conventional phenol/chloroform method) by using fecal specimens with known concentration of *G. duodenalis* cysts was performed. FTA filter paper was the most effective method, which could detect *G. duodenalis* in fecal specimens with the concentration of at least 0.6 cysts/PCR reaction mixture. The sensitivities of 5 previously described PCR protocols, using five different genotyping primer sets, were also compared in *Giardia* DNA derived from both trophozoites and cysts. The results showed that RH11/RH4, GiarF/GiarR primer set that amplified SSU-rRNA gene of this organism was the most sensitive primer. A blind diagnostic test to compare PCR and immunofluorescent assay for the detection of *G. duodenalis* in stool specimens was also conducted. FTA filter paper for DNA extraction together with the PCR method using the primer set RH11/RH4, GiarF/GiarR primer set showed 97.30% sensitivity and 100% specificity for the detection of *G. duodenalis* in stool specimens, while the immunofluorescent assay gave a sensitivity of 91.90% and a specificity of 100%. There were no statistically significant differences (p=0.61) between these two methods.

KEY WORDS: *GIARDIA DUODENALIS*/ PCR/ IMMUNOFLUORESCENT ASSAY

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การเปรียบเทียบความไวของ PCR วิธีต่างๆในการแยกสายพันธุ์ของเชื้อจิอาร์เดียดูโอดีนาลิส (COMPARISON OF THE SENSITIVITIES OF PCR METOHDS FOR GENOTYPING OF *GIARDIA DUODENALIS*)

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บทคัดย่อ

Giardia duodenalis เป็นโปรโตซัวเซลล์เดียวที่อาศัยอยู่ภายในลำไส้เล็กของมนุษย์และสัตว์เลี้ยงลูกด้วยนมหลายชนิด การวินิจฉัยโรคซึ่งเกิดจากเชื้อชนิดนี้โดยทั่วไปยังใช้วิธีการตรวจด้วยกล้องจุลทรรศน์ การพัฒนากล้องจุลทรรศน์ชนิดฟลูออเรสเซ้นเพื่อนำมาใช้สำหรับการวินิจฉัยโรคสามารถเพิ่มความไวในการตรวจวินิจฉัยได้ อย่างไรก็ตามทั้งสองวิธีดังกล่าวไม่สามารถใช้จำแนกสายพันธุ์ของเชื้อชนิดนี้ได้ จึงมีการพัฒนาวิธีการเพิ่มสารพันธุกรรมขึ้นเพื่อแก้ปัญหาดังกล่าว แม้ว่าวิธีการเพิ่มสารพันธุกรรมและวิธีการสกัดสารพันธุกรรมหลายวิธีได้ถูกพัฒนาขึ้นสำหรับการตรวจหาเชื้อชนิดนี้ แต่ยังไม่เคยมีการศึกษาเปรียบเทียบความไวของวิธีการเหล่านี้

งานวิจัยนี้มีจุดประสงค์เพื่อเปรียบเทียบความไวของวิธีสกัดสารพันธุกรรม 3 วิธี ได้แก่ ชุดสกัดสารพันธุกรรมชนิดสำเร็จรูป 2 ชนิด คือ FTA assay และ QIAamp stool mini kit และวิธีสกัดสารพันธุกรรมแบบดั้งเดิมด้วยสารฟีนอลคลอโรฟอร์ม จากการทดลองพบว่าวิธี FTA assay มีความไวที่สุด โดยสามารถสกัดสารพันธุกรรมของเชื้อในตัวอย่างอุจจาระได้ถึงแม้ว่ามีเชื้อเพียง 0.6 cysts เท่านั้น เนื่องจากความไวสูงของวิธี FTA assay ดังนั้นวิธีนี้จึงถูกนำมาใช้ในการประเมินความไวของของไพรเมอร์ในการสังเคราะห์สารพันธุกรรมของเชื้อในตัวอย่างอุจจาระและเชื้อจากการเพาะเลี้ยงในห้องทดลอง พบว่าไพรเมอร์ชุด RH11/RH4, GiarF/GiarR ซึ่งใช้จำเพาะกับ SSU gene เป็นไพรเมอร์ที่ให้ผลดีที่สุด และยังสามารถนำไปใช้วิเคราะห์หาสายพันธุ์ของเชื้อได้อีกด้วย นอกจากนี้ในการศึกษาครั้งนี้ได้เลือกวิธีการสกัดสารพันธุกรรมและไพรเมอร์ที่มีความไวสูง เพื่อนำมาใช้ในการเปรียบเทียบความไวและความจำเพาะระหว่างวิธีการเพิ่มปริมาณสารพันธุกรรมและวิธีกล้องจุลทรรศน์ชนิดฟลูออเรสเซ้น จากการศึกษาพบว่าวิธีการเพิ่มปริมาณสารพันธุกรรมมีความไวสูงถึง 97.30% และมีความจำเพาะ 100% ในขณะที่วิธีวิธีกล้องจุลทรรศน์ชนิดฟลูออเรสเซ้นมีความไว 91.9% และมีความจำเพาะ 100% เช่นเดียวกัน ซึ่งทั้งสองวิธีไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (p=0.61)

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LIST OF ABBREVIATIONS

/	per
%	Percent
bp	Base pair
ºC	Degree Celsius
CIE	Counterimmunoelectrophoresis
DFA	Direct fluorescence antibody
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ef	Elongation factor
ELISA	Enzyme-linked immunosorbent assay
et al.	*et. alii* (Latin), and others
g	Acceleration gravity
g	Gram
GDH	Glutamate dehydrogenase
h	Hour
i.e.	id est (Latin), that is
Ig	Immunoglobulin
IFA	Immunofluorescent assay
kDa	KiloDalton
kg	Kilogram
l	Liter
mg	Milligram
min	Minute
ml	Milliter
mm	Millimeter
mM	Millimolar
µl	Microliter

LIST OF ABBREVIATIONS
(continued)

µm	Micrometer
ng	Nanogram
nm	Nanometer
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	Picogram
sec	Second
SDS	Sodium dodecyl sulfate
SSU	Small subunit
TBE	Tris/borate/EDTA buffer
TPI	Triosephosphate isomerase
UV	Ultra violet
vol	Volume

CHAPTER I
INTRODUCTION

Giardia is an intestinal flagellate that infects a broad range of vertebrate hosts. Based on the morphology and ultrastructure of their trophozoites, the genus currently comprises of six species, namely: *Giardia agilis, Giardia ardeae, Giardia duodenalis, Giardia microti, Giardia muris, and Giardia psittaci* (1). However, *Giardia duodenalis* (syn. *G. intestinalis, G. lamblia*) is the only species found in humans. It is also found in other mammals, including pets and livestock. This protozoan produces environmentally resistant cysts, which are voided in the feces of infected host and transmitted directly through fecal/oral contact, or by ingestion of contaminated water and food (2).

Giardia duodenalis has global distribution causing an estimated 2.8×10^8 cases per annum, and is the most common intestinal parasite of humans in developed countries. Approximately 200 million people in Asia, Africa and Latin America suffer from symptomatic giardiasis, with some 500,000 new cases each year (3). In Thailand, the prevalence of giardiasis has been studied in different groups of population such as school students and children in an orphanage. The prevalence in these groups of population varies from 2.21 to 37.7% (4-7).

The diversity within the *G. duodenalis* morphological group has been demonstrated by comparing data on the antigens, isoenzymes, and DNA sequences from isolates of human and animal origin, which were propagated either *in vitro* (axenization) or *in vivo*. However, it is well known that axenization is a highly selective process and many isolates cannot be cultured. Therefore, PCR-based methods for direct typing of *Giardia* cysts present in fecal and environmental samples have been developed. These methods eliminate the need for *in vitro* culture and allow unbiased characterization of genotypes that occur in nature (3). From previous studies, *G. duodenalis* have been classified at least into 7 assemblages (A to G) using PCR-based methods to amplify the conserved genetic loci such as rDNA and a variety of housekeeping genes including those coding for glutamate dehydrogenase (GDH),

elongation factor 1-alpha (ef1-α), triosephosphate isomerase (TPI). Those results have revealed that only assemblages A and B are detected in humans. However, these two assemblages are also found to infect a wide range of other mammalian hosts. On the contrary, the assemblages C to G are likely to be host-specific (8-14).

The PCR amplification using stool specimens for the detection of *G. duodenalis* could be insensitive because of PCR inhibitors and the difficulty of cyst disruption. To raise the sensitivity of PCR to detect *G. duodenalis* infection, an effective DNA extraction method is needed. Commercial DNA extraction kits such as the QIAamp stool mini kit (QIAGEN, Hilden, Germany), DNeasy Tissue Kit (Qiagen, Valencia, CA), and FTA filter paper (Whatman Bioscience, Cambridge, United Kingdom) have been used for isolation of *Giardia* DNA. However, these DNA extraction methods have never been compared. Thus, the efficiency of three DNA extraction methods, i.e., conventional phenol/chloroform extraction, FTA filter paper assay, and QIAamp stool mini kit were evaluated in this study.

In recent years, many PCR-based techniques for differentiating *G. duodenalis* of human origin and *G. duodenalis* of animal origin have been developed. These techniques are based on the polymorphic nature of *G. duodenalis* that infects humans and most animals at the small-subunit (SSU) rRNA, glutamate dehydrogenase (GDH), elongation factor1-alpha (ef1-α), triosephosphate isomerase (TPI), and β-giardin sequences (2,8-17). However, the comparative performance of these genotyping tools has not been evaluated. In this study, five genotyping protocols (2,8-14) for detection and differentiating *G. duodenalis* were compared. Moreover, the sensitivities and specificities of the most sensitive PCR method using the most sensitive DNA extraction method were compared with results obtained from immunofluorecent assay (IFA).

THE SPECIFIC OBJECTIVES

1. To compare the sensitivities of genotyping primers previously described for *G. duodenalis*

2. To compare the efficiencies of three DNA extraction methods including conventional phenol/chloroform extraction, FTA filter paper assay, and QIAamp stool mini kit for PCR amplification of *G. duodenalis*

3. To compare the sensitivities and specificities of PCR amplification with direct immunofluorescent assay for the detection of *G. duodenalis*

CHAPTER II
LITERATURE REVIEW

1. *GIARDIA*

1.1 Taxonomy

1.1.1 Currently recognized species

During the first half of 20th century, the taxonomy of *Giardia* at the species level was complicated and confused. Numerous species of this organism had been described, mainly on the basis of host occurrence rather than differences in morphology (18,19). Until 1952, Filice proposed that species of *Giardia* could be divided into only three morphologically distinct groups. Differentiation was based primarily on the shape of the median bodies, as well as body shape and length (Figure 1 and Table 1) (20). The first group of organisms, *G. duodenalis*, has pyriform shaped trophozoites which possess a distinctive claw-hammer median body and cause disease in a variety of mammals, including humans. Members of the second group, *G. muris*, have rounded median bodies, a rounder trophozoite shape, and primarily infect rodents. Trophozoites of the third group, *G. agilis*, are characterized by long, narrow bodies with relatively short adhesive discs, long, club-shaped median bodies, and have only been isolated from amphibians (20).

In addition to the three species described by Filice (1952), two additional species occurring in birds have been proposed by using ultrastructural features identified by scanning electron microscopy (1,20,21). The first species, *G. psittaci*, was described from bugerigars (*Melopsittacus undulatus*). Trophozoites of *G. psittaci* are morphologically similar to *G. duodenalis*, i.e. possessing claw-hammer median bodies and pyriform trophozoite shape, but they lacked the ventrolateral flange and a marginal groove which is a channel formed by the flange (20,22). Another species, *G. ardeae*, share morphological features with *G. duodenalis* and *G. muris* (23). The trophozoites of *G. ardeae* have been observed to possess either rounded or claw-hammer median bodies, although the orientation of the claw-hammer is different from that observed for *G. duodenalis*. The shape of *G. ardeae* trophozoites is similar to *G.*

duodenalis trophozoites. However, other features such as the size of the ventral disc, a notch in the caudal region of the ventral disc, and a single caudal flagellum are similar to *G. muris* trophozoites. The nuclei of *G. ardeae* are long, slender and tear-dropped in shape, unlike those of either *G. duodenalis* or *G. muris*, which are rounded (20,23).

An additional species, *Giardia microti*, has been described on the basis of cyst morphology (20,21,24). The cysts of *G. microti* contain two differentiated trophozoites with mature ventral discs, whereas the cysts of *G. duodenalis* contain a single trophozoite which has four nuclei and lacks a ventral disc. Although *G. microti* was originally isolated from prairie vole (*Microtus ochrogaster*), it has also been recovered from other rodents (25). The recognized species and host of *Giardia* species are summarized in Table 2 (21).

1.1.2 Variation in *G. duodenalis*

Although Filice (1952) proposed that species of *Giardia* could be divided into only three morphological groups. He was also aware that the *G. duodenalis* morphological group contained forms that varied phenotypically in characteristics other than morphology (20).

Until recently, it has been difficult to interpret the extensive genetic variability within *G. duodenalis* in a taxonomic perspective. Although the ability to axenize and amplify isolates of *G. duodenalis* in laboratory culture can provide sufficient material for genetic characterization using procedures such as allozyme electrophoresis, not all isolates of the parasite can be established in axenic *in vitro* culture. This includes a significant proportion of human isolates. Isolates from some species such as dogs are also largely refractory to *in vitro* culture. As a result, it has not been possible to genetically characterize many isolates of *G. duodenalis*. Therefore, much of the available genetic data were based on a small pool of culture-selected isolates that could not be considered to be representative of the extensive gene pool existing in nature (3).

The recent application of PCR-based procedure has eliminated the need for *in vitro* culture and amplification. This procedure can directly characterize the parasite from faecal and environmental samples, and enable the characterization of previously inaccessible genotypes (8,26). Using PCR-based procedure in association with analysis of conserved genetic loci such as rDNA and a variety of house keeping genes

including that coding for glutamate dehydrogenase (GDH), elongation factor 1-apha (ef1-α), and triosephosphate isomerase (TPI) combined with much larger data sets, it has been possible to elucidate the fundamental genetic divisions within the *G. duodenalis* group (Table 3) (2,3,18). This shows that *G. duodenalis* is not a uniform species but it is a species complex comprising a variety of genetically and phenotypically distinct, yet morphologically similar genotypes which also exhibit differences in host specificity (3).

Giardia isolates recovered from humans fall into one of the two major genotypic grouping or assemblages (Table 3). These two assemblages comprising of several genetic subgrouping are now widely known as Assemblage A and Assemblage B. Assemblage A consists of isolates that can be grouped into two distinct clusters; AI consists of a mixture of closely related animal and human isolates which are geographically widespread, and most attention regarding the zoonotic potential of *Giardia* has focused on this AI subgroup. In contrast, the second group, AII consists entirely of human isolates. Assemblage B comprises of two subgroups, BIII and BIV. Subgroup BIV appears to be human-specific (3,27).

Some of the genotypes, or assemblages, are genetically quite uniform and appear to be confined to specific animal hosts. *Giardia* genotypes that exhibit a limited host range include genotypes recovered from cats, dogs, rats, voles/muskrats and hoofed animal (Table 3). In addition of these available assemblages, the two novel *Giardia* genotypes have also been reported in quenda (*Isoodon obesulus*) and Tasmanian devil in Australia (28,29).

1.2 Biology

1.2.1 Morphology

The trophozoite of *Giardia duodenalis* is half-pear-shaped (with one flat and one rounded surface) with a broad anterior and a narrow posterior. Its size is approximately 12 to 15 μm long and 5 to 9 μm wide (Figure1). The ventral side of trophozoite is occupied by the unique adhesive disc, whereas there are no apparent features on the dorsal surface of the parasites (1,30). It has four pairs of flagella (anterior, posterior, caudal, and ventral) and a pair of distinctive median bodies. Although their function is unknown, median bodies are unique to *Giardia* and are comprised mainly of microtubules (30). The two trophozoite nuclei are nearly

identical in appearance. Both of them are transcriptionally active, and replicate at approximately the same time. *Giardia* trophozoite has a simple intracellular organization, with no mitochondria or peroxisomes. Golgi complexes become visible in encysting trophozoites but have not been confirmed to be present in vegetative trophozoites. However, stacked membranes suggestive of Golgi complexes have been demonstrated (1,3,30)

Cyst of *Giardia* can be round or oval shape. Its size is approximately 11 to 14 × 7 to 10 μm (Figure1). It has four nuclei and contains axonemes and median bodies (31). Cyst is covered by a wall that is 0.3 to 0.5 μm thick and composed of an outer filamentous layer and an inner membranous layer with two membranous.

1.2.2 Life cycle

Life cycle of *Giardia* is simple including two major stages which are infectious cyst stage and the intestinal trophozoite stage (1,32). Cyst stage of *Giardia* has a hyaline wall that protect its from extremes of temperature and other environmental stress. This stage is not killed uniformly by chlorination of water, and it also may be resistant to boiling water at high altitude. Cyst stage may survive in water for up to 3 months (33). Infection of a host is initiated when the cyst is ingested with contaminated water or, less commonly, food or through direct fecal-oral contact. After the cyst is ingested and exposed to the acidic environment of the stomach, cyst excysts into trophozoites in the proximal small intestine (1). Excystation yields the release of two motile trophozoites, which replicate by longitudinal binary fission. Trophozoites do not invade the epithelium; instead, they appear to adhere to the epithelial surface by using its unique ventral adhesive disc (30,33). After exposure to biliary fluid, some of the trophozoites encyst in the jejunum. These cysts are passed in the feces of infected host to continue further transmission of the disease (1,33) (Figure 2).

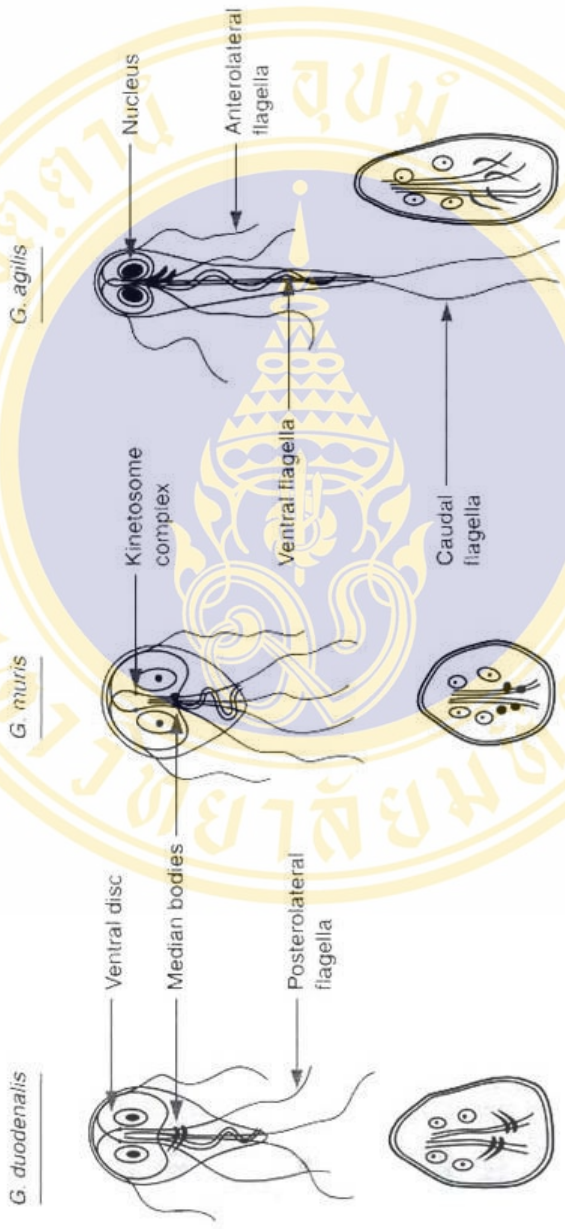


Figure 1. Trophozoite and cyst morphology of *Giardia duodenalis, G. muris* and *G. agilis* (20)

Table 1. Morphological characters used to identify species of *Giardia* (20)

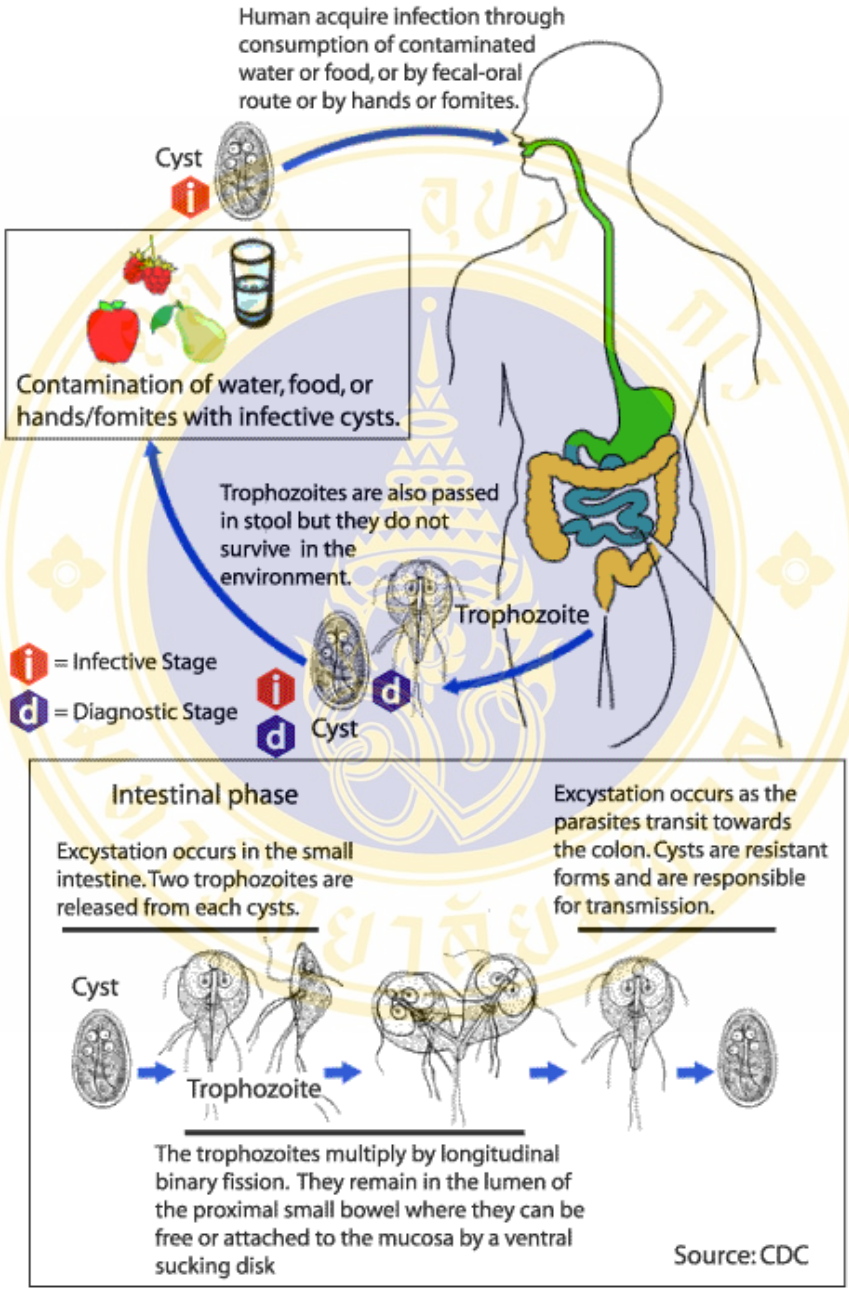
Feature	*G. agilis*	*G. muris*	*G. ardeae*		*G. psicatti*	*G. duodenalis*
Median bodies						
Shape	Long club shaped rods	Small rounded	Small rounded	Claw hammer	Claw hammer	Claw hammer
Location	Anterior third of trophozoite parallel with longitudinal axis	Centre of body	Centre of body	Parallel with longitudinal axis	Transversely across body	Transversely across body
Trophozoite shape	Long, narrow tear drop	Pyriform, short, broad	Pyriform, short/medium		Pyriform, medium	Pyriform, medium
Ventrolateral disk	1/5 of body length	> 1/2 of body length, deeply notched posterior end	> 1/2 of body length, deeply notched posterior end		< 1/2 body length, incomplete ventrolateral flange (posterior end)	< 1/2 body length
Caudal flagellae	Paired	Single	Single		Paired	Paired
Shape of nuclei	Oval	Oval	Tear-drop		Oval	Oval

Table 2. Recognized species and host of *Giardia* species (21)

Species	Host
G. duodenalis (syn. *G. intestinalis*, *G. lamblia*)	Most mammals including humans, livestock, pets
G. muris	Mice
G. microti	Voles, muskrats
G. psicatti	Budgerigars
G. ardeae	Heron, ibis
G. agilis	Frogs

Table 3. Genotype and host range of isolates within the *Giardia duodenalis* morphological Groups (3)

Genotype/Assemblage	Host range
Zoonotic/A	Humans, livestock, cats, dogs, beavers, guinea pig, slow loris
Zoonotic/B	Humans, slow loris, chinchillas, dogs beavers, rats, siamang
Dog/C,D	Dog
Livestock/E	Cattle, sheep, pigs
Cat/F	Cat
Rat/G	Domestic Rats
Muskrats/Vole	Wild rodents



(www.dpd.cdc.gov)

Figure 2. Life cycle of *Giardia*

1.3. Transmission

Transmission of giardiasis is predominantly by fecal-oral contamination. Therefore, levels of infection are highest under conditions of poor hygiene and sanitation, particularly in tropical and subtropical environments. Under such conditions, transmission of giardiasis can be accomplished with only minute quantities of infective material (34). As few as 10 cysts appear to be able to initiate an infection (35). Thus explaining why ingestion of very small amounts of fecal material may be sufficient for transmission of giardiasis (34). Other predisposing factors may enhance the frequency of fecal-oral transmission. For example, conditions conductive to fecal-oral contamination are common in day care centers and high prevalence rates of *Giardia* infection have often been observed in children attending such centers, particularly in those under 3 years of age (36,37).

Another common transmission method is the ingestion of cyst contaminated food. A number of outbreaks of foodborne giardiasis related to food preparation have been documented, probably caused by infected food handlers or contact by food handlers with infected people, particularly children (38,39). For example, an outbreak of giardiasis occurred after a family party for 25 people. Nine people who ate fruit salad at the party became ill. The person who prepared the fruit salad had a diapered child and a pet rabbit at home who were both positive for *G. duodenalis* (40). In 1990, an outbreak among insurance company employees resulted in 18 laboratory-confirmed and nine suspected cases of giardiasis. Raw sliced vegetables served in the employee cafeteria and prepared by a food handler infected with *G. duodenalis* were the probable cause of the outbreak (41). However, no outbreaks of foodborne giardiasis related to industrially manufactured foods have been reported (38).

Numerous cases of *Giardia* infection have been associated with contaminated water and, in the United States, giardiasis is the most frequently diagnosed waterborne disease (34). Waterborne outbreaks of giardiasis have also been reported in Canada (42) and Europe (43,44). The potential for waterborne transmission has been recognized in Australia (34) and in New Zealand surveys have identified *Giardia* in a number of water supplies (45). Waterborne transmission is also a well documented cause of giardiasis in travelers who usually contract infection from drinking local tap water (34). Investigations of endemic waterborne giardiasis in the USA have usually

found the contamination of water supply to have resulted from inadequate water treatment, ineffective filtration, or contamination with human sewage (46,47). Filtration is necessary to remove *Giardia* as chlorination alone is insufficient without high concentrations of chlorine and long contact times (47). However, the fact that human infections with *Giardia* have been tracted to the consumption of water from streams in rural areas away from urban environments has implicated a number of species of wild animals, particularly beavers and muskrats, as reservoirs of infection (34).

Because *Giardia duodenalis* can infect a wide range of mammalians species including humans, this vast assortment of host species has raised great concerns regarding the zoonotic potential of this parasite. However, the evidence for giardiasis being a zoonosis is still limited (21).

1.4 Clinical features

G. duodenalis can produce a spectrum of manifestations, including asymptomatic cyst passage, acute self-limited diarrhea, and a chronic syndrome with diarrhea and malabsorption. However, the majority of infected individuals are asymptomatic (48,49).

In acute infection, symptoms usually begin with a feeling of intestinal uneasiness, followed by nausea and anorexia. Low-grade fever and chills may also be early symptoms. Subsequent symptoms may include explosive, watery, foul-smelling diarrhea; marked abdominal gurgling and distention associated with the passage of foul gas; and perhaps belching, with a foul taste. Other clinical presentations such as upper or mid-epigastric cramps and blood or mucus in the stool may also occur (48,50).

Although the spontaneous clearing may occur in some acute infection, the chronic infection, often with the features of steatorrhea, may develop. Weight loss can be profound under these circumstances with losses of 10% to 20% of the usual or ideal body weight (49). A number of studies reported that intestinal malabsorption occurs in patients with giardiasis with about 50% of symptomatic patients having biochemical evidence of fat malabsorption (51-55). Malabsorption of other nutrients such as vitamin A and vitamin B_{12} have also been reported (51,52). Severe macrolytic anemia because of folate deficiency also occurs (49). Other symptoms such as urticaria,

cholecystitis, and pancreatitis have also been reported with *Giardia* infection. In addition, this chronic infection in children may present as failure to thrive (50).

1.5 Disease mechanism

Pathogenesis of giardiasis is not known but is probably multifactorial. An early hypothesis about pathogenesis suggested that the parasite disturbs the intestine by functioning as a mechanical barrier to absorption (56). This is unlikely because the size of the parasite (~12×5 μm), the infectious dose (10 to 100 cysts) and the generation time (~6-12 h *in vitro*) suggest that the area colonized after six to ten days would be extremely small compared with the intestinal surface, which is estimated to be the size of a tennis court. The *Giardia* genotype has been proposed to be important for induction of symptoms (9,57) but there is no consensus concerning the connection between genotype and virulence (58).

Giardia infections in mice and gerbils damage the mucosal surface by microvillus and crypt atrophy, and affect the activity of digestive enzymes such as lipase, proteases and disaccharidases (56,59,60). However, in 567 human patients with *Giardia*-positive biopsies, only a small fraction (3.7%) showed villus shortening and little inflammation (61). In an experimental infection of ten human volunteers with *Giardia* trophozoites genotype B, infection was established in all subjects, with five developing symptoms (62). Only two of the five symptomatic volunteers showed brush border abnormalities. These results indicate that microvillus shortening and inflammation are not strictly correlated to symptoms and subsequent clearance in human giardiasis (58).

In vitro experiments have indicated that *G. duodenalis* rearranges cytoskeletal F-actin and α-actinin in human duodenal cell and that this reduces transepithelial electrical resistance (63). Furthermore, *in vitro* coincubation showed that *Giardia* induces apoptosis that correlates with the loss of epithelial barrier function via the disruption of tight junction ZO-1 and the subsequent increase in permeability (64). Infection of mice with *Giardia muris* confirmed the F-actin rearrangement and tight-junction disruption, including and increase of small-intestinal permeability (65), but clinical observation in humans did not confirm the tight junction disruption (66).

1.6 Diagnosis

1.6.1 Microscopy

The diagnosis of *Giardia* infection is based on the detection of cysts or trophozoites in feces under light microscopy. Trophozoites survive for relatively brief periods in stools and are usually found only when diarrhea is severe and watery and when the examination can be made soon after the stool has been passed. Cysts can be detected in saline wet mounts or following concentration techniques using formalin-ethyl acetate or zinc sulfate. Cyst preparations should be stained with trichrome or iron hematoxylin. Fluorescent labeled monoclonal antibodies to cyst proteins can be used to aid microscopic detection and to help distinguish *Giardia* cysts from other cysts or cyst-like bodies in stools. Due to the periodic nature of cyst shedding, three fecal specimens which are taken on nonconsecutive days are required for reliable assessment of the *Giardia* appearance (49,67,68).

In some cases unable to be confirmed by stool examinations, examination of fluid from the area of the duodenal-jejunal junction may reveal *Giardia* trophozoites. Fluid can be obtained by endoscopy or, more simply, by using the duodenal string test (Entero-Test). The Entero-Test is a gelatin capsule containing a length of nylon string to which a small weight is attached. On ingestion, the capsule dissolves, the nylon string is released, and peristaltic action pulls the weighted string down into the duodenal area, where the trophozoites attach. After 4 h to overnight, the string is withdrawn and inspected microscopically for trophozoites (31,50).

In an occasional case of giardiasis that connot be confirmed by the above methods, diagnosis may be made by small intestinal biopsy in the area of the duodeno-jejunal junction or from multiple duodenal and jejunal sites. The morphology of the organisms in these presentations may be considerably more distorted than in routine, permanently stained, fecal smears. *G. duodenalis* is rarely recognized as a mucosal invader; therefore, parasites are more likely to be found on the microvillus border, particularly in the crypts (50).

These more invasive approaches to diagnosis may be appropriate when the diagnosis is strongly suspected clinically but when fecal specimens are repeatedly negative.

1.6.2 Immunodiagnosis

Two immunodiagnostic approaches have been used for the diagnosis of giardiasis: the detection of *Giardia* antigens in fecal specimens and serological assay.

1.6.2.1 Fecal Antigen Detection

A variety of immunologic approaches have been used to detect *Giardia* specific antigens in fecal specimens such as enzyme-linked immunosorbent assay (ELISA), immunofluorescent assay (IFA), and counterimmunoelectrophoresis (CIE). However, the most commonly used antigen detection immunoassays for *Giardia* are enzyme-linked immunosorbent assay (ELISA), which detect soluble stool antigens, and immunofluorescent assay, which detect intact organisms in stool specimens (49,69,70).

One of the most interesting *Giardia*-specific antigens identified by ELISA is the GSA 65 (20,71,72). GSA 65 is the glycoprotein identified in cysts and trophozoites of *Giardia*. It has an approximate molecular mass of 65 kDa. This antigen is the main antigen found in the feces of individuals infected with *G. duodenalis* and very specific to this parasite (71- 73). The ELISA-GSA 65 is available commercially as a kit, and its sensitivity and specificity are comparable to those of microscopic examination for cysts in the stool (73,74). Many studies with the ELISA-GSA 65 have reported a greater sensitivity of the immunodiagnosis assay over the microscopic examination of a single specimen (73,75-78). The sensitivity of the assay varies between 95 and 100%, and 100% specificity has been reported when it was used with stool from patients infected with other intestinal parasites (78,79). It has also been reported that the ELISA-GSA 65 can detect *Giardia* infection in at least 30% more cases than the microscopic examination (73).

The stool ELISA is a useful tool. It may especially be helpful as an epidemiologic tool for mass screening of multiple stools from multiple patients, as in the day care center setting, or for controlling outbreaks of giardiasis. It is also useful in the diagnosis of giardiasis in patients who have three negative stool microscopic examinations (80).

Another widely used immunodiagnosis for *Giardia* detection is Immunofluorescent assay. This technique utilizes fluorescein-labeled antibodies against cell wall antigens of *Giardia* cysts and allows visualization of intact parasites

under fluorescence microscopy, providing a definitive diagnosis (69). Nowadays, commercial kits for immunofluorescent assay are available. The examples of these kits are MERIFLUOR DFA (Meridian Bioscience, Cincinnati, Ohio, USA) and *Giardia*-CEL IF (Bradsure Biologicals, Leicestershire, UK) (81,82). The sensitivity and specificity of this technique have been compared to those of microscopic examination for cysts in stool specimens (81,83-87). Several studies have reported a greater sensitivity of immunofluorescent assay over the microscopic examination (81,83-87). The sensitivity of this assay varies between 99.2% and 100% and its specificity varies between 98.5 and 100% (84-87). Furthermore, the study of Morimoto *et al.* indicated that immunofluorescent assay is a useful tool for detecting *G. duodenalis* cysts in fecal samples that have undergone long-term storage (88) and this technique is also proved to be an effective technique for the detection of *Giardia* cysts in frozen stool samples (89).

1.6.2.2 Serological Assay

Detection of anti-*Giardia* antibodies in serum has not proven to be a major contributor to diagnosis (33,49). Anti-*Giardia* IgG is found in infected and noninfected individuals in endemic areas, presumably because of continuous exposure to the parasite. Thus, it is unhelpful in distinguishing past from present infection (48,49,90). However, specific anti-*Giardia* IgM titers are usually increased only in individuals with ongoing infection, with antibody concentrations falling rapidly once infection has been cleared (62,91). Anti-*Giardia* IgM has been shown to be useful in identifying individuals with acute giardiasis even in endemic areas such as India and The Gambia (92). Approximately one third of patients develop specific serum anti-*Giardia* IgA response (92), which similar to IgM is relatively short-lived and may be of value in the diagnosis of ongoing infection in those patients with raised IgA titers. A negative serologic test does not exclude infection (21).

1.6.3 Molecular Methods

A number of molecular techniques including PCR-based procedure have been developed for detection of giardiasis. PCR-based methods have greater sensitivity and specificity than conventional diagnostic methods that rely on microscopy and/or immunodiagnosis. In addition to their excellent sensitivity and specificity, such procedures are quick and amenable to large sample throughput.

Another advantage of this type of molecular technique is that it can provide information on the genotype or species of *Giardia* parasite. Because of many excellent advantages of this procedure, many primers that amplify conserved genetic loci such as rDNA and a variety of housekeeping genes have been developed for *Giardia* detection (3).

A polymerase chain reaction-based method for genotyping *G. duodenalis* isolates using a polymorphic region near the 5' end of the small subunit ribosomal RNA gene was described by Hopkins *et al*. (8). The specificity of this primer pair, RH11 and RH4, was tested with human DNA, human fecal DNA, bacterial species including *Escherichia coli, Staphylococus aureus* and *Serpulina hyodysenteriae,* and protozoa DNA isolated from *Giardia*, *Cryptosporidium parvum*, and *Trichomonas foetus*. A positive amplification was shown only with *Giardia* template. The specificity of the PCR reaction was further confirmed by probing the amplification products with an internal *Giardia*-specific SSU-rDNA oligonucleotide, RH12. The probe hybridized only the 292-bp PCR product present in the lanes containing *Giardia* DNA. The authors also reported that this primer pair can reveal 4 different genetic groups of *G. duodenalis* (groups 1-4) from 13 human and 9 dog isolates. In 2002, Read *et al*. developed a nested PCR procedure that amplify a region of the SSU-rRNA gene using the primer RH11 and RH4 as previously described by Hopkins *et al*. (8) and the newly designed primer pair GiarF and GiarR (9).

Besides the primers that amplify the SSU-rRNA gene, other primers used to amplify a variety of genetic loci such as glutamate dehydrogenase (GDH), elongation factor 1- alpha (ef1-α), β-giardin, and triosephosphate isomerase (TPI) genes are also utilized for detection and genotyping of *Giardia* parasite (2,10-14).

To detect *Giardia* by using PCR-based procedure, the important step is the DNA isolation. Due to the difficulty of lysing cysts and the large amount of inhibitory substances present in fecal specimens (82), many protocols of *Giardia* DNA isolation are developed. *Giardia* DNA can be extracted from stool samples or *in vitro* cultures by conventional procedures such as freeze/thaw cycles, proteinase K digestion or lysis buffer incubation followed by phenol/chloroform extraction, and ethanol precipitation (8,93-95). Moreover, many commercial kit developed for *Giardia* DNA extraction are also widely used, for example, QIAamp stool mini kit (QIAGEN, Valencia, CA,

USA), DNeasy Tissue Kit (QIAGEN, Valencia, CA, USA), and Puregene kit (Gentra systems, Minneapolis, MN,USA) (96-99).

1.7 Treatment

There are several medications approved or commonly used to eradicate the organism in the intestine for the treatment of giardiasis, for example, metronodazole, quinacrine, albendazole, and nitazoxanide.

Metronidazole (Flagyl®), the nitroimidazole derivative, is the mainstay drug for giardiasis treatment (100). Metronidazole utilizes the anaerobic pathways present in *Giardia*. This drug enters the trophozoite, and once within cell, electron transport protein ferredoxins of the parasite donate electrons to the nitro group of the drug. The drug becomes activated by reduction of this nitro group. Reduced metronidazole serves as a terminal electron acceptor which binds covalently to DNA macromolecules. This results in DNA damage in form of loss of helical structure, impaired template function, and strand breakage, with subsequent trophozoite death. In addition to this effect, metronidazole inhibits trophozoite respiration. The reductive activation of metronidazole may also lead to toxic radicals, which react with essential cellular components (67).The recommended dosages of metronidazole are 250 mg three times a day for 5-7 days for adults and 5 mg/kg three times a day for 5-7 days for children (67,101). Metronidazole has been shown to be slightly less effective than quinacrine, but it is generally more tolerated (22). The most common side effects of this drug include headache, vertigo, nausea, and a metallic taste in the mouth. The patient should avoid alcohol while under treatment with this drug. Besides metronidazole, the single dose of other nitroimidazole derivative including tinidazole, ornidazole, and secnidazole are also used for giardiasis treatment.

Quinacrine (Atabrine®) was first introduced as an antimalarial agent in 1930 and became the antimalarial of choice for allied troops in World War II because of its greater availability and better tolerance compared with quinine. After the world war, it soon became an important agent against *G. duodenalis*. The mechanism of action of quinacrine is not completely understood. The antiprotozoal activity of this drug may involve the binding of this agent to *Giardia* DNA. The dosages are 100 mg three times a day for 5-7 days for adults and 2 mg/kg three times a day for children. Quinacrine-

associated side effects include headache, dizziness, nausea, vomiting, and yellow or orange discoloration of skin and mucous membrane (67).

Alternative anti-*Giardia* drugs include albendazole (Albenza®) and Nitazoxanide (Alinia®). The adult dosage of albendazole is usually 400 mg per day for 5 days and usual dosage in children is 15 mg/kg per day for 5 to 7 days (67). The excellence activity *in vitro* against *G. duodenalis* of albendazole has been reported. Albendazole had a lower IC_{50} values compared with that of metronidazole in adherence assay (102). In an ultrastructural analysis of the effects of albendazole on *Giardia* trophozoites, parasite morphology was distorted and attachment was impaired. It also mimicked the effect of natural cell death by causing microtubules and microribbons to scatter, trophozoites to swell, and the ventral disk to fracture (32). Albendazole has also been found to be effective in clinical trials when given for 5 or more days (67). Nitazoxanide (Alinia®) has broad-spectrum antiparasitic activity. It is active against many intestinal protozoa and helminthes and possibly some anaerobic bacteria. In United States, this drug has been approved for the treatment of diarrhea caused by *Giardia* and *Cryptosporidium* in children aged 1-11 years (101).

Other alternative anti-*Giardia* therapeutic agents such as furazolidone and paromomycin are also available. Although, there are several drugs providing for giardiasis treatment, the report about the developing of resistant organism has emerged (103). For this reason, the diverse classes of new drugs are clearly needed (67).

1.8 Prevention

The prevention of giardiasis including general hygiene principles such as hand washing before handling food, after using the toilet, after changing diaper, and after any contact with animals or their feces. The potentially contaminated water including recreational water, untreated drinking water from shallow wells, lakes, rivers, ponds, and streams should not be drinking. However, if using or drinking water that may be contaminated cannot be avoided, the water should be treated by heating it to a rolling boil for at least 1 minute or filtering the water through the filter that has a pore size of at least 1 μm. If water cannot be filtered or boiled, it can also be treated with chlorination or iodination. However, the efficiency of these chemical methods is less against *Giardia* than boiling or filtering because they are dependent on the

temperature, pH, and cloudiness of the water. Eating hot, cooked foods is also recommended to prevent infection from *Giardia* (50,104).

CHAPTER III
MATERIALS AND METHODS

1. Sources of *Giardia*

1.1 Axenic *G. duodenalis* trophozoites

Axenic *G. duodenalis* trophozoites provided from the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University were used for DNA extraction using QIAamp stool mini kit (QIAGEN, Germany) to determine the sensitivities of selected primers.

1.2 Fecal samples

A survey of intestinal parasitic infections was performed in 317 army personels, War Dog Center, Department of Veterinary, the Royal Thai Army at Nakhon Ratchasima province in June, 2005. Stool specimens were examined for *G. duodenalis* under light microscopy using wet preparations and floatation technique at Department of Parasitology, Phramongkutklao College of Medicine. Positive *G. duodenalis* cysts were concentrated by floatation technique, and followed by cyst counting. This concentrated cysts was used for determining the sensitivities of selected primers and the efficiencies of DNA extraction methods.

1.3 Preparation of concentrated *G. duodenalis* cysts for DNA extraction

Approximately 2 g of fresh stool were added into 2 ml of saturated sodium nitrate and mixed until homogenized. The mixture was filtrated with double layer of gauze. Then, the filtrate was added with 20 ml of saturated sodium nitrate and incubated at room temperature for 20 min. The surface of mixture was transferred into a centrifuged tube and 12 ml of phosphate buffer saline (PBS) was added to the mixture. The mixture was centrifuged at 500-1000 *g* for 10 min. The sediment was mixed with 12 ml of phosphate buffer saline until homogenized and centrifuged at 500-1000 *g* for 10 min. This step was repeated at least 3 times. Finally, the sediment from centrifugation was dissolved in 1.5 ml of phosphate buffer saline and used for cyst counting.

1.4 Cyst counting

G. duodenalis cyst counting was performed by using hemocytometer. Approximately 10 µl of dilute specimen were transferred to a hemocytometer and the number of cysts was determined by counting cysts in the W area of hemocytometer under the light microscope at x40 magnification. Cyst counting was done on 8 areas of the W area. The mean of cysts on 8 areas was calculated to obtain the number of cysts per ml. The specimen was kept at 4 ºC for the comparison of the efficiencies of different DNA extraction methods and the sensitivities of selected primers.

2. Determination the sensitivities of genotyping primers to detect *G. duodenalis* using trophozoite DNA

To determine the sensitivities of genotyping primers in *G. duodenalis* trophozoite DNA, genomic DNA was extracted from *G. duodenalis* axenic trophozoites by using QIAamp stool mini kit (QIAGEN, GERMANY). After extraction, the concentration of trophozoite DNA sample was determined by UV absorption at 260 nm. The different concentrations of *G. duodenalis* trophozoite DNA were performed by 1:10 serial dilution (100,000 , 10,000 , 1,000 , 100 , 10 , 1 pg/µl, respectively). These different concentrations of trophozoite DNA were used for PCR amplification using different primer pairs. The most sensitive primer pair was defined as the primer pair that can amplify DNA of *G. duodenalis* at the lowest trophozoite DNA concentration.

3. Evaluation of DNA extraction methods using known number of cysts

To determine the efficiencies of the three DNA extraction methods i.e. FTA filter paper (Whatman Bioscience, UK), QIAamp stool mini kit (QIAGEN, Germany), and conventional phenol/chloroform method, known number of *G. duodenalis* cysts were used. After counting, the sample was 1:5 serially diluted to obtain varying concentration of cysts i.e. 16,842 , 3,368 , 674 , 135 cysts/ml, respectively. In addition, 1:2 serial dilution of sample containing 674 cysts/ml was performed (337 and 168 cysts/ml). These solutions containing different number of cysts were used for three DNA extraction methods. Since only 200 µl of each dilution were used for DNA isolation by QIAamp stool mini kit and conventional method and only 10 µl of each dilution was used as template in PCR amplification, the number of cysts per PCR reaction were 168 , 34 , 7 , 3 , 2 , 1.3 cysts/reaction, respectively. Because only 15 µl of each dilution was used for DNA extraction by FTA and only one piece from four

pieces of the filter was used in PCR amplification, hence the number of cysts per PCR reaction were 63 , 13 , 3 , 1.3 , 0.6 cysts/reaction, respectively. The most efficient extraction method was defined as the method that can extract DNA from the lowest cyst numbers and give positive band of *G. duodenalis* in PCR amplification using the primers (RH11, RH4 and GiarF, GiarR) (8) which were the most sensitive primers for *G. duodenalis* trophozoite DNA.

4. Determination the sensitivities of genotyping primers to detect *G. duodenalis* using cyst DNA

To determine the sensitivities of genotyping primers in *G. duodenalis* cysts DNA**,** the different numbers of cysts performed by 1:5 and 1:2 serial dilutions were used for DNA extraction. The *G. duodenalis* cyst DNA was extracted by using FTA filter paper assay which is the most efficient method for extraction DNA from *G. duodenalis* cysts. Genomic DNA from *G. duodenalis* cysts was used as template for PCR amplification using different genotyping primers. The most sensitive primer pair was defined as the primer pair that can amplify DNA of *G. duodenalis* at the lowest numbers of cyst.

5. Molecular techniques

5.1 DNA extraction methods

5.1.1 FTA filter paper

A six-mm diameter disks were punched out from FTA filter paper (Whatman Bioscience, UK) and placed in a 1.5 ml microcentrifuge tubes. Fifteen µl of samples were applied onto both sides of FTA disk, and the filters were dried at room temperature. The disks were cut into 4 pieces and only one piece of them was used in a PCR amplification. FTA disks were washed twice with 200 µl of FTA purification buffer (Life technologies, Gaitherburg, MD.) for 15 min, followed twice with 200 µl of 10 mM Tris (pH 8.0) containing 0.1 mM EDTA for 5 min and again air-dried at room temperature. These washed filters were used directly as the source of DNA template for PCR amplification.

5.1.2 QIAamp stool mini kit

The *G. duodenalis* DNA was prepared by using QIAamp DNA stool mini kit (QIAGEN, Germany) following the manufacturer's instruction. Two hundred microliters of each stool sample was added with 1,400 µl of buffer ASL and vortexed

continuously for 1 min. The suspension was heated for 5 min at 70 ºC and vortexed again for 15 sec. The suspension was centrifuged at 20,000 *g* for 1 min to sediment stool particles. One thousand and two hundred microliters of the supernatant was transferred into 2 ml microcentrifuge tube and the pellet was discarded. The sample was added with 1 inhibitEX tablet and then vortexed immediately and continuously for 1 min. The suspension was incubated for 1 min at room temperature to allow inhibitors to adsorb to the inhibitEX matrix. The sample was centrifuged at 20,000 *g* for 3 min to pellet inhibitors bound to inhibitEX. All the supernatant was transferred into 1.5 ml microcentrifuge tube, re-centrifuged at 20,000 *g* for 3 min. Two hundred µl of supernatant from the last centrifugation was added into the 1.5 ml microcentrifuge tube containing 15 µl of Proteinase K. Then, 200 µl of buffer AL was added to the mixture. The mixture was vortexed for 15 sec and incubated at 70 ºC for 10 min. Two hundred µl of absolute ethanol was added into the lysate and mixed again by vortexing. The complete lysate was transferred into the QIAamp spin column without moistening the rim. The QIAamp spin column was centrifuged at 20,000 *g* for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube and the tube containing the filtrare was discarded. The QIAamp spin column was opened and added with 500 µl of buffer AW1 and centrifuged at 20,000 *g* for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube. The collection tube containing the filtrate was discarded. The QIAamp spin column was opened and added with 500 µl of buffer AW2 and re-centrifuged at 20,000 *g* for 3 min. The collection tube containing the filtrate was discarded. The QIAamp spin column was transferred into 1.5 ml microcentrifuge tube and added with 200 µl of buffer AE directly onto the QIAamp membrane. The QIAamp spin column was incubated for 1 min at room temperature and then centrifuged at 20,000 *g* for 1 min to elute DNA. The extracted DNA of each sample was frozen at -20 ºC until used.

5.1.3 Conventional phenol/chloroform method

Two hundred microliters of floated stool samples were freezed-thawed 5 times in liquid nitrogen, suspended in 600 µl of SDS lysis buffer (1% SDS, 20 mM Tris-HCl, 20mM EDTA, 50 mM NaCl, pH 7.5), and incubated at 56 ºC for 2-3 hr. DNA was extracted by shaking with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) 3 times and with chloroform/isoamyl alcohol (24:1) one time. The

top aqueous phase containing the DNA was collected into a new tube. Then this solution of DNA was added with 0.1 vol of 3 M sodium acetate pH 5.0 and mixed by flicking the tube several times. After this step, 2 vol of precooled absolute ethanol was added to the solution. DNA was collected by centrifugation at 10,000 *g* for 20 min. Then ethanol is removed and the pellet was collected after washing with 1 ml of room temperature 70% ethanol. The DNA was dried at room temperature with lid open. DNA-containing pellet was dissolved in 200 µl of sterile distilled water and used as DNA template in PCR (8).

5.2 PCR amplification

The nested PCR was carried out by using *G. duodenalis* genomic DNA and species specific primer pairs amplifying 5 different genetic loci of *G. duodenalis* which are small subunit-rRNA gene (SSU-rRNA), β-giardin gene, glutamate dehydrogenase gene (GDH), triosephosphate isomerase gene (TPI), and elongation factor 1-alpha gene (ef1-α) (Table 4).

The first primers were **RH11** (5'CAT CCG GTC GAT CCT GCC3'), **RH4** (5'AGT CGA ACC CTG ATT CTC CGC CAG G3') (8) and **GiarF** (5' GAC GCT CTC CCC AAG GAC3'), **GiarR** (5' CTG CGT CAC GCT GCT CG3') (9) amplifying 130 bp fragment of *G. duodenalis* small subunit-rRNA gene with the condition previously described by Hopkins *et al.*(8) : initial denaturing at 96 ºC for 2 min; then 35 cycles, each comprising denature at 96 ºC for 20 sec, annealing at 59 ºC for 20 sec, extension at 72 ºC for 30 sec followed by a final extension at 72 ºC for 7 min. The second primers were **G7** (5'AAG CCC GAC GAC CTC ACC CGC AGT GC3'), **G759** (5'GAG GCC GCC CTG GAT CTT CGA GAC GAC3') (2) and **forward primer** (5'GAA CGA GAT CGA GGT CCG3'), **reverse primer** (5'CTC GAC GAG CTT CGT GTT3') (14). These primer pairs amplified 511 bp fragment of *G. duodenalis* β-giardin gene. Both primary and nested PCR of these primer pairs were amplified with the PCR protocol described by Lalle *et al.* (14). The primary PCR initiated with denaturation step of 15 min at 95 ºC and followed by 35 cycles of 30 sec at 95 ºC, 30 sec of annealing at 65 ºC, and 60 sec at 72 ºC, with a final extension of 7 min at 72 ºC. For the nested PCR, the process initiated with denaturation step of 15 min at 95 ºC; then 35 cycles, each comprising denaturation at 95ºC for 30 sec, annealing at 55 ºC for 30 sec, and extension at 72 ºC for 60 sec followed by a final

extension of 7 min at 72 ºC. The third primers were **GDHeF** (5'TAC ACG T(CT)A A(CT)C G(CT)G G(CT)T TCC GT3'), **GDHiF** (5'CAG TAC AAC TC(CT) GCT CTC GG3') and **GDHiR** (5'GTT (AG)TC CTT GCA CAT CTC C3') (13) amplifying 432 bp fragment of glutamate dehydrogenase gene with the condition described by Read *et al.* (13). The PCR initiated with 1 cycle of 94 ºC for 2 min, 56 ºC for 1 min, and 72 ºC for 2 min, followed by 55 cycles of 94 ºC for 30 sec, 56 ºC for 20 sec, and 72 ºC for 45 sec, and a final extension at 72 ºC for 7 min. The fourth primer pairs were **AL3543** (5'AAA TIA TGC CTG CTC GTC G3'), **AL3546** (5'CAA ACC TTI TCC GCA AAC C3') and **AL3544** (5'CCC TTC ATC GGI GGT AAC TT3') and **AL3545** (5'GTG GCC ACC ACI CCC GTG CC3') (12). These primer pairs generated 530 bp PCR products from triosephosphate isomerase gene. Both primary and nested PCR were performed as PCR protocol described by Sulaiman *et al.* (12): initial denaturing at 94 ºC for 5 min; then 35 cycles, each comprising denaturing at 94 ºC for 45 ºC, annealing at 55 ºC for 30 sec, extension at 72 ºC for 60 sec followed by a final extension at 72 ºC for 10 min. The last primer pairs were **EF1AR** (5'AGC TC(CT) TCG TG(AG) TGC AT(CT) TC3'), **GLONGF** (5'GCT C(GC)T TCA AGT ACG CGT GG3') (10) and **RTef1-αF** (5'GCC GAG GAG TTC GAC TAC ATC3'), **RTef1-αR** (5'GAC GCC (GC)GA GAT CTT GTA GAC3'(11). The primary PCR was performed as described by Monis *et al*. (10): initial denaturing at 94 ºC for 2 min, then 35 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec, and 72 ºC for 1 min, with a final 72 ºC extension for 7 min. The nested PCR was performed as described by Traub *et al.* (11) initiating with 94 ºC for 2 min, 64 ºC for 1 min, and 72 ºC for 2 min, followed by 50 cycles of 94 ºC for 30 sec, 64 ºC for 30 sec, and 72 ºC for 30 sec and 1 cycle of 72 ºC for 7 min. These primer pairs generated PCR product size 191 bp of elongation factor 1-alpha gene.

5.3 Agarose gel electrophoresis

PCR product was electrophoresed in 2% agarose gel in 1x TBE buffer. The melt agarose was casted and allowed to harden at least 15 min and totally submerged in 1x TBE buffer. The minicell electrophoresis set will be used throughout this study. DNA samples will be mixed in a ratio of 1:5 with dye loading buffer. The gel was run at a constant voltage of 100 volts at room temperature for at least 30 min or until the loading dye reached 1 cm from the other rim of the gel. Then, the gel was removed

from the tray and stained with ethidium bromide solution for 10-15 min and destained with water for 10-15 min. The DNA was visualized with a UV light and photographed using UVI TECH (EEC). The size of DNA fragments was estimated by using 100 bp DNA ladder (New England BioLab, USA).

6. Comparison of diagnostic methods using direct immunofluorescent assay and PCR techniques

6.1 Stool samples. Seventy stool specimens were used to determine the sensitivities and specificities of the immunofluorescent assay (IFA) and PCR technique. Within seventy specimens, there were thirty three negative stool specimens and thirty seven positive stool specimens diagnosed by light microscopy using the concentration technique. All specimens were floated using saturated sodium nitrate. Then, all specimens were randomly blinded to test with PCR and IFA.

6.2 Direct immunofluorescent assay. Floated stool specimens were tested for the detection of the *G. duodenalis* by using *Giardia* Cel IF test (CeLLabs, UK) following the manufacturer's instruction. Twenty microliters of the stool specimens were placed on the microscope slides which have 6-8 mm diameter wells. The specimens were allowed to completely air dry. These slides were fixed for 5 min in acetone, and were allowed to air dry. Twenty-five microliters of *Giardia* Cel Reagent (RG1) were added to the fixed specimens and provided positive control slide (G). The slides were incubated at 37ºC in humid chamber for 30 min, and were rinsed gently in a bath of PBS for 1 min. Then, the slides were drained, and excess moisture around wells was removed with tissue. A drop of mounting fluid (RMG) was added to each slide well. A coverslip was placed on the top of the drop. The entire specimens were scanned by using a fluorescent microscope initially at x200 magnification, then at x400 for confirmation.

6.3 PCR. The most sensitive primers and DNA extraction method were used to detect *G. duodenalis* in blinded stool specimens. The sensitivity and specificity of PCR were compared to those obtained from immunofluorescent assay.

6.4 Data analysis. To determine the sensitivities and specificities of PCR and immunofluorescent assay, the 2×2 table was performed using Epi info version 6.04. Chi-square test was used to determine the significance of the difference between 2

proportions for sensitivities and specificities of the two diagnostic methods at 95% confidence interval.

Table 4. Different target gene loci and primer pairs using for detection of *Giardia duodenalis* in this study

Primers	Amplicon size (bp)	Target gene loci	Primer names	References
RH 11 RH 4 GiarF GiarR	130	Small subunit rDNA (SSU rDNA)	5'CAT CCG GTC GAT CCT GCC3' 5'AGT CGA ACC CTG ATT CTC CGC CAG G3' 5' GAC GCT CTC CCC AAG GAC3' 5' CTG CGT CAC GCT GCT CG3'	Hopkins *et al.* 1997 (8) Read *et al.* 2002 (9)
G7 G759 forward reverse	511	β-giardin	5'AAG CCC GAC GAC CTC ACC CGC AGT GC3' 5'GAG GCC GCC CTG GAT CTT CGA GAC GAC3' 5'GAA CGA GAT CGA GGT CCG3' 5'CTC GAC GAG CTT CGT GTT3'	Caccio *et al*. 2002 (2) Lalle *et al*. 2005 (14)
GDHeF GDHiF GDHiR	432	Glutamate dehydrogenase (GDH)	5'TAC ACG T(CT)A A(CT)C G(CT)G G(CT)T TCC GT3' 5'CAG TAC AAC TC(CT) GCT CTC GG3' 5'GTT (AG)TC CTT GCA CAT CTC C3'	Read *et al*. 2004 (13)
AL3543 AL3546 AL3544 AL3545	530	Triose phosphate isomerase (TPI)	5'AAA TIA TGC CTG CTC GTC G3' 5'CAA ACC TTI TCC GCA AAC C3' 5'CCC TTC ATC GGI GGT AAC TT3' 5'GTG GCC ACC ACI CCC GTG CC3'	Sulaiman *et al.* 2003 (12)
EF1AR GLONGF RTef1-αF RTef1-αR	191	Elongation factor 1-alpha (ef1-α)	5'AGC TC(CT) TCG TG(AG) TGC AT(CT) TC3' 5'GCT C(GC)T TCA AGT ACG CGT GG3' 5'GCC GAG GAG TTC GAC TAC ATC3' 5'GAC GCC (GC)GA GAT CTT GTA GAC3'	Monis *et al.* 1999 (10) Traub *et al*. 2003 (11)

CHAPTER IV
RESULTS

1. The sensitivities of genotyping primers to detect *G. duodenalis* using trophozoite DNA

To determine the sensitivities of selected genotyping primer sets in *G. duodenalis* trophozoite DNA, the different concentrations of DNA extracted from axenic culture trophozoites were submitted to the experiment. The results illustrated that RH11/RH4 and GiarF/GiarR primer set, amplifying the SSU-rRNA gene, was the most sensitive primer set that could detect as low as 10 pg of DNA/PCR reaction mixture. The G7/G759 and forward/reverse primer set, amplifying β-giardin gene, detected 100 pg of DNA/PCR reaction mixture, was less sensitive than RH11/RH4 and GiarF/GiarR primer set which amplify the SSU-rRNA gene. The GDHeF/GDHiR, GDHiF primer set and AL3543/AL3546, AL3544/AL3545 primer set, which amplify glutamate dehydrogenase gene (GDH), triosephosphate isomerase gene (TPI) genes, respectively, had the same sensitivities. The least sensitivity of primers tested in this study was the EF1AR/GLONGF and RTef1-αF/RTef1-αR primer set which detect elongation factor 1-alpha gene (ef1-α) (Table 5).

Table 5. Comparison of the sensitivities of 5 genotyping primer sets for the detection of *G. duodenalis* in axenic culture trophozoites.

Primers	Target gene loci	Amplicon size (bp)	References	*G. duodenalis* trophozoite DNA concentrations					
				1 pg	10 pg	100 pg	1 ng	10 ng	100 ng
RH11/RH4 and GiarF/GiarR	Small subunit rDNA (SSU rDNA)	130	Hopkins *et al.* (8) Read *et al.*(9)	-	+	+	+	+	+
G7/G759 and forward/reverse	β-giardin	511	Caccio *et al.* (2) Lalle *et al.* (14)	-	-	+	+	+	+
GDHeF/GDHiR and GDHiF	Glutamate dehydrogenase (GDH)	432	Read *et al.* (13)	-	-	-	+	+	+
AL3543/AL3546 and AL3544/AL3545	Triosephosphate isomerase (TPI)	530	Sulaiman *et al.* (12)	-	-	-	+	+	+
EF1AR/GLONGF and RTef1-αF/RTef1-αR	Elongation factor 1-alpha (ef1-α)	191	Monis *et al.* (10) Traub *et al.* (11)	-	-	-	-	+	+

2. Efficiencies of DNA extraction methods

To evaluate the efficiencies of the three DNA isolation methods, known number of cysts were used for the experiment. Table 6 shows cyst numbers in PCR reaction mixture that could be detected by FTA filter paper assay, QIAamp stool mini kit, and conventional phenol-chloroform method. FTA disk could detect the cysts at least 2.4 cysts/FTA disk. Since only one-fourth of FTA disk was used for each PCR reaction, the sensitivity of the FTA disk could detect at least 0.6 cysts/PCR reaction mixture. QIAamp stool mini kit could detect the cysts at the concentration of 140 cysts/200 µl stool dilution. Since 10 µl of extracted DNA was used for each PCR reaction mixture, the sensitivity of QIAamp stool mini kit was equivalent to 7 cysts/PCR reaction mixture. The sensitivity of phenol/chloroform extraction method was equivalent to QIAamp stool mini kit which was 7 cysts/PCR reaction mixture. The comparison showed that FTA filter paper assay was the most efficient DNA extraction method compared to QIAamp stool mini kit and conventional phenol/chloroform extraction method.

Table 6. Comparison of the efficiencies of three DNA extraction methods for detection of *G. duodenalis* in stool specimens

Extraction methods	Number of cysts per PCR reaction mixture							
	0.6	1.3	2	3	7	13	34	168
FTA filter paper	+	+	ND	+	ND	+	ND	ND
QIAamp stool mini kit	ND	-	-	-	+	ND	+	+
Phenol/chloroform	ND	-	-	-	+	ND	+	+

ND = Not determined

3. The sensitivities of genotyping primers to detect *G. duodenalis* using cyst DNA

FTA filter paper assay was used for comparison of the sensitivities of PCR protocols of 5 genotyping primer set shown in Table 7. The results demonstrated that the protocol of RH11/RH4 and GiarF/GiarR primer set was the most sensitive primer set that could detect at least 2.4 cysts/FTA disk. Since only one-fourth of FTA disk was used for each PCR reaction, RH11/RH4 and GiarF/GiarR primer set could amplify the DNA of *G. duodenalis* at the equivalent of at least 0.6 cysts/PCR reaction mixture (Figure 3). The protocols which used GDHeF/GDHiR and GDHiF primer set and AL3543/AL3546 and AL3544/AL3545 primer set could detect *G. duodenalis* at the equivalent of 1.3 (Figure 5) and 13 cysts/PCR reaction mixture (Figure 6), respectively. Following the techniques of Caccio *et al*/Lalle *et al*. (2,14) and Traub *et al*./Monis *et al*. (10,11), using the G7/G759 and forward/reverse primer set (Figure 4) and EF1AR/GLONGF and RTef1-αF/RTef1-αR primer set (Figure 7), respectively, could not amplify the DNA of *G. duodenalis* at 13 cysts per PCR reaction.

Table 7. Comparison of the sensitivities of 5 genotyping primer sets for the detection of *G. duodenalis* cysts in stool specimens

Primers	Target gene loci	Amplicon size (bp)	References	Number of cyst per PCR reaction mixture[a]															
				0.6				1.3				3				13			
				1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
RH11/RH4 and GiarF/GiarR	Small subunit rDNA (SSU rDNA)	130	Hopkins *et al.* (8) Read *et al.*(9)	-	-	+	+	-	-	-	+	-	-	+	+	-	+	+	+
G7/G759 and forward/reverse	β-giardin	511	Caccio *et al.* (2) Lalle *et al.* (14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GDHeF/GDHiF and GDHiR	Glutamate dehydrogenase (GDH)	432	Read *et al.* (13)	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+	-
AL3543/AL3546 and AL3544/AL3545	Triosephosphate isomerase (TPI)	530	Sulaiman *et al.* (12)	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
EF1AR/GLONGF and RTef1-αF/RTef1-αR	Elongation factor 1-alpha (ef1-α)	191	Monis *et al.* (10) Traub *et al.* (11)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a PCR amplification was done three times for each cyst concentration.

4. Comparison of diagnostic methods using direct immunofluorescent assay and PCR techniques

A blinded evaluation of immunofluorescent assay and PCR for detection of *G.duodenalis* was performed. FTA filter paper assay was used as DNA templates for PCR amplification using RH11/RH4 and GiarF/GiarR primer set to evaluate their sensitivities and specificities for detection of *G. duodenalis* in 70 blinded stool specimens. Examination of clinical specimens by immunofluorescent assay showed 34 specimens were positive for *Giardia* cysts. Using PCR method, 36 specimens were positive for *G. duodenalis*. Two specimens, which were positive by PCR, were negative for *Giardia* cysts by immunofluorescent assay. Table 8 shows the sensitivities and specificities of PCR method and immunofluorescent assay. The sensitivities of PCR method and immunofluorescent assay were 97.3% (95% CI, 87.39-99.86) and 91.9% (95% CI, 79.50-97.89), respectively. The specificities of both PCR and immunofluorescent assay were 100% (95% CI, 91.32-100.00). There were no significantly differences between PCR and immunofluorescent assay in terms of their sensitivities (p=0.61) and specificities.

Table 8. Comparison of PCR and immunofluorescent assay for the detection of *G. duodenalis* in blinded 70 stool specimens

Methods	Result	Number and (%)		
		Positive by concentration	**Negative by concentration**	**Investigated**
PCR*	Positive	36 (97.3)	0 (0)	36 (51.4)
	Negative	1 (2.7)	33 (100)	34 (48.6)
IFA*	Positive	34 (91.9)	0 (0)	34 (48.6)
	Negative	3 (8.1)	33 (100)	36 (51.4)
PCR or IFA	Any	37 (52.8)	33 (47.2)	70 (100)

*Done in duplicate

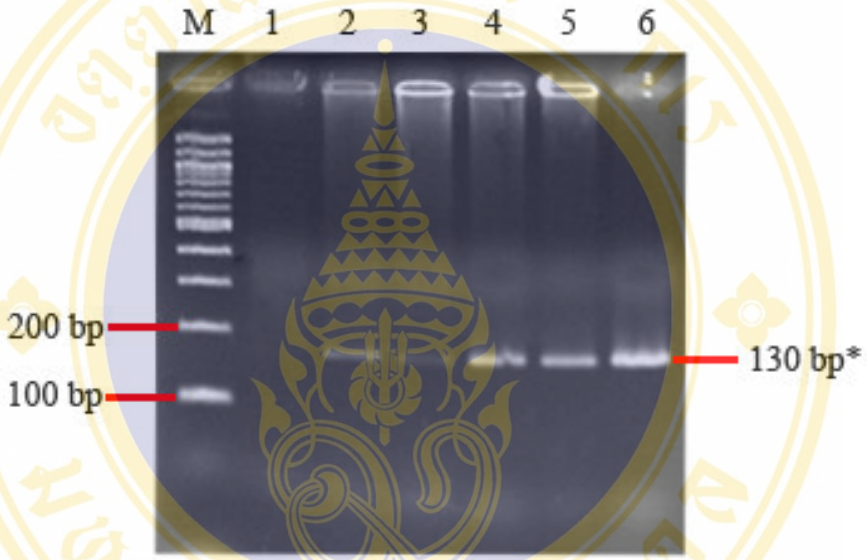


Figure 3 PCR analysis using the RH11/RH4 and GiarF/GiarR primer set specific to the SSU rRNA gene of *G. duodenalis*. Lane M, molecular markers of 100-bp DNA ladder; lane 1, negative control; lane 2 to 5, different concentrations of cysts (0.6, 1.3, 3, and 13 cysts/PCR reaction mixture, respectively)**;** lane 6, positive control

* Expected size of PCR product

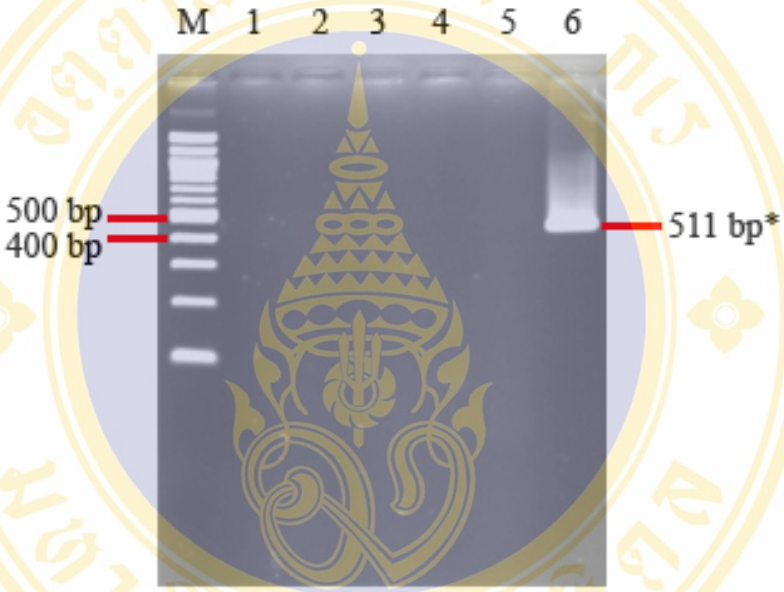


Figure 4 PCR analysis using the G7/G759 and forward/reverse primer set specific to the β-giardin gene of *G. duodenalis*. Lane M, molecular markers of 100-bp DNA ladder; lane 1, negative control; lane 2 to 5, different concentrations of cysts (0.6, 1.3, 3, and 13 cysts/PCR reaction mixture, respectively)**;** lane 6, positive control

* Expected size of PCR product

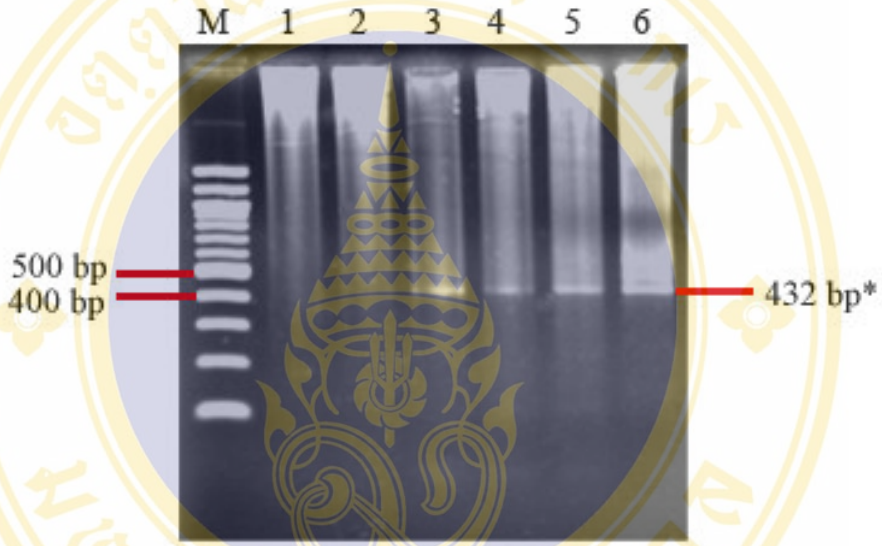


Figure 5 PCR analysis using the GDHeF/GDHiR and GDHiF primer set specific to the glutamate dehydrogenase (GDH) gene of *G. duodenalis*. Lane M, molecular markers of 100-bp DNA ladder; lane 1, negative control; lane 2 to 5, different concentrations of cysts (0.6, 1.3, 3, and 13 cysts/PCR reaction mixture, respectively)**;** lane 6, positive control

* Expected size of PCR product

Figure 6 PCR analysis using the AL3543/AL3546 and AL3544/AL3545 primer set specific to the triosephosphate isomerase (TPI) gene of *G. duodenalis*. Lane M, molecular markers of 100-bp DNA ladder; lane 1, negative control; lane 2 to 5, different concentrations of cysts (0.6, 1.3, 3, and 13 cysts/PCR reaction mixture, respectively)**;** lane 6, positive control

* Expected size of PCR product

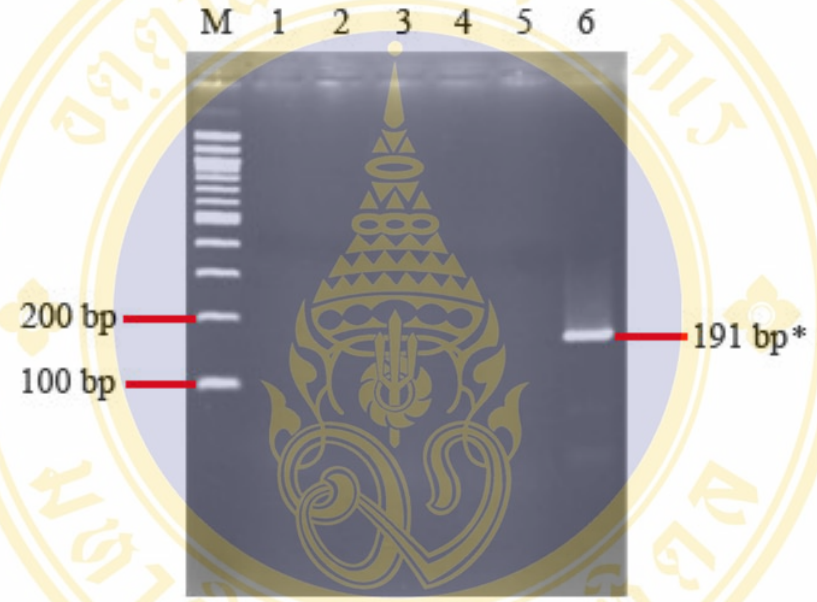


Figure 7 PCR analysis using the EF1AR/GLONGF and RTef1-αF/RTef1-αR primer set specific to the elongation factor1-alpha (ef1-α) gene of *G. duodenalis*. Lane M, molecular markers of 100-bp DNA ladder; lane 1, negative control; lane 2 to 5, different concentrations of cysts (0.6, 1.3, 3, and 13 cysts/PCR reaction mixture, respectively)**;** lane 6, positive control

* Expected size of PCR product

CHAPTER V
DISCUSSION

1. Evaluation of DNA extraction methods

A highly effective DNA extraction method is necessary for the detection of protozoa parasite in stool specimens by PCR because of the presence of inhibitors in stools. To choose an effective DNA extraction method for the PCR detection of *G. duodenalis* DNA in stool specimens, three DNA extraction methods; FTA filter paper assay, QIAamp stool mini kit, and conventional phenol/chloroform method were evaluated. The results showed that the detection varied from 0.6 to 168 cysts/PCR reaction mixture, depending on the extraction method. The conventional phenol/chloroform method was shown to be as effective as QIAamp stool mini kit procedure which could detect *G. duodenalis* cysts in stool specimens at the same concentration of 7 cysts/PCR reaction mixture. Although the conventional phenol/chloroform method can detect low number of *G. duodenalis* cysts, this method is time-consuming with several steps involved. Another disadvantage of this method is that it requires many laboratory's equipments. Thus, this method is unsuitable for field-work studies that may involve a large number of specimens.

The QIAamp stool mini kit procedure involves digestion of protein, elimination of inhibitor, binding DNA to silica-gel membrane and elution of DNA by spin column. Firstly, in this study, floated stool specimens were incubated at 70ºC for 5 min with lysis buffer for efficient cyst lysis before extraction. The results showed that incubation at this temperature could detect *G. duodenalis* cyst at only 34 cysts/PCR reaction mixture. This could be due to the nature of *G. duodenalis* cyst that is resistant to disrupt and lyse. For this reason, the incubation temperature of this step was raised up to 95ºC. The results revealed that increasing of incubation temperature could increase the efficiency of *G. duodenalis* detection in stool samples. However, the QIAamp stool mini kit still involved several steps and took approximately 1 h to be completed; thus, limiting the numbers of specimens that could be handled at one time.

FTA filter paper is an extraction-free, filter-based template which is impregnated with denaturants, chelating agents, and a free radical trap. Lysis of organisms occurs upon contact with the FTA filter paper, and then DNA is trapped on the matrix. Cell remnants, samples debris, and other factors that may interfere with PCR are effectively removed by washing reagent. The FTA filter paper has been used for DNA isolation in various organisms including bacteria and parasitic protozoa in food, environmental and clinical specimens (105-109). A high efficiency was demonstrated when compared to other methods. In this study, the FTA filter paper has been shown to be an effective tool for DNA preparation of *G. duodenalis* from clinical stool specimens as it could detect *G. duodenalis* at least 0.6 cysts/PCR reaction mixture.

The application of stool samples onto the FTA filter is very simple. The whole procedure, including drying and washing to get the DNA template ready for PCR amplification, takes less than 3 h after spotting the specimens. Hence, a large number of samples in field epidemiological studies can be prepared simultaneously by untrained personnel with less technical equipments. The dried FTA disks can be stored at room temperature without changing the DNA properties so that they are easy to handle and transport for further analysis. Furthermore, the modification of the FTA filter paper by using individual hole punch to get an FTA disk with a 6-mm diameter for tested samples can reduce the cost. Thus, for long-term use, FTA filter is a low-cost method with high efficiency, which makes them a good choice for use with large number of samples.

Although FTA filter paper has many advantages, the major disadvantage of using this procedure is the non-homogeneous of DNA template on the filter paper. Because the small size of PCR tube, it can contain only one to two fourth of FTA disk. Some parts of the disk may contain more DNA template than other parts. This can affect the result of PCR amplification. To handle this problem, at least two PCR amplifications per one disk of FTA filter paper were recommended.

2. Comparison of five genotyping primer sets

A number of researchers have developed PCR-based techniques for differentiating *G. duodenalis* of human origin and *G. duodenalis* of animal origin. These techniques are based on the polymorphic nature of *G. duodenalis* strains that infect humans and most animals at the small-subunit (SSU) rRNA, glutamate

dehydrogenase (GDH), elongation factor1-alpha (ef1-α), triosephosphate isomerase (TPI), and β-giardin sequences (12,13,44,78-82,84-86). However, the comparative performance of these genotyping tools has not been evaluated. In this study, the nested PCR protocols rather than single-step PCR protocols were evaluated because the nested PCR protocols are more specific and sensitive than single-step PCR protocols.

FTA filter paper was used as DNA preparation to evaluate the sensitivities of five different genotyping primer sets i.e. RH11/RH4 and GiarF/GiarR, G7/G759 and forward/reverse, GDHeF/GDHiR and GDHiF, AL3543/AL3546 and AL3544/AL3545, EF1AR/GLONGF and RTef1-αF/RTef1-αR (12,13,44,78-82). The results illustrated that the RH11/RH4 and GiarF/GiarR primer set that amplify the SSU-rRNA sequence was the most sensitive primer set to detect *G. duodenalis* in both axenic culture trophozoites and stool specimens. The sensitivity of this primer set may be due to the high copy numbers of SSU-rRNA gene in the organism. Approximately 60-130 copies of rRNA gene per nucleus of *G. duodenalis* arranged in tandem repeat have been reported (110,111).

Although, G7/G759, forward/reverse and AL3543/AL3546, AL3544/AL3545 primer sets could amplify the specific target sequences when tested with trophozoite DNA, these primer sets gave different results when tested with cyst DNA. To improve the sensitivities of these primer sets for detection cyst DNA, many conditions of these PCR protocols were modified, for example, modification of Mg^{2+} concentration, modification the amount of DNA template and modification the amount of *Taq* polymerase enzyme. Unfortunately, even modified many conditions and repeated the experiment several times, the results were still the same. The reasons why these PCR method showed different results when tested with trophozoite and cyst DNA are still unexplainable.

3. Sensitivities and specificities of PCR and immunofluorescent assay for the detection of *G.duodenalis*

The development of direct immunofluorecent microscopy has generally improved the sensitivity of detecting and quantitating fecal *Giardia* cysts and may allow for accurate determination of prevalence rates and cyst excretion intensities compared to conventional microscopy (112). However, this method is not possible to discriminate between morphologically identical, or similar, organisms that are genetically different

(10). To solve this problem, many PCR-based procedures have been developed for the detection and discrimination of *Giardia* parasite (12,13,44,78-82,84-86). Although the immunofluorescent assay and PCR have been widely used, the comparisons of sensitivities and specificities between these two procedures in stool specimens have not been evaluated. However, some studies conducted in calves stool specimens indicated that PCR was likely more sensitive than immunofluorescent assay (113-114).

In this study, the sensitivities and specificities of PCR and immunofluorescent assay for the detection of *G. duodenalis* in stool specimens were evaluated. The most effective techniques to detect *G. duodenalis*, i.e., FTA filter paper for DNA extraction and the RH11/RH4 and GiarF/GiarR primer set for PCR amplification were used. Although immunofluorescent assay seemed to be less sensitive than the PCR method, there are no statistically significant differences (p=0.61) between these two methods. The nonsignificant difference might be due to the high experience of the microscopist. However, immunofluorescent assay gave high sensitivity of 91.9% (95% CI, 79.50-97.89) and specificity of 100% (95% CI, 91.32-100.00), this method cannot provide the information about genetically different of the organism. Hence, immunofluorescent assay is proper for screening of this infection. The PCR method using RH11/RH4 and GiarF/GiarR primer set together with FTA filter paper for DNA extraction gave 97.30% (95% CI, 87.39-99.86) sensitivity and 100% (95% CI, 91.32-100.00) specificity for the detection of *G. duodenalis* in stool specimens. Thus, this method is a good diagnostic method for evaluation clinical specimens and also a meaningful tool for epidemiological study of this infection. Another advantage of this method is that the PCR product can be used for sequence analysis to provide information about genotype of the organism. Understanding the genetic variations of *G. duodenalis* among the population will be useful for exploring the source, transmission, and pathogenesis of this organism.

CHAPTER VI
CONCLUSION

In conclusion, the extraction-free, FTA filter paper was the most effective method for DNA extraction of *G. duodenalis* from stool specimens compared to QIAamp stool mini kit and conventional phenol/chloroform method. FTA filter paper was ease to use, rapid, and required inexperience persons to handle specimens. Comparison of the 5 PCR protocols to detect both trophozoite and cyst DNA showed that PCR amplification using RH11/RH4, GiarF/GiarR primer sets, detecting SSU-rRNA gene, was the most sensitive method for the detection of *G. duodenalis* in this study. There was no statistically significant difference between IFA and PCR using the most efficient DNA extraction method and suitable primer set. However, PCR-based procedure had the advantage over the IFA in which it can provide the information about the genetic variation in *G. duodenalis*. Understanding the genetic variations of this organism among the population will be useful for exploring the source, transmission, and pathogenesis of this organism.

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