ZOONOTIC CANINE GIARDIASIS IN TEMPLE-RELATED COMMUNITIES IN BANGKOK, THAILAND

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Thesis

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ZOONOTIC CANINE GIARDIASIS IN TEMPLE-RELATED COMMUNITIES IN BANGKOK, THAILAND

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ABSTRACT

Giardia duodenalis is a flagellate protozoan that inhabits the small intestines of humans and other mammals. In Thailand, religious and cultural practices lead to unwanted pet dogs being abandoned in temples, where monks are obliged to care for them. The close relationship shared with the dogs, coupled with overcrowded conditions, place monks and the surrounding underdeveloped communities at increased risk of acquiring *Giardia* infections from dogs.

Fecal samples were collected from 162 humans and 200 dogs from 18 different temples in Bangkok, as well as communities in the surrounding areas. Both human and dog stool samples were examined for *Giardia* using zinc sulfate flotation and microscopy. The prevalence of *Giardia* in dogs was 9%, and 3% in humans. However, when a highly sensitive and species-specific nested PCR was utilized to screen for *Giardia* in this study, the prevalence of *Giardia* was found to be 75% in dogs and 60% in humans. Nine of 20 PCR-positive, but microscopy-negative *Giardia* samples were shown positive by immunofluorescence (IF). All samples microscopy-positive for *Giardia* were genotyped. The majority of the dog population was placed in Assemblage A, followed by Assemblages B, D, and C respectively, while human isolates were placed in Assemblages A and B. Therefore, dogs in temple communities were shown to pose a potential zoonotic risk to humans for transmission of *Giardia*, especially Assemblage A genotypes. Multivariate risk factor analysis revealed that the dogs belonging to the temple, as well as dogs that were allowed outside houses into the temple compound, were significantly more likely to be infected with *Giardia*.

KEY WORDS: GIARDIA / HUMAN / DOG / TEMPLE / PCR

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การศึกษาโรคสัตว์สู่คนของเชื้อพยาธิโปรโตซัว *Giardia* จากสุนัขในวัดและรอบๆบริเวณวัดในกรุงเทพมหานคร (ZOONOTIC CANINE GIARDIASIS IN TEMPLE-RELATED COMMUNITIES IN BANGKOK, THAILAND)

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บทคัดย่อ

Giardia duodenalis เป็นเชื้อพยาธิโปรโตซัวที่อาศัยในลำไส้เล็กของมนุษย์และสัตว์เลี้ยงลูกด้วยนมชนิดอื่นๆ ในประเทศไทยตามคำสอนทางศาสนาและประเพณีเชื่อว่าการทำลายสัตว์เป็นบาปทำให้มีการนำสุนัขไปปล่อยทิ้งไว้ในวัดเพราะเชื่อว่าพระสงฆ์สามารถดูแลสุนัขเหล่านี้และสุนัขจะไม่ถูกฆ่า จากปัญหาดังกล่าวเมื่อพระสงฆ์และประชาชนที่อาศัยอยู่รอบๆบริเวณวัดมีการสัมผัสกับสุนัขเหล่านี้ทำให้มีโอกาสเสี่ยงที่จะติดเชื้อนี้จากสุนัขได้

การศึกษาในครั้งนี้ทำการเก็บอุจจาระจากมนุษย์ 162 คนและสุนัขจำนวน 200 ตัว จากวัด 18 วัด ในเขตกรุงเทพมหานคร จากนั้นทำการตรวจอุจจาระโดยวิธีการลอยตัวด้วยสาร zinc sulfate และตรวจหาเชื้อด้วยกล้องจุลทรรศน์ ผลที่ได้พบว่าความชุกของการพบเชื้อนี้ในสุนัขเท่ากับร้อยละ 9 และในมนุษย์เท่ากับร้อยละ 3 อย่างไรก็ตามเมื่อทำการตรวจโดยวิธี nested PCR พบว่าความชุกของการพบเชื้อนี้ในสุนัขเท่ากับร้อยละ 75 และในมนุษย์เท่ากับร้อยละ 60 หลังจากนั้นทำการสุ่มตัวอย่างจำนวน 20 ตัวอย่างที่ให้ผลบวกโดยวิธี PCR แต่ให้ผลลบจากการตรวจด้วยกล้องจุลทรรศน์แล้วทดสอบโดยวิธี immunofluorescence (IF) พบว่าตัวอย่าง 9 ตัวอย่างให้ผลบวกโดยวิธีนี้ ผลการ genotype ตัวอย่างที่ให้ผลบวกเชื้อพยาธิ *Giardia* จากการตรวจด้วยกล้องจุลทรรศน์พบว่าตัวอย่างของเชื้อนี้ที่ได้จากสุนัขส่วนใหญ่เป็นชนิด Assemblage A ตามด้วยชนิด Assemblage B D และ C ตามลำดับ ขณะที่มนุษย์เป็นชนิด Assemblage A และ B ดังนั้นสุนัขในชุมชนวัดมีความเสี่ยงที่จะถ่ายทอดการติดต่อของเชื้อนี้สู่มนุษย์ได้โดยเฉพาะอย่างยิ่งชนิด Assemblage A ผลการวิเคราะห์ปัจจัยเสี่ยงที่อาจทำให้เกิดการติดต่อของเชื้อนี้พบว่าสุนัขที่อาศัยอยู่ในวัดและสุนัขที่ออกจากบ้านมาบริเวณวัดมีโอกาสที่จะติดเชื้อนี้มากกว่าสุนัขกลุ่มอื่นๆอย่างมีนัยสำคัญทางสถิติ

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LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
^0C	Degree Celsius
sec	Second
min	Minute
h	Hour
ml	Milliliter
%	Percent
mg	Milligram
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
PBS	Phosphate Buffer Saline
ID_{50}	Infectious Dose Fifty
µl	Microliter
rpm	Rounds per minute
g	Gravity
et al.	et alli
syn	Synonym
µm	Micrometer
gm	Gram
cm	Centimeter
CI	Confidence Interval
PCR	Polymerase Chain Reaction
tpi	Triose Phosphate Isomerase
bp	Base pair
$MgCl_2$	Magnesium Chloride
DNA	Deoxyribonucleic acid

LIST OF ABBREVIATIONS (CONT.)

Abbreviation or symbol	Term
RNA	Ribonucleic acid
mM	Millimolar
nM	Nanomolar
pM	Picomolar
U	Unit
dNTP	Deoxynucleoside triphosphate
ρmol	Picomole
µg	Microgram
ef1-α	Elongation factor 1-alpha
SSU	Small subunit
ELISA	Enzyme-linked immunosorbent assay
RFLP	Restriction fragment length polymorphism
RAPD	Random amplified polymorphic Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
gdh	Glutamate dehydrogenase
ITS	Internal Transcribed Spacer
CELISA	Coproantigen Enzyme-linked immunosorbent assay

CHAPTER I
INTRODUCTION

Giardia duodenalis (syn: *Giardia lambia*, *Giardia intestinalis*) is a flagellated protozoan that inhabits the small intestine of human and other mammals. It is distributed worldwide and considered the most commonly detected intestinal parasite in humans in developed countries (Schantz, 1991). *Giardia* is commonly accepted as a zoonotic agent (Milstein and Goldsmid, 1997: Thompson *et al*., 2000). The organism is transmitted faecal-orally producing environmentally resistant cysts that are voided in the faeces and transmitted via water or food or directly via person-to-person or animal-to-person contact. About 200 million people suffer from symptomatic giardiasis in Asia, Africa and Latin America with some 500,000 new cases each year (World Health Organization, 1996). In developed countries, it is considered a re-emerging infectious agent because of its role in outbreaks of diarrhoea in child-care centers (Thompson, 2000). The potential for zoonotic transmission of *Giardia* from domestic dogs to humans remains largely unknown (Ashford and Snowden, 2001). If zoonotic transmission is possible, then both domestic as well as stray dogs may constitute a potential source of infection for humans. Recent surveys of gastrointestinal parasites of dogs have demonstrated high levels (prevalence of 7.2-22.1%) of *Giardia duodenalis* in stray and domestic dogs both in developing and developed countries (Bugg *et al*. 1999; Itoh et al. 2001; Jacobs, Forrester and Yang, 2001; Oliveira Sequeira *et al*. 2002, Traub *et al*., 2004). In Thailand, recent surveys addressed *Giardia* infection only in humans with a prevalence of 5.3-14.36% (Waikagul *et al*. 2002; Nuchprayoon *et al*. 2002; Sirivichayakul *et al*. 2003). The prevalence of *Giardia* in animals, however, is unknown.

PROJECT BACKGROUND

It is common in Thailand to leave or abandon unwanted dogs in temple grounds. Since temples are public places of worship and to donate gifts, the owners trust that their animals will be fed and will be taken care of by monks and good-hearted temple visitors. Furthermore, temple grounds in Thailand are generally sizeable with free access to the public, therefore it seems to be a perfect place for unwanted animals. At a minimum, the dogs could live on the left-over food of the monks. In general, there are quite a few reasons why dogs become "unwanted". Either, the dam produces too many puppies or the dogs become aggressive and attack family members. Further, when children grow up, they are also no longer interested in their pets. In addition, when animals are sick and become burdensome to look after, or when the owners relocate or pass away, they may no longer be wanted by their owners. Therefore, under these circumstances many zoonoses such as rabie, hookworm, *Toxocara* and *Giardia* can be transmitted from those "dislocated" dogs to monks and people leaving nearby the surrounding communities.

Many countries in Europe, Asia and Australia are concerned about zoonotic giardiasis (Hopkin *et al*. 1997; Capelli *et al*. 2003; Sulaiman *et al*. 2003; Traub *et al*. 2004). They have been studying this subject extensively and have developed tools for testing and diagnosing infections with the organism. However, there has been no study undertaken on this subject in Thailand, so far.

Information regarding the epidemiological status of zoonotic canine giardiasis in dogs and humans in Thailand is needed. The results of this study should therefore provide information on the transmission of giardiasis resulting in the subsequent establishment of proper control and preventive measures against this zoonotic agent.

CHAPTER II

OBJECTIVES

SPECIFIC OBJECTIVE

1. To study the epidemiology of zoonotic canine giardiasis in temple-related communities in Bangkok, Thailand.
2. To genotype *Giardia duodenalis* isolates from humans and dogs living in temple-related communities in Bangkok, Thailand.

SIGNIFICANCE OF THE RESEARCH

1. Determine the prevalence of *Giardia* in dogs and humans residing in the temple-related communities
2. Determine whether canine *Giardia duodenalis* isolates are potentially zoonotic and evaluate the risks of transmission to humans.
3. Utilize the results as input for establishing of preventive measures to prevent *Giardia* transmission from dogs to human.

CHAPTER III
LITERATURE REVIEW

Giardia is a flagellated protozoan parasite belonging to the Class of Zoomastigophora, Order Diplomonadida. They represent a group of parasitic diplomonads in the Family Hexamitidae. Representatives of this genus have been described in the intestinal tract of all classes of vertebrates.

The life cycle of *Giardia* consists of two stages, trophozoites and cysts. The trophozoites feed and multiply whilst the cysts originate from trophozoites by forming a cyst wall to resist the adverse environment outside the host.

Giardia trophozoites measure 9-21 μm in length and 5-15 μm in width. The 2-4 μm thick body has a pear like appearance, which is split lengthwise with a broad rounded anterior, a large ventral adhesive disk, two oval nuclei dorsal to the adhesive disk, central microtubular structures called median bodies, and four pairs of flagellae which originate in a kinetosomal complex. The movement of trophozoites resembles falling leaves. *Giardia*, like other genera in the Family Hexamitidae, lacks mitochondria and Golgi stacks. *Giardia* trophozoites feed by pinocytosis and numerous digestive vacuoles have been described near the dorsal surface (Meyer, 1994). *Giardia* cysts are surrounded by a homogenous wall which is closely apposed to the cell membrane.

In the first half of 20th century, the taxonomy of *Giardia* at the species level was complicated and confused. Numerous species of dubious validity had been described, principally on the basis of host occurrence rather than marked differences in morphology. In 1951, a landmark study was published by Filice in which he suggested that only three morphologically district species should be recognized: *G. duodenalis* was proposed as the form of *Giardia* occurring in most mammals, including humans, their companion animals and livestock; *G. agilis* in amphibians and *G. muris* in rodents. An additional two morphologically distinct species have been

recognized recently in bird (Thompson *et al*., 2000). Today 5-6 morphologcally district species are recognized (Table 1).

Table 1. Recognized species in the genus *Giardia* (Thompson *et al*., 2000).

Species	Host	Morphological characteristics
G. duodenalis	Wide range of domestic and wild mammals including humans	Pear-shaped trophozoites with claw-shaped median bodies
G. agilis	Amphibians	Long, narrow trophozoites with club-shaped median bodies
G. muris	Rodents	Rounded trophozoites with small round median bodies
G. psittaci	Birds	Pear-shaped trophozoites, with no ventrolateral flange. Claw-shaped median bodies
G. ardeae	Birds	Rounded trophozoites, with prominent notch in ventral disc and rudimentary caudal flagellum. Median bodies round-oval to claw shaped

Molecular techniques are particularly useful for studying the taxonomy, population structure, zoonotic potential of animal isolates, and the correlation between the genetic variability of the parasite and the range of clinical symptoms observed in humans.

Although morphologically identical isolates of *G. duodenalis* have been shown to be phenotypically and genetically heterogeneous (Thompson *et al*., 2000), *Giardia* isolates recovered from humans have been shown to fall into one of the two major genetic groups or assemblages, each of which contains a number of genetic subgroupings. Assemblage A Group I has been shown to consist of a mixture of closely related animal (including dog) and human isolates that appear to have undergone recent global dispersion (Mayrhofer *et al*., 1995). Assemblage A Group II has, to date, been shown to comprise human isolates and more recently dog isolates

(Traub *et al*., 2004). Assemblage B comprises a range of genetically diverse groups, predominantly human isolates, but also some animal (including dog) isolates (Monis *et al*., 1996; Monis *et al*., 1998). A number of other distinct genotypes have also recently been identified within the *G. duodenalis* morphological group, and they all appear to be confined to individual species of mammalian host. The non-human, host-specific genotypes found in dogs (Assemblage C/D) and cats (Assemblage F) are quite distinct from those found in assemblage A and B. In contrast, the genotypes identified in hoofed livestock (Assemblage E) and cats (Assemblage F) appear to be closely related to isolates in the major assemblages, suggesting a much more recent divergence.

Table 2. Genotypic groupings of *Giardia duodenalis* (Olson *et al*, 2004).

Assemblage (genotypic grouping)	Host range
Assemblage A (groupI)	Humans and other mammals; zoonotic
Assemblage A (groupII)	Mainly humans, dogs; zoonotic
Assemblage B (groupIII)	Humans and other mammals; zoonotic
Assemblage B (groupIV)	Humans
Assemblage C/D	Dogs
Assemblage E	Livestock
Assemblage F	Cats

LIFE CYCLE

The life cycle of *Giardia* is well known and is shown in detail in Fig. 1. The trophozoite attaches to the small intestinal epithelium of the vertebrate host by an adhesive disk. There, binary fission repeatedly takes place, the eventual result of which is the establishment of enormous numbers of the protozoa, around the mucosa of the host small intestinal epithelium. Normally, *Giardia* protozoa are supposed to be noninvasive. However, invasion of the host has been described (Meyer, 1974). As trophozoites detach from the intestinal epithelium, they may be flushed out by the fecal stream and be excreted. The transformation of *Giardia* trophozoites to cysts occurs in the host intestinal tract. Each of the two nuclei within the cysts undergoes a single division which results in a quadrinucleate cyst (infective stage). The cycle

continues when a suitable host ingests the cyst. Stomach acidity and other factors trigger the excystation process, which usually takes place in the host small intestine.

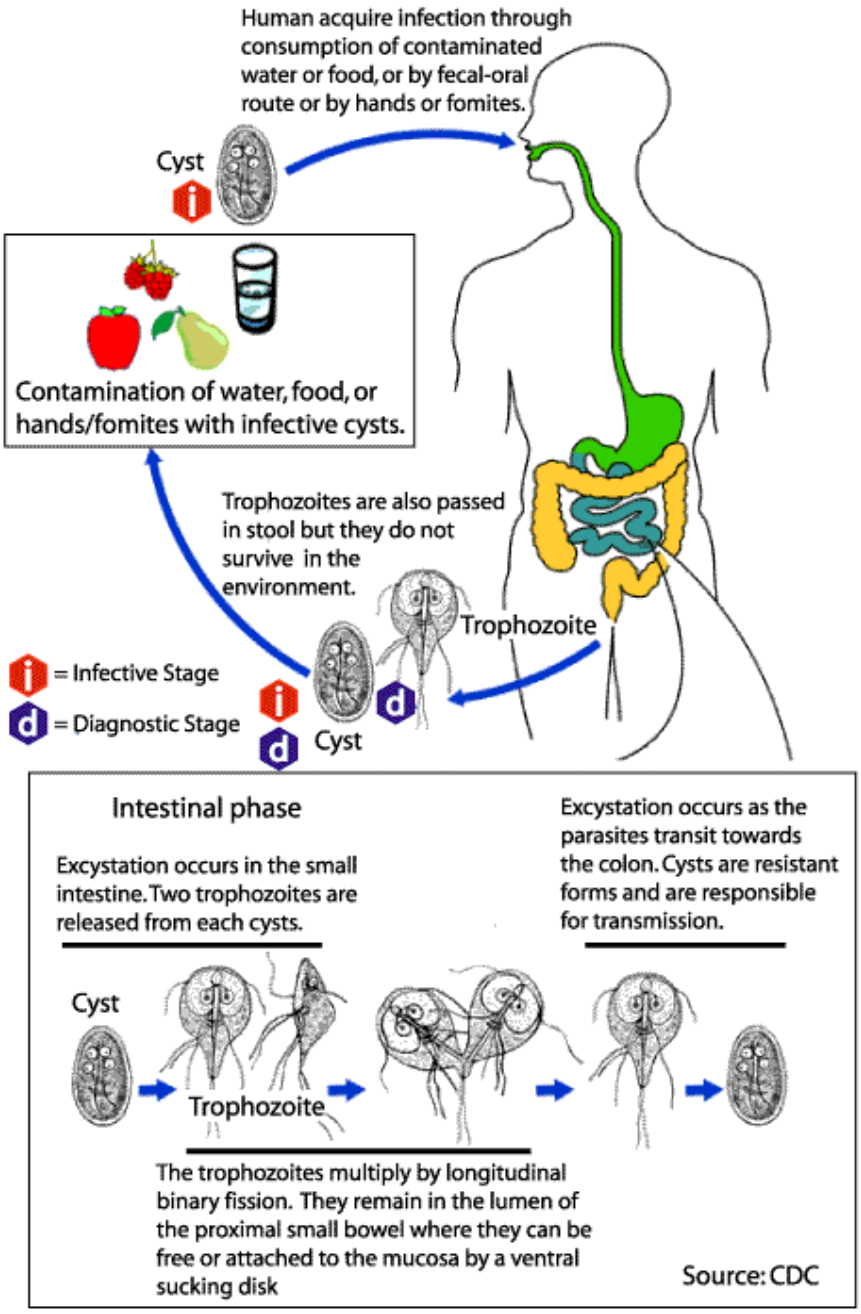


Figure 1. The life cycle of *Giardia spp* (CDC).

(http://sprojects.mmi.mcgill.ca/tropmed/disease/giardia/image/GiardiaLifeCycle.gif).

Health Effects

The prepatent time for giardiasis is generally between 6-16 days and the minimal infective dose can be as low as 1-10 cysts. Although there are large differences between the different isolates of the parasite in virulence and antigenic diversity. The ID_{50} (number of cysts ingested resulting in 50% of the test subjects becoming infected) was found to be 19 cysts using human-source cysts in human, but can be as high as 543 for human-source *Giardia* in gerbils (Rendthorff, 1978). *Giardia* strains that are well adapted to their hosts can frequently infect with 50 cysts or less. Exposure to the parasite results in partial or total immunity for a period of up to 21 weeks in mice (Robert *et al*., 1976 and Belosevic and Faubert, 1983).

The host-parasite relationship between *Giardia* and its hosts is complex. Olson *et al*. (1994) observed lower cysts output and a greater weight gain in kittens immunized subcutaneously. Humoral immune response is revealed by increased levels of circulating IgG and IgM antibody and secretion of IgA in milk, saliva and possibly intestinal mucus. Very little is known about cellular immunity, but spontaneous killing of trophozoites by human peripheral blood monocytes has been described (denHollander *et al*., 1988).

The symptoms of giardiasis vary widely. Characteristic symptoms, range from mild to moderate abdominal discomfort, abdominal distention due to increased intestinal gas, sulfurous or "rotten egg" burps, highly offensive flatulence, and mild to moderate diarrhea. Stools are soft (but not liquid), bulky, and foul smelling. They have been described as steatorrhea, and they float on the surface of water. Nausea, weakness, and loss of appetite may occur and also deficiency of fat soluble especially in children. Studies have shown that giardiasis can be suspected when the illness lasts seven or more days with at least two of the above symptoms. However, most infected individuals have are asymptomatic. In one incident studied by the CDC, disruption in a major city's water disinfection system allowed the entire population to consume water heavily contaminated with *Giardia*. Yet only 11 percent of the exposed population developed symptoms even though 46 percent had organisms in their stools (Rockwell, 2002). The pathogenesis of *Giardia* appears to be a result of villous atrophy and damage to the microvilli which is associated with an inflammatory

response (Williamson *et al.*, 2000). The pathophysiology of giardiasis implicates a diffuse loss of epithelial microvillus length, which in turn contributes to the impairment of brush border enzymes, and to malabsorption of electrolytes, nutrients and water. These changes may occur in the absence of villus atrophy or delayed enterocyte maturation, despite concurrent increases in epithelial cell turnover. Live *Giardia* trophozoites as well as their secretory-excretory products alter cytoskeletal proteins such as F-actin, villin, ezrin and α–actinin. These alterations to enterocyte ultrastructure and function are associated with a loss of intestinal epithelial barrier function, and ultimately lead to the production of diarrhea in the infected host (Buret *et al*., 2003). The risk factors for clinical giardiasis and duration of infection is poorly understood but undoubtedly comprises both host factors such as age and immune status as well as the virulence or pathogenicity of the *Giardia* 'strain' (Boreham, 1991).

Giardiasis has been called a disease of "somes." Some people do not contract it even from heavily contaminated sources. Some infestations vanish with no treatment at all. Some patients become asymptomatic cysts passers for a period and have no further clinical manifestations. Some evidence suggests that some people acquire a natural immunity to some strains. Some strains seem to be more virulent than others.

Previous epidemiological surveys of *Giardia* in humans and dogs

Itoh*, et al*. (2001) reported the prevalence of *Giardia lamblia* infection in household dogs in Japan. A total of 1,035 household dogs were examined. Out of those, 151 (14.6%) stool samples were positive for *Giardia*.

Jacobs *et al*. (2001) reported the prevalence of *Giardia* in dogs presented to Canadian veterinary practices. One thousand and two hundred sixteen fecal samples were collected from dogs presented to 15 veterinary practices across Canada and tested for *Giardia* by ELISA for group-specific antigen. If positive, the presence of *Giardia* was confirmed by microscopy. Eighty-eight submissions tested positive by ELISA and microscopy. The overall prevalence rate of *Giardia* was 7.2%.

Oliveira-Sequeira *et al*. (2002) reported the prevalence of intestinal parasites in dogs from Saint Paulo State, Brazil. The prevalence of gastrointestinal parasites was

investigated in stray dogs and dogs with owners by fecal examinations from 271 dogs employing sedimentation, simple flotation and centrifugation-flotation methods. The prevalence of *Giardia* was 12.2% and higher in stray dogs when compare with dogs with owners ($P < 0.05$).

Hackett *et al*. (2003) reported the prevalence of enteric pathogens in dogs of north-central Colorado. Fecal samples were obtained from client-owned dogs presented to the Veterinary Teaching Hospital at Colorado State University for evaluation of acute small-bowel, large-bowel, or mixed-bowel diarrhea (n=71) and from age-matched, client-owned, healthy dogs (n=59). The prevalence of *Giardia* was 5.4%in that study.

McGlade *et al*. (2003) determined the prevalence of *Giardia* in 40 faecal samples from domestic cats in the Perth metropolitan area by using microscopy, PCR and *Giardia* CELISA test. The results showed the prevalence of *Giardia* was 5, 80 and 60% by the tests, respectively and demonstrated that more sensitive techniques such as PCR maybe necessary, and may yield more reliable results, in the detection of low levels of *Giardia* in animals.

Kaur*, et al*. (2002) investigated the prevalence of intestinal parasites in children suffering from diarrhea in Delhi, India. Stool specimens of 127 children were examined for intestinal parasites by the direct smear technique. *Giardia intestinalis* parasites were found in 14 of those specimens, which correspond to a prevalence of 11% in that population.

Capelli *et al*. (2003) reported the prevalence of *Giardia* spp. in dogs and humans in Northern and Central Italy. A total of 916 faecal samples were examined, 616 from dogs and 300 from humans, in order to detect *Giardia* cysts. By applying various techniques like centrifugation and flotation combined with sedimentation the overall prevalence was determined to be 21.3% in dogs and 2% in humans, respectively.

Verle *et al*. (2003) examined the prevalence of intestinal parasitic infections in northern Vietnam. 526 households of six ethnic groups: Muong, Kinh, Dao, Thai, Tay and Hmong were surveyed. Eggs or cysts of at least one parasite species were

detected in 88% of all the stool samples (n = 2522). *Giardia lamblia* was found in all districts and among all groups and the prevalence of infection was estimated to be 3%.

Meloni *et al*. (1993) reported the prevalence of *Giardia* in children, dogs and cats from Aboriginal communities in the Kimberley. Feacal samples were collected from 385 humans, 182 dogs and 33 cats. The prevalence of *Giardia* was 30.9% and 17.0% in humans and dogs, respectively. Cats were not found to be infected with *Giardia* in this communities. In humans, *Giardia* infection was highest in the age group two to five years and most infections occurred in the fourth years of life.

In Thailand, the prevalence of giardiasis has reported only in humans so far. Although, there is no prevalence data available for *Giardia* in animals in Thailand, especially dogs. The potential importance of dogs in Thailand as zoonotic transmitters of *Giardia* is unknown.

Waikagul *et al*. (2002) investigated intestinal parasitic infections among school children in Nan province, Northern Thailand by using the formalin-ether sedimentation technique. A total of 1,010 faecal samples were examined and *Giardia lamblia* protozoa were found in 5.3%.

Nuchprayoon *et al*. (2002) reported the prevalence of intestinal parasitic infection among Thai patients at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The authors collected the data of 6231 individuals examined for parasite infections.. Evidence of parasitic infections was found in 557 (8.94%) and *Giardia* was the most common protozoan infection (14.36%).

Eight percent of Thai people with mental handicaps were shown to be infected with *Giardia* (Sirivichayakul *et al*. 2003).

Methods for *Giardia* detection

Diagnosis of *Giardia* in humans is most commonly made by correlating clinical signs with stool examination for *Giardia* cysts or trophozoites. Antigen detection tests such as ELISA techniques or anti-*Giardia* fluorescein-labelled monoclonal antibodies in a direct fluorescence assay (DFA) have also been frequently utilised due to their superior sensitivity, speed and ease of detection (Goldin *et al.*, 1993, Hopkins *et al.*, 1993, Hill, 2001).

Mochizuki *et al*. (2001) reported the epidemiological status of *Giardia* infection in Japan. A total of 95 rectal swab specimens were taken from dogs and examined by enzyme-linked immunosorbent assay (ELISA). The samples comprised 85 diarrheal and 10 normal stools, were also examined for *Giardia* cyst by a zinc sulfate centrifugation flotation method. More than 40% of both diarrheal and normal feacal specimens were found to be positive by the ELISA and *Giardia* parasite were observed in 20.6% of diarrheal feces by microscopic examination after the zinc sulfate centrifugal flotation.

In addition Beltran *et al*. (2002) reported the detection of *Giardia duodenalis* antigen in human faecal elutes by using the enzyme-linked immunosorbent assay and polyclonal antibodies. 196 samples of human feces were tested by parasitologic diagnosis: 69 were positive for *Giardia* cysts, 56 had no *Giardia* parasites at all, and 71 revealed parasites other than *Giardia*. The optimal concentration of polyclonal antibodies for antigen capture was about 40 μg/ml and the optimal conjugate dilution was 1:100. The parameters of the ELISA test for *Giardia* antigen detection were: sensitivity 100% and specificity 95%.

Genetic characterization of a total of 15 *Giardia lamblia* isolates, 8 from China (all from purified cysts) and 7 from Korea (2 from axenic cultures and 5 from purified cysts) was performed by PCR amplification and sequencing of a 295-bp region near the 5′ end of the small subunit ribosomal DNA. *G. lamblia* isolates from humans in China and Korea are divided into 2 major lineages, all Korean isolates were grouped into assemblage A, whereas 4 Chinese isolates were grouped into assemblage A and 4 into assemblage B (Yong *et al*., 2000).

Amar *et al*. (2002) reported a sensitive PCR-restriction fragment length polymorphism assay for the detection and genotyping of *Giardia duodenalis* in human faeces. *Giardia* cysts from patients in England and Wales with sporadic cases of giardiasis between September 1995 and March 2000 were isolated the DNA amplified and sequenced. The same procedure was done in a nursery outbreak of giardiasis that occurred in North Wales during April 2000. Among 35 DNA samples extracted from whole feces from patients with confirmed sporadic giardiasis, the *tpi* gene could be amplified in 33 cases which corresponds to 94%. Of those, nine (27%) samples

contained assemblage A group II, 21 (64%) contained assemblage B and 3 (9%) contained a mixture of assemblage A group II and assemblage B. The *tpi* gene of *G. duodenalis* assemblage B was amplified from 21 of 24 (88%) DNA samples extracted from whole feces from patients with confirmed cases of infection in the nursery outbreak.

Read *et al*. (2002) reported a correlation between the genotype of *Giardia duodenalis* and diarrhoea. Monthly faecal samples were obtained from children under 5 years of age attending 10 day-care centers from February 1998 to May 2000. The samples were initially screened by microscopy for the presence of *Giardia* cysts and genotyping was subsequently carried out using a nested PCR procedure, amplifying a region of the SSU-rDNA using the primer RH11, RH4, GiarF and GiarR. A total of 1306 samples were screened for *Giardia* from 353 children over the course of the study and 7.6% (27) of the children samples were positive for *Giardia.* Sequence information was obtained for 36 isolates of *Giardia* from 23 different children. Thirty percent (7/23) of those children were infected with isolates of *G. duodenalis* that were genotyped as Assemblage A, and 70% (16/23) had isolates that were genotyped as Assemblage B.

Sulaiman *et al*. (2003) utilised a PCR based on the the triose phosphate isomerase gene for characterizing the potential zoonotic transmission of *Giardia duodenalis* The *tpi* gene was amplified in 37 human isolates, 15 dog isolates, 8 muskrat isolates, 7 isolates each from cattle and beavers and in 1 isolate each from rat and rabbit. Distinct genotypes of *Giardia* were found in humans, cattles, beavers, dogs, muskrat and rat.

McGlade *et al*. (2003) determined the prevalence of *Giardia* in 40 faecal samples from domestic cats in the Perth metropolitan area by using microscopy, PCR and *Giardia* CELISA test. The results showed the prevalence of *Giardia* was 5, 80 and 60% by the tests, respectively and demonstrated that more sensitive techniques such as PCR maybe necessary, and may yield more reliable results, in the detection of low levels of *Giardia* in animals.

Read *et al*. (2004) developed a PCR-RFLP based on the glutamate dehydrogenase gene capable of discriminating all genotypes of *Giardia duodenalis.*

DNA was extracted from cultured *Giardia* trophozoites, *Giardia* cysts purified from faeces and directly from whole faeces. Eight human, 12 cat, 9 dog and 16 cattle faecal isolates were genotyped. Analysis of the *gdh* sequences for those samples revealed a 100% match with GenBank reference sequences with the exception of the three-point mutations in two of the cattle isolates, and two point mutations in one cat isolate.

Evidence for zoonotic transmission of canine *Giardia*

Numerous experimental cross-species transmission studies of *Giardia duodenalis* were undertaken to determine the zoonotic potential of *Giardia* between the 1970s to mid 1990s. Although conflicting, most of these experiments were successful in establishing human isolates of *G. duodenalis* in a variety of animals (Davies and Hibler, 1979) including dogs (Hewlett *et al.*, 1982) cats (Kirkpatrick and Green, 1985), sheep, mice, beavers and gerbils (Majewska, 1994).

Further evidence to support the potential of zoonotic transmission was demonstrated by establishing infection in human volunteers with an isolate of *Giardia* recovered from a wild rat (Majewska, 1994). However, many of these cross-transmission experiments showed conflicting results and provided little evidence on the host or zoonotic specificity of *G. duodenalis.* Poor experimental design and the vast genetic heterogeneity that exists within *G. duodenalis* were implicated as contributing factors for the inconclusive results obtained (Thompson *et al.*, 1990).

The advent of PCR-based procedures has allowed *Giardia* isolates from humans and various animals to be characterized genetically which has lead to the improved understanding of the epidemiology and zoonotic potential of this protozoan. More recently, PCR-based tests capable of amplifying and characterizing isolates of *Giardia* directly from cysts in faeces has lead to a more comprehensive range of genotypes to be examined from humans and animals (Hopkins *et al.*, 1997, Monis *et al.*, 1998, Amar *et al.*, 2002, Read *et al.*, 2002).

The major limiting factor in understanding and interpretation of the genetic heterogeneity within the *G. duodenalis* groups prior to advent of the aforementioned PCR-based tests, has been the refractory growth *in vitro* of many isolates of *Giardia,*

including a significant portion of human as well as dog isolates (Meloni and Thompson, 1987).

Two studies to date have conducted epidemiological studies to ascertain the zoonotic potential of canine *Giardia* by directly comparing isolates of *Giardia* recovered from dogs and humans in the same community.

Hopkins *et al*. (1997) reported that ribosomal RNA sequencing revealed differences between the genotypes of *Giardia* isolates recovered either from humans or from dogs living at the same locality. Analysis was done by recovering *Giardia* cysts directly from feces. Isolates were collected from humans and dogs living in Aboriginal communities. Comparison of the SSU-RNA sequences from 13 human and 9 dog isolates revealed 4 different genetic groups. Group A and B contained all of the human isolates, whereas group C and D consisted entirely of *Giardia* samples recovered from dogs.

Traub *et al*. (2004) shown strong epidemiological and molecular evidence to support the zoonotic transmission of *Giardia* among humans and dogs living in the same community. Feacal samples were collected from 328 humans and 101 dogs from 3 tea estates in Assam, India. The prevalence of *Giardia* in humans was 8.8% using microscopy alone, while in dogs it was 3% by microscopy and 20% by PCR. For epidemiological result, this study has provided the first direct evidence of zoonotic transmission of *Giardia* between dogs and humans, by finding the same genotype of *Giardia* in people and dogs, not only in the same village, but also in the same household. *Giardia* isolates were characterized at three difference loci: the SSU-rRNA, elongation factor 1-alpha (ef1-α) and *tpi* gene. Evidence for zoonotic transmission was supported by strong epidemiological data showing a highly significant association between the prevalence of *Giardia* in humans and the presence of a *Giardia* positive dog in the same household. A major finding of this study was the importance of using multiple loci when inferring genotypes to *Giardia* in epidemiological investigations.

The direct genotypic characterization of *Giardia* isolates has made a major contribution to our understanding of the host specificity of different genotypes. Some *Giardia* isolates appear to have a limited host range, whereas others appear to be

infective to a wide range of host species. This has also allowed a predictive assessment to be made about determining sources of infection in outbreak situations, as well as zoonotic transmission and the likely interaction between life cycles. The greatest zoonotic risk should come from those genotypes of *Giardia* in assemblage A, particularly those in cluster A-I, and to a lesser extent, genotypes in assemblage B. Although the clinical consequences of *Giardia* infections in dogs appear to be minimal, there has been much speculation about the public health significance of such infections in pets. Recent research has shown that some genotypes of this parasite may be shared between humans and dogs, particularly in rural areas (Bugg *et al*., Traub *et al*, 2004).

In this study we apply PCR to amplify and sequence segments of two separate genes encoding the SSU rRNA and triose phosphate isomerase (tpi) from *G. duodenalis* recovered from humans and dogs living in the same locality, in temple including communities in the surrounding area under study in order to ascertain the zoonotic potential of canine *Giardia* under natural conditions.

Treatment

Giardiasis can be treated using a number of drugs including metronidazole, quinacrine, furazolidone, tinidazole, ornidazole and nimarazole. The success of treatment programmes as a means for controlling giardiasis depends on several factors, including the therapeutic efficacy of the drug, the total number of cases that are to be treated, the rate of reinfection after successful treatment, the endemicity of the infection and the sensitivity of the diagnostic technique. In developing countries with high population density and endemic giardiasis, mass diagnosis of cases followed by selective chemotherapy may not be a practical solution, because of limited diagnostic and treatment facilities available in rural areas. However, diagnosis and treatment of symptomatic cases may be undertaken by the health workers at the primary health care level.

CHAPTER IV
MATERIALS AND METHODS

Study site

This study was carried out at 18 temples in Bangkok including communities in the surrounding area. The laboratory works were performed at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University and at the World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections, School of Veterinary and Biomedical Sciences, Murdoch University, Western Australia.

Study period

The study took place from March 2004 to January 2005.

Sample size

Based on a total population of 20,000 dogs and 50,000 humans, a total sample size of 381 humans and 370 dogs were needed for a prevalence of 50% to be predicted within 5% accuracy. Faecal samples were collected from a total of 162 humans and 200 dogs from 18 temples, which also included people living in the community in the surrounding area.

Methods

After informed consent (Appendix A), three questionnaire were administered to the appropriate personnel.

1. Form 1, this questionnaire was concerned with obtaining general information for the temples (Appendix B).
2. Form 2, this questionnaire was concerned with persons who lived in households for getting individual data (including age, sex, gender, defaecation

practices, socio-economic status, crowding). If their child was less than 12 years of age, their parent answered the questionnaire on their behalf (Appendix C).

3. Form 3, this questionnaire was for persons who took care of dogs Information about the dog including their age, sex, habit, diet and also deworming and vaccination (Appendix D) was obtained.

1. Stool collection from humans and dogs was as follows:

1.1 Collection of faecal samples from humans.

1.1.1 After questionnaire, faecal collection container was given to the interviewee, for faecal collection the following day.

1.1.2 The faecal collection procedure was as follows:

1.1.2.1 The faecal sample was collected in a clean container or on clean paper and transferred to a suitable container by applicator sticks at least 5 gm and place into a faecal collection pot and an icebox immediately.

1.1.2.2 Feces deposited on soil are not satisfactory since contaminants from soil will cause confusion in diagnosis.

1.1.2.3 Feces should not be contaminated with urine as this will destroy eggs and larvae and specimen, protected from drying is adequate.

1.1.2.4 The container with the faecal sample was labeled with the interviewee's name (or number code) and date of collection.

1.1.3 All samples were transported to Department of Protozoology, Faculty of Tropical Medicine, Mahidol University for further processing. Faeces were preserved separately in 5% formalin for microscopic screening and immunological tests and 20 %DMSO for molecular testing.

1.2 Collection of faecal samples from dogs.

1.2.1 Dog was restrained with a soft rope leash prior to muzzling for safety.

1.2.2 Approximately 5 gm of faeces was collected per rectum and place into a faecal collection pot and an icebox immediately.

1.2.3 All pots were labeled with identical stickers to the one on the corresponding questionnaire.

1.2.4 All samples were transported to the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University for further processing. Faeces were preserved separately in 5% formalin for microscopic screening and immunological tests and 20 %DMSO for molecular testing.

2. Parasitological methods.

All stool samples were processed as follows:

2.1 Zinc Sulfate flotation.

2.1.1 Feacal suspension in a container was prepared by thoroughly mixing a stool sample about the size of a pea (1 gm) with about 10 times its volume of tap water.

2.1.2 The suspension was strained though a small funnel lined with two layers of wet gauze into a 15 ml centrifuge tube filling it two-third full.

2.1.3 This preparation was centrifuged for two minute at 3,000 x g, making certain that the machine is properly balanced.

2.1.4 The supernatant fluid was poured off.

2.1.5 After pouring off the final washing, zinc sulfate solution was added sufficient to fill the tube half full.

2.1.6 An applicator was used to break up the packed sediment thoroughly.

2.1.7 Additional zinc sulfate solution was added to fill the tube to within one half inch of the top.

2.1.8 This suspension was centrifuged for two minutes at 3,000 x g.

2.1.9 Without shaking or spilling, the tube was placed in a rack carefully.

2.1.10 After waiting one or two minutes, a wire loop was touched to the surface film and place several loopfuls on a clean microscopic slide.

2.1.11 A coverglass was put on, avoiding air bubbles by drawing one edge of the coverslip slightly into the suspension and lowering it almost to the slide before letting it fall.

2.1.12 Cysts were looked under light microscope.

2.2 Purification of *Giardia* cyst using saturated salt and glucose .

All positive samples were processed as follows:

2.2.1 One gram of faeces were placed in a 50 ml faecal pot jar. Then 1 X Phosphate Buffered Saline (PBS) was added to emulsify and liquefy the faeces.

2.2.2 A four-layer thick square piece of surgical gauze (approximately 8 X 8 cm) was placed onto a new 50 ml specimen jar and compressed in the middle. The faecal suspension was poured through the gauze and rinse with 1X PBS to remove any excess material and squeeze with fingertips.

2.2.3 The filtered faecal suspension was poured into a 10 ml centrifuge tube and centrifuge at 3,000 x g for 4 minutes at 20º C.

2.2.4 The supernatant was removed down to the pellet and discarded. Re-suspend the pellet in PBS till the 10 ml mark and mix by inverting the tube and re-centrifuge at 3,000 x g for 4 minutes at 20º C.

2.2.5 The supernatant was removed down to the pellet and discarded. The pellet mixed thoroughly in 2 ml of PBS (If the sample contained lipids, then the pellet will be re-suspended in 8 ml of PBS and 2 ml of ether. Shake the tube vigorously and centrifuged for 8 minutes at 4,000 x g. Then the supernatant was removed down to the pellet and discarded. Add two ml of PBS.

2.2.6 Four milliliters of saturated salt and D-glucose (specific gravity 1.33) were placed into a new 10 ml tube up to the 4 ml mark. Slowly and gently placed 2 ml of washed faecal material on top of the gradient, being careful not to disturb it.

2.2.7 The gradient was centrifuged at 4,000 x g for 20 minutes at 20º C. The braking mechanism was turned off as it will result in disruption of the interphase layer (middle layer).

2.2.8 The cysts were removed from the interphase layer (middle layer) in a circular motion with a disposable pipette and placed into a new 10 ml tube.

2.2.9 The cysts were diluted in 10 ml of PBS and re-centrifuged at 3,000 x g for 5 minutes.

2.2.10 The supernatant was removed into 2 ml mark and put PBS up to 10 ml and mixed by inverting a tube and centrifuged at 3,000 x g for 5 minutes.

2.2.11 The supernatant was removed and allowed the cysts to be re-suspended in 1 ml of PBS in a 1.5 ml tube.

2.2.12 The tube was centrifuged at 8,000 x g for 1 minute and then removed the supernatant down to 300 µl.

3. Molecular methods.

3.1 Isolation of DNA.

All positive samples were extracted using QiAMP DNA Mini Stool Kit (Qiagen GMBH, Hilden, Germany) for isolation DNA. The procedures are as follows:

3.1.1 The eppendorf tubes containing the purified samples were centrifuged at 20,000 x g for 3 min.

3.1.2 The supernatant was removed, discarded, and 1.4 ml of buffer ASL was added to each 20 µl sample and the tube was vortexed.

3.1.3 The sample was freezed-thawing by placing the tubes in boiling water (100^0C), follow by immediately placing the tubes in liquid nitrogen and then boiling for 5-10 min, for 3-5 times per sample in order to rupture cysts.

3.1.4 The sample was incubated in boiling water for a further 10-15 min.

3.1.5 The sample was vortexed for 15 sec and centrifuged at 20,000 x g for 1 min to pellet stool particles.

3.1.6 One point two milliliter of the supernatant was pipetted into a new 2 ml microcentrifuge tube and discarded the pellet.

3.1.7 One InhibitEX tablet was added to each sample and vortexed immediately and continuously for 1 min or until the tablet is completely suspended. The suspension was incubated for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

3.1.8 The sample was centrifuged at 20,000 x g for 3 min to pellet inhibitors bound to InhibitEX.

3.1.9 All the supernatant was pipetted into a new 1.5 ml microcentrifuge tube and discarded the pellet. The sample was centrifuged at 20,000 x g for 3 min.

3.1.10 Fifteen microliters (µl)of Proteinase K was pipetted into a new 1.5 ml microcentrifuge tube.

3.1.11 Two hundred (µl) of supernatant from step 9 was pipetted into the 1.5 ml microcentrifuge tube containing Proteinase K.

3.1.12 Two hundred microliter of buffer AL was added and vortexed for 15 sec.

3.1.13 The sample was incubated at 70^0C for 10 min.

3.1.14 Two hundred microliter of absolute ethanol was added to the lysate, and mixed by vortexing.

3.1.15 The lid of a new QIAamp spin column was labeled and placed in a 2 ml collection tube. The complete lysate from step 14 was carefully applied to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at20,000 x g for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, and discarded the tube containing the filtrate.

3.1.16 The QIAamp spin column was opened carefully and added 500 µl of buffer AW1. The QIAamp spin column was centrifuged at 20,000 x g for 1 min and placed in a new 2 ml collection tube, and discarded the collection tube contraining the filtrate.

3.1.17 The QIAamp spin column was opened carefully and added 500 µl of buffer AW2. The QIAamp spin column was centrifuged at 20,000 x g for 3 min and placed in a new 2 ml collection tube, and discarded the collection tube contraining the filtrate.

3.1.18 The pellets were dried by placing the tubes with the lid open in a vacuum trap until dry.

3.1.19 The QIAamp spin column was transferred and labeled into a new 1.5 ml microcentrifuge tube and pipetted 35 µl of buffer AE directly onto the QIAamp membrane. The QIAamp spin column was incubated for 5-10 min at room temperature, and then centrifuged at 20,000 x g for 1 min to elute DNA.

3.2 PCR Amplification

2 loci were used in this study for all positive samples.

3.2.1 The 18S-rDNA (Sulaiman *et al.*, 2003).

Primary PCR

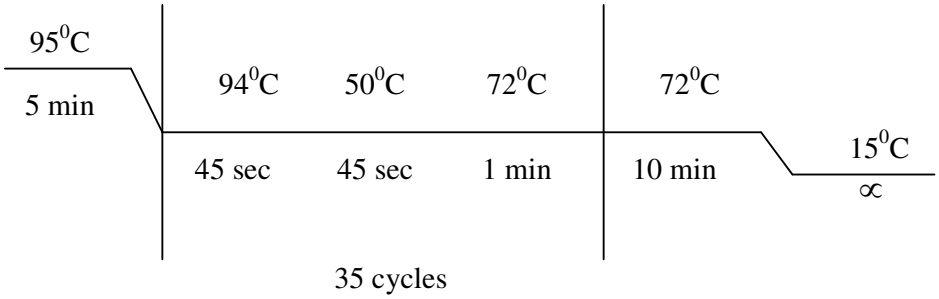
3.2.1.1 A PCR product of 300 bp was amplified by using primers:

- AL4303, forward primer [5′-ATCCGGTCGATCCTGCCG-3′]
- AL4305, reverse primer [5′-AGGATCAGGGTTCGACT-3′]

3.2.1.2 A PCR reaction was comprised of 25 µl reactions

- 200 µM each of deoxynucleoside triphosphate (dNTP).
- 2.5 µl 10X DNA polymerase buffer (Biotech International).
- $MgCl_2$ 1.5 mM (Biotech International).
- 1.5 U of T*th* plus DNA polymerase (Biotech International).
- 12.5 ρmol of RH11 forward and RH4 reverse external primers.
- 1.25 µl of dimethyl sulfoxide (DMSO).
- PCR Grade Ultra Pure Water (Biotech International).
- 1 µl of DNA.

3.2.1.3 The reaction was performed on a Perkin-Elmer GeneAmp 2400 (Perkin-Elmer) thermalcycler as follows:



Secondary PCR

3.2.1.4 The condition for the secondary PCR was same as the primary PCR reaction mixture with 1 exception. The external primers were replaced with

- AL4304 [5-CGGTCGATCCTGCCGGA-3′]
- AL4306 [5′-TGGGACTAAGAGGCGG -3′]

3.2.1.5 After the first round of amplification of the primary tubes, a 1.0 μl aliquot from the primary tubes were added to the corresponding secondary tubes as the template and amplified in a final volume of 25 μl.

3.2.1.6 A 100-bp molecular mass ladder (BioLabs, New England) and positive and negative controls were included in each gel.

3.2.1.7 PCR products were analyzed by 1.5% (w/v) agarose gel in TAE buffer electrophoresis and were stained with ethidium bromide (10 μg/ml) for visualization.

3.2.2 Triose phosphate isomerase gene (Sulaiman *et al*., 2003).

Primary PCR

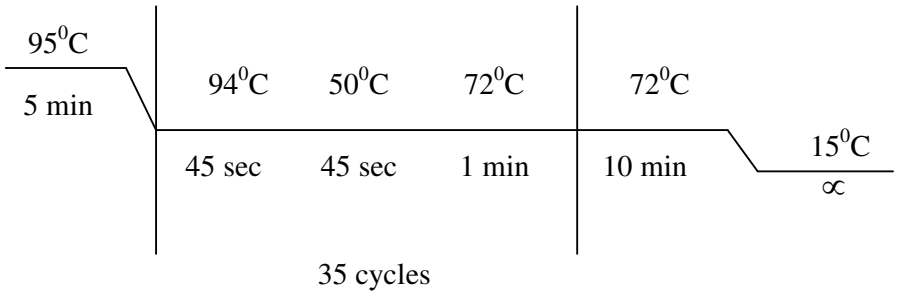
3.2.2.1 A PCR product of 605 bp was amplified by using primers

- AL3543 [5′-AAATIATGCCTGCTCGTCG-3′]
- AL3546 [5′-CAAACCTTITCCGCAAACC-3′]

3.2.2.2 PCR reaction was comprised of a 25 µl reactions

- 200 µM each of deoxynucleoside triphosphate (dNTP)
- 2.5 µl 10X PCR buffer (Perkin Elmer, Wellesley, MA)
- $MgCl_2$ 3.0 mM
- 1.5 U of Taq polymerase (GIBCO BRL, Frederick, MD)
- 12.5 pM of each primer
- PCR Grade Ultra Pure Water (Biotech International).
- 1 µl of DNA

3.2.2.3 The reaction was performed as follow:



Secondary PCR

3.2.2.4 A fragment of 530 bp was amplified by using 1 µl of primary PCR product and primers were used

- AL3544 [5′-CCCTTCATCGGIGGTAACTT-3′]
- AL3545 [5′-GTGGCCACCACICCCGTGCC-3′]

The condition for the secondary PCR was identical to the primary PCR

3.2.2.5 PCR products were analyzed by 1.5% agarose gel electrophoresis and were stained with ethidium bromide for visualization.

3.3 Sequencing of PCR products.

All positive PCR products from 18s-rDNA and *tpi* gene were sequenced according to manufacturer's instructions (GeneWorks, Australia)

3.3.1 DNA band from a TAE agarose gel were cut and determined gel band weight. The following procedures should be carried out directly in the spin filter (for up to 0.2 gm of gel).

3.3.2 Gels were placed in the spin filter basket. Besure gel is resting on the filter. The maximum capacity per spin filter is 0.2 gm.

3.3.3 Three volumes of Gel bind were added to the gel slice for example 0.1 gm agarose require 0.3 ml of Gel bind, then incubated for 2 minutes at 65 ^{0}C

3.3.4 Samples were inverted once, then incubated one minute more or until gel was melted.

3.3.5 Samples were inverted once to mix, then centrifuged spin filter 10 sec at 10,000 x g.

3.3.6 The spin filter was removed and vortex the collection tube for 5 sec to mix the flow though.

3.3.7 All the liquid from collection tube were reloaded back onto the spin filter and centrifuged 10 sec at 10,000 x g.

3.3.8 Liquid were discarded and replaced spin filter basket, then added 300 µl of GelWash buffer.

3.3.9 All samples were spin 10 sec at 10,000 x g, then discarded flow though and spin again for 30 sec at 10,000 x g.

3.3.10 Filter basket were carefully transferred to a clean collection tube, then added 50 µl of Elution buffer directly onto the center of the white spin filter membrane.

3.3.11 Samples were centrifuged 30 sec at 10,000 x g then discarded filter basket.

3.3.12 Samples were run in 1% agarose gel for calculated quantity of DNA to adjust the volume of mixture to use in sequence reaction.

3.3.13 A sequence reaction was comprised 10 µl reactions

3.3.13.1 Quarter reaction

- 2 µl of Dye Terminator reaction mix.
- 1 µl of 5X PCR buffer (Perkin Elmer, Wellesley, MA)
- 1 µl of each primer (Forward/Reward)
- 4.5 µl PCR Grade Ultra Pure Water (Biotech International).
- 1.5 µl of DNA

3.3.13.2 Half reaction

- 2 µl of Dye Terminator reaction mix.
- 1 µl of 5X PCR buffer (Perkin Elmer, Wellesley, MA)
- 1 µl of each primer (Forward/Reward)
- 3 µl PCR Grade Ultra Pure Water (Biotech International).
- 3 µl of DNA

3.3.14 The sequence reaction was performed as follows:

96^0C	96^0C	50^0C	60^0C	
2.20 min	10 sec	5 sec	4 min	15^0C ∝

48 cycles

3.3.15 The reaction was then centrifuged briefly and transferred to a 600 µl tube.

3.3.16 Twenty-five microlitter of 95% cold ethanol and 1 µl of 3M sodium acetate was added to the reaction, mixed well and placed on ice for 20 minutes to allow the DNA to precipitate.

3.3.17 The reaction was spin at 20,000 x g for 30 minutes to pellet the DNA.

3.3.18 The ethanol was then removed and the pellet washed with 70% cold ethanol, re-centrifuged for 10 minutes, the ethanol removed, and pellet vacuum dried.

3.4 Sequence analysis and genotyping.

3.4.1 Sequence results were analysed using Seq Ed (Applied Biosystems).

3.4.2 Miscalled bases were corrected by analyzing chromatogram peaks and comparing these to published sequences.

3.4.3 The unknown sequences were aligned with known published sequences of the major genotypes using Clustal W (Thompson *et al*., 1994).

3.4.4 The genotypes of samples were determined based on this comparison and phylogenetic analysis were used Mega 2.1 (Kumar *et al*, 2001), then Tamura-Nei distance estimates of aligned nucleotide sequences derived from PCR products of SSU-rDNA fragment.

3.5 PCR Amplification to screening for *Giardia*

100 dogs and 80 human that negative for *Giardia* on microscopy were screened randomly using PCR. The procedures are as follows:

3.5.1 The 18S-rDNA (Read *et al.*, 2002).

Primary PCR

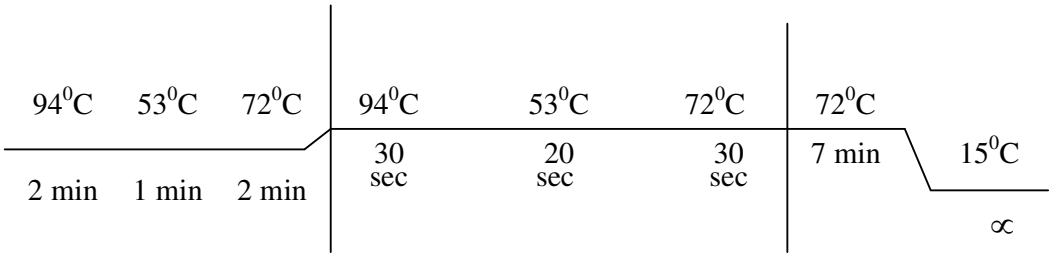
3.5.1.1 A PCR product of ~130 bp will be amplified by using primers:

- RH11, forward primer [5′-CATCCGGTCGATCCTGCC-3′]
- RH4, reverse primer[5′AGTCGAACCCTGATTCTCCGCCAGG-3′]

3.5.1.2 A PCR reaction will be comprised 25 μl.

- 200 μM each of deoxynucleoside triphosphate (dNTP).
- 2.5 μl of 10X DNA polymerase buffer (Biotech International).
- $MgCl_2$ 1.5 mM (Biotech International).
- 1 U of T*th* plus DNA polymerase (Biotech International).
- 12.5 ρmol of RH11 forward and RH4 reverse external primers.
- 1.25 μl of dimethyl sulfoxide (DMSO).
- PCR Grade Ultra Pure Water (Biotech International).
- 1 μl of DNA template.

3.5.1.3 The reaction will be performed on a Perkin-Elmer GeneAmp 2400 (Perkin-Elmer) thermo-cycler as follows:



Secondary PCR

3.5.1.4 After the first round of amplification of the primary tubes, a 1.0 μl aliquot from the primary tubes was added to the corresponding secondary tubes as the template and amplified in a final volume of 25 μl.

3.5.1.5 The conditions for the secondary PCR were the same as the primary PCR reaction mixture but the following primers were used. The external primers were replaced with

- GiarF [5′-GACGCTCTCCCCAAGGAC-3′]
- GiarR [5′-CTGCGTCACGCTGCTCG-3′]

3.5.1.6 A 100-bp molecular mass ladder (BioLabs, New England) and positive and negative controls were included in each gel.

3.5.1.7 PCR products were analyzed by 1.0% (w/v) agarose gel in TAE buffer electrophoresis and were stained with ethidium bromide (10 μg/ml) for visualize.

3.6 Immuno-fluorescence test to screening for *Giardia* (O'Handley *et al*., 1999)

Twenty samples positive for *Giardia* by PCR but that were negative by microscopy were randomizly selected to screen for *Giardia* by this method. The procedures are as follows:

3.6.1 Faecal sample was filtered though a surgical gauze sponge, then washed with PBS and expressed from the gauze to yield approximately 7 ml of filtrate.

3.6.2 The filtrate was layered over 5 ml of 1M sucrose (specific gravity 1.13) to clarify the sample.

3.6.3 The sample was then centrifuged at 800 x g for 5 minutes in a fixed rotor centrifuge to concentrate parasite cyst at the sucrose-water interface.

3.6.4 The interface and upper layer of liquids were transferred by pipette to a clean tube and centrifuged at 800 x g for 5 minutes.

3.6.5 The supernatant was decanted, and the pellet was suspended in PBS to a volume of 1 ml.

3.6.6 Two drop of 15 μl samples of the concentrate were then spotted on a microscope slide 2 spots (duplicated), and the slide was air-dried for 30 minutes on a 37 ^{0}C slide warmer.

3.6.7 The slide was then fixed with acetone for 1 minute and left to dry.

3.6.8 A *Giardia*-specific fluorescein isothiocyanant-labeled monoclonal antibody solution (2 µl) was placed on 1 of the spotted samples.

3.6.9 The slide was incubated in a humidity chamber at 37^0C for 45 minutes, excess antibody was gently washed off with PBS, and the slide was left to air-dry.

3.6.10 Slides were mounted with a fluorescent antibody mounting fluid and a cover slip.

3.6.11 Cysts were examined and enumerated at 100X and 400 X magnifications, respectively, using an epifluorescence microscope.

3.7 Statistical Analysis

Uni-variate risk factors for the prevalence of *Giardia* in dogs and humans and host, behavioral and environmental factors were initially made using SPSS version 12.0. Only variable significant at $p \leq 0.25$ in the uni-variate analyses were considered eligible for multivariate risk factors (SPSS version 12.0).

CHAPTER V
RESULTS

Population structure of dogs in the study

1. Age of dogs

The majority of dog population surveyed was generally adult between 1 to 7 years of age, followed by young adult dog, puppy and older dog respectively. The ages of immature dogs could be determined by dental examination. Most owners could not provide the exact age of mature dogs. Ninety-one percent of dogs belonged to temple dogs and 9% belonged to households.

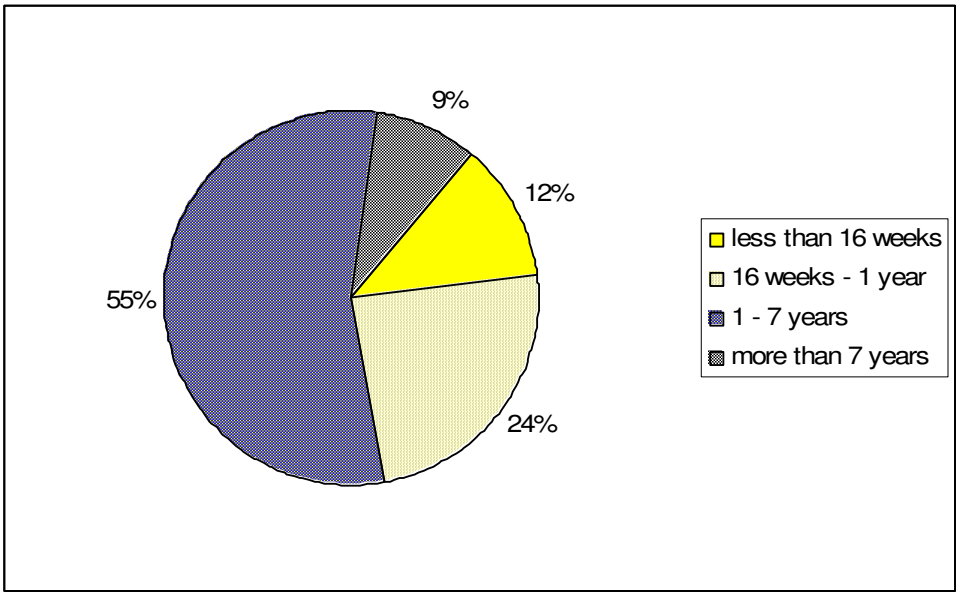


Figure 2. The relative proportion of dogs in each age group that participated in the survey.

2. Sex of dogs

The overall percentages of dogs sample were 63% females and 37% males in this study. Entire male and female dogs sample were 30.5% and 47% respectively. One percent of female dogs were lactating.

3. Dog breeds

Ninety-five percent of dogs that participated in this study were of mixed breeds. Of the purebred dogs, two were Rottweilers, one was a Miniature Poodle and another one was a Miniature pinscher that belonged to temple dogs. Only 3 dogs, two that were Shih-Tzus and one that was a Miniature Schuauzer belonged to a household in the surrounding area.

Results of the questionnaire

1. Questionnaires aimed at human data

The majority of the human populations surveyed were adults. Males comprised 73.5% and female 26.5% females in this study. Most of them were monks (73%)**,** and 28% were people in the surrounding area. The level of education in this study was 9% below primary school, 40% primary school, 28% secondary school and 23% tertiary school. For lavatory facilities, most of them defecated indoor (99%), only 1% defecated out door. Sixty-four percent of humans drunk bottled water while 36% drunk tap water but only 21% that drunk tap water was boiled water all the time before used. The majority of people did not swim in the river (93%). Most of them had never been dewormed (65%). Most people thought that they had a good health (79%) followed by very healthy (9%) and 12% claimed that they are unhealthy.

2. Questionnaires aimed at dog data

Most dogs (71%) defaecated within the temple complex and most of the owners (71%) disposed of the faeces daily and most of them disposed of the faeces in the rubbish bin (74%). Over 40% of dogs were allowed to scavenge for food. The majority of dogs (82%) were vaccinated against rabies but only 11% were dewormed.

3. Knowledge of participants about zoonotic diseases

Forty - eight percent of individuals admitted having contact with dogs on a regular basis but only 31% of individuals were aware that parasites could be transmitted from dogs to human.

Parasite prevalence results

1. Results of microscopic screening of *Giardia*

The overall prevalence of *Giardia* in dogs was 9% (95%CI = 5.07%-12.93%). Age was found to be the only significant risk factor for infection in dogs. Dogs < 1 year old were more likely to be infected with *Giardia* than dogs > 1 year old (OR= 3.1, 95%CI = 1.15%-8.5%, p=0.02). The overall prevalence *Giardia* in humans was 3% (95%CI = 0.1%-4.9%). All *Giardia* positive individuals were children below age of 6 years of age. Apart from one child suffering from anorexia no other children complained of any associate clinical signs of giardiasis.

2. Results of the nested PCR technique for screening canine *Giardia*

Seventy-five percent (95%CI = 69.03%-80.97%) and 60% (95%CI = 52.46%-67.54%) of dogs and humans were found to be positive for *Giardia* by PCR compared to 9% (95%CI = 5.07%-12.93%) and 3% (95%CI = 0.1%-4.9%) by microscopy, respectively. No samples found negative by PCR were positive using microscopy.

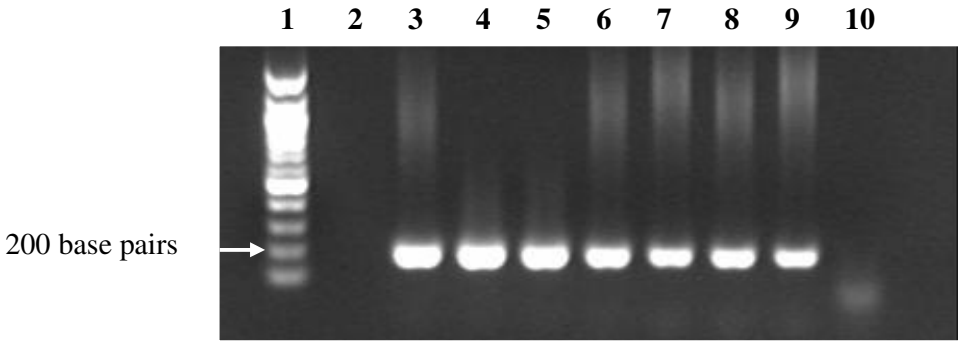


Figure 3. Agalose gel electrophoresis of PCR products for *Giardia* by 18S nested PCR (Read *et al*, 2002), stained with ethidium bromide. Lane 1, ladder; lane 2-8,sample number 1-7 (Note: sample in lane 2 was negative while sample in lane 3-8 were positive) ; lane 9, positive control; lane 10, negative control.

3. Result of the Immunofluorescence test to screening for *Giardia*

Nine from 20 microscopy negative but PCR positive samples were positive for *Giardia* by IF. However most of them were very low number of cysts in one slide (20 μl).

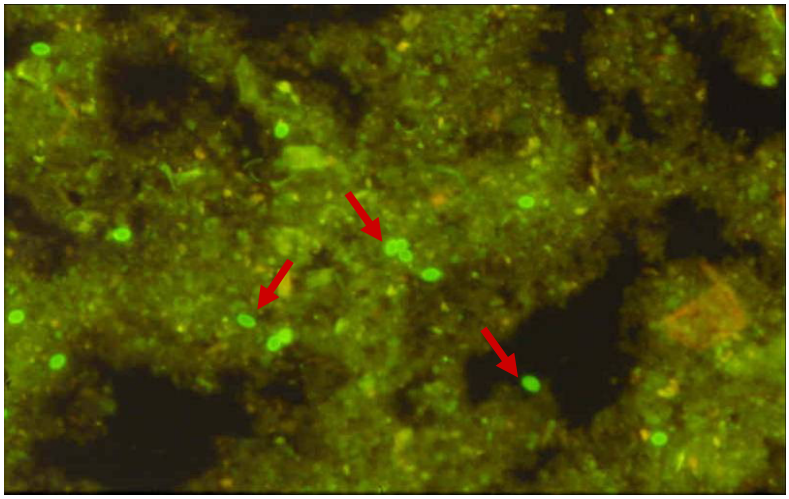


Figure 4. Immunofluorescence positive for *Giardia*; arrows shown *Giardia* cyst complemented with monoclonal antibody.

Result on *Giardia* incidence VS time to collect sample.

Samples were collected from 18 temples including communities in the surrounding area from June to September. Prevalence results based on the aforementioned PCR results of both humans and dogs were graphed over time of collection of the faecal samples. The prevalence of *Giardia* before the onset of the monsoon season was found to be low compared to the days following onset of the rainy season. There was a sudden and rapid increase in the prevalence of *Giardia* in both dogs and humans approximately a week after the onset of the monsoon season.

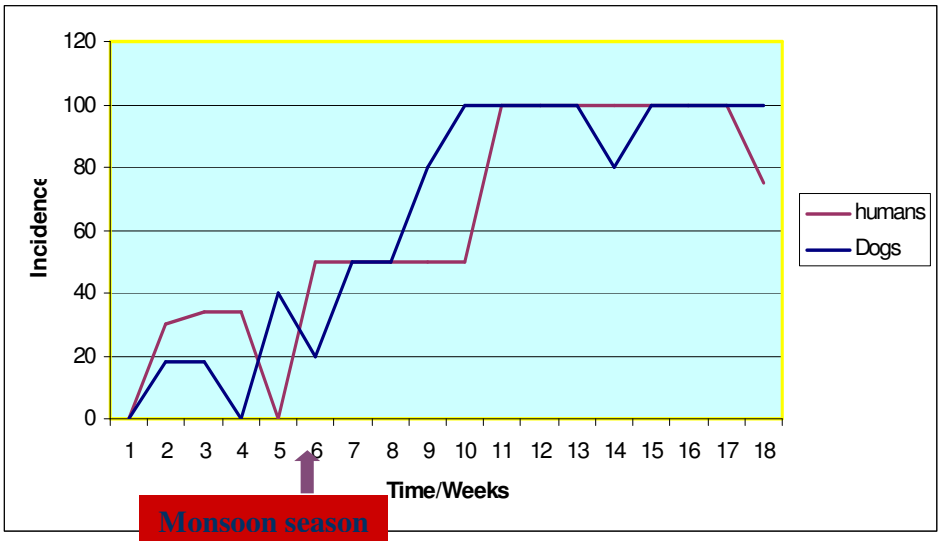


Figure 5. Graph shown the prevalence of *Giardia* positive by PCR were increased after monsoon was started in both humans and dogs populations; X axis, incidence of *Giardia* positive by PCR; Y axis, time to collect sample in each temple.

Molecular characterisation and phylogenetic analysis of *Giardia* isolates found in humans and dogs

For the SSU- rDNA gene, human isolates were grouped into Assemblages A, BIII while dog isolate were grouped into Assemblages A, B (zoonotic genotypes) and C, D (dog genotypes)

Table 3 summarises the amplification and genotyping results for the *Giardia* positive human samples at the 18S loci used in this study. Of the 4 *Giardia* positive human samples, the SSU-rDNA was amplified and genotypes characterised from 3 (75%) samples.

Table 3. Summary of genotype results of *Giardia* isolates recovered from humans at 18S loci.

Human isolate	SSU-rDNA
T8P7	A
T8P8	A, BIII
T9H22P1	A

Table 4 summarises the amplification and genotyping results of the 18 *Giardia* positive dog samples amplified at the SSU-rDNA. Genotypes were characterised in 13 (72%) of these dogs.

Table 4. Summary of genotype results of *Giardia* isolates recovered from dogs at 18S loci.

Dog isolate	SSU-rDNA	Human isolate
T2D1	D	
T2D2	A, BIII	
T4D1	A	
T4D5	A, BIII	
T5D10	A	
T5D11	A	
T8D10	A	T8P7, T8P8
T9D2	D	
T11D3	A	
T11D7	D	
T13D3	C	
T13D14	A, BIII	
T13D15	A	

Phylogenetic analysis of the SSU-rDNA data placed all of the dog isolates into the Assemblage A, B, C and D clusters and human isolates into Assemblage A and B cluster but was poor at resolving the relationships of the various assemblages (Figure 4). The majority of dog population were Assemblage A following by Assemblage B, D and C respectively. Three dog had mixed infection with Assemblage A and BIII (Table 4) and only T8D10 were associated with human isolates. The majority of human *Giardia* isolates clustered into Assemblage A, only T8P8 had mixed infection with Assemblage A and BIII (Table 3).

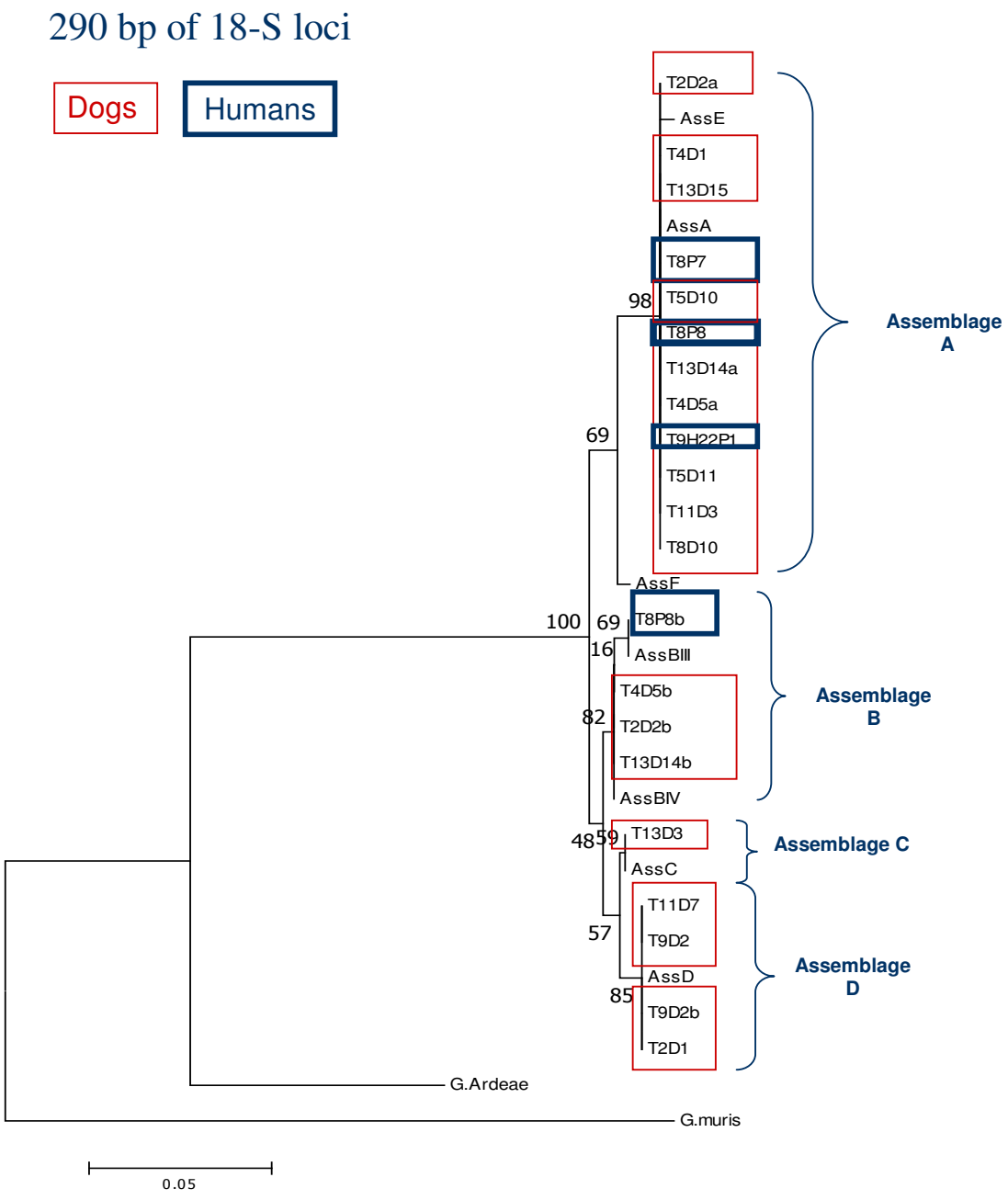


Figure 6. Phylogeny of the *Giardia* isolates inferred by distance based analysis using Tamura-Nei distance estimates of alined nucleotide sequences derived from the PCR products of the SSU-rDNA gene.

Risk factor analysis based on PCR results

Multi-variate risk factor analysis revealed that dogs belonging to the temple were significantly more likely to be infected with *Giardia* than household dogs (OR= 17, 95%CI = 1.35%-97.84%, p=0.01) (Table 5). Dogs allowed outside the house into the temple compound were more likely to be infected with *Giardia* when compare with dogs confined indoors (OR= 5.6, 95%CI = 1.65%-10.26%, p=0.01) (Table 6). No clinical signs correlated with positive humans and dogs with *Giardia* infection. No risk factors of significance were found for humans positive for *Giardia* using PCR technique.

Table 5. The SPSS analysis result according to *Giardia* PCR technique when temple dogs compare with household dogs.

Place	*Giardia* result		Odd ratio	P-value
	positive	Negative		
Dogs belonged to Temple	81	102	17	0.01
Dogs belonged to household	1	16		

Table 6. The SPSS analysis result according to *Giardia* PCR technique when dogs allowed outside the house into the temple compound compare with dogs confined indoor.

Pattern	*Giardia* result		Odd ratio	P-value
	positive	Negative		
Dogs allowed outside the house into the temple compound	77	95	5.6	0.01
Dogs confined indoor	5	23		

CHAPTER V
DISCUSSION

Based on a total population of 20,000 dogs and 50,000 humans, a total sample size of 381 humans and 370 dogs were needed for a prevalence of 50% to be predicted within 5% accuracy. Faecal samples were collected from only 162 humans and 200 dogs and therefore the targeted sample sizes could not be successfully collected, from either dog or the human participants.

The primary reasons for non-response in humans included:

- Embarrassment.
- Suffering from constipation.
- Uncooperative and forgetful.

The primary reasons for non-response in dogs included:

- Managing to escape restraint during the collecting time.
- Empty or bone-filled rectum unsuitable for faecal collection.
- Non-response on behalf of the monk or household members.
- Dog was too aggressive or distressed to be restrained for faecal collection.

  Another reason was the time limitation of this study

In present study, under microscopy the overall prevalence of *Giardia* was 3% (95%CI = 0.1%-4.9%) among humans and 9 % (95%CI = 5.07%-12.93%) among dogs, however 75% (95%CI = 69.03%-80.97%) and 60% (95%CI = 52.46%-67.54%) of dogs and humans respectively were found to be positive for *Giardia* by PCR technique. This is a first study to reveal the highly endemic prevalence of *Giardia* in humans and dogs in a developing community using PCR.

The high levels of PCR positives and lack of confirmation by microscopy may have arisen from contamination. However, this was considered unlikely since a nested PCR procedure (Read *et al*., 2002) and appropriate controls were used. The second possible explanation was that low numbers of cysts were present in the faeces and microscopy was not sensitive enough to detect these low levels. Similar observations have been reported by Collins *et al*, (1987). The limitation of microscopy techniques, with respect to sub-clinical levels of *Giardia* infection, was illustrated in their study when a skilled microscopist detected only 2 *Giardia* positive samples out of 32 samples positive for *Giardia* using PCR technique. For both samples positive by microscopy, there was only one cyst detected on the slide (McGlade *et al*., 2003).

In a similar study McGlade *et al.* (2003) determined the prevalence of *Giardia* in 40 faecal samples from domestic cats in the Perth metropolitan area by using microscopy, PCR and *Giardia* CELISA test. The results showed the prevalence of *Giardia* was 5, 80 and 60% by the tests, respectively and demonstrated that more sensitive techniques such as PCR maybe necessary, and may yield more reliable results, in the detection of low levels of *Giardia* in animals.

PCR-based tests capable of amplifying and characterizing isolates of *Giardia* directly from cysts in faeces has lead to a more comprehensive range of genotypes to be examined from humans and animals (Hopkins *et al.*, 1997, Monis *et al.*, 1998, Amar *et al.*, 2002, Read *et al.*, 2002). The major limiting factor in understanding and interpretation of the genetic heterogeneity within the *G. duodenalis* groups prior to advent of the aforementioned PCR-based tests, has been the refractory growth *in vitro* of many isolates of *Giardia,* including a significant portion of human as well as dog isolates (Meloni and Thompson, 1987). This is a first study to prove and break-though of using PCR to amplify *Giardia* cysts directly from faeces and provide a superior result in detecting *Giardia* from faeces.

This study can suggest that PCR could be as gold standard for screening *Giardia* because of the primer was very sensitive and specific however IF and

CELISA should be used to back up the result. Also diagnostic methods utilized depend highly on purpose of the study. If the aim of study is to determine morbidity of giardiasis in clinically affected individuals (eg. children, elderly, immunologically naieve) then the intensity of *Giardia* cysts may be a better indicator and microscopic screening would be an appropriate diagnostic tool.

In this study immunofluorescence method was used to support the PCR results. Nine from 20 samples were positive to this test. From this result could tell that this PCR result was true positive but the majority of dogs and humans were shedding low cyst numbers. However, irregular cyst excretion may partially account for this finding and therefore dogs may still provide a significant zoonotic risk.

Based on result of *Giardia* incidence versus time to collect sample. The prevalence of *Giardia* before the onset of the monsoon season was found to be low compared to the days following onset of the rainy season. There was a sudden and rapid increase in the prevalence of *Giardia* in both dogs and humans approximately a week after the onset of the monsoon season. Therefore the onset of the rainy season may be a significant risk factor for the prevalence of *Giardia* in this study. However this is only preliminary result and further sampling during this season (summer-monsoon) is required to further validate these results.

The genetic characterisation of *Giardia* isolates recovered from dogs and humans provides supporting evidence for the occurrence of both zoonotic and non-zoonotic transmission in this localised endemic focus. Analysis of the SSU-rDNA sequence data showed dog populations have 2 cycles of *Giardia* transmission. The first was a zoonotic cycles in which *Giardia* isolates belonging to Assemblages A and B were cycling among dogs and presumably humans, similar to previous study in the tea growing communities in India, where it was found that human genotype predominates in Assemblage A and B (Traub *et al.,* 2004). The second was dog specific cycles which dogs isolates of *Giardia* placed within Assemblage C and D

similar to previous study in Aboriginal communities in Australia, where it was found that the dog genotype predominates in infected dogs (Hopkins *et al.*, 1997).

This study has both zoonotic and non-zoonotic cycles of *Giardia* circulating among dogs in this community. Dogs in the temple community roamed within packs with a high level of dog to dog contact. Dogs in this group were therefore shedding and transmitted *Giardia* Assemblage C and D from dogs to dogs. Secondly dogs also had close contact with human populations within this community and therefore were cycling *Giardia* isolates from Assemblage A and B both to and from humans and also from dogs to dog. Therefore, this study is different from Hopkins *et al.* (1997) because dogs in Aboriginal communities in Australia tend to stay together in packs for much of the time enhancing possibilities for dog to dog transmission. On the other hand, Traub *et al*. (2004) showed that because dogs in the tea growing communities had predominantly dog-human contact and also had access to human faeces (Traub *et al.*, 2002), zoonotic forms of *Giardia* predominated within the dog population.

Despite efforts of optimization, the TPI loci failed to amplify Giardia from microscopy positive faecal samples and sequence information could therefore not be analysed and compared with SSU-rDNA gene. The possible reasons for failure included:

- Poor primer and PCR design – primer length too short, conserved.
- Low sensitivity.

  TPI gene was a gene in genome that coded for an enzyme and therefore had low copy numbers in the genome (vs ribosomal genes that have high copy numbers).
- Low specificity – cross reaction with many other faecal elements producing non-specific bands. Preferential primer usage for amplifying non-specific products, annealing temperature too low, $MgCl_2$ too high, poor primer design – too conserved, primer length too short.

- Inhibition from faecal contents.

  This study we extracted DNA from faeces and despite purification and extraction using QiAMP DNA Mini StoolKit (Qiagen), molecules such as salts and bilirubin etc. were present that could interfere and inhibit the PCR.

When looking at the molecular characterization, the majority of dogs were cluster into Assemblage A following by Assemblage B, D and C respectively, while humans were placed into Assemblage A and B. Then dogs in temple communities posing moderate zoonotic risk to humans with regards to transmission of *Giardia,* especially Assemblage A (Table 4). Interestingly, Traub *et al*. (2004) also found *Giardia* isolates from dogs within Assemblage A to be the most significant zoonotic risk.

In the present study multivariate risk factor analysis revealed that dogs belonged to temple significantly more likely to be infected with *Giardia* than household dogs and also dogs allowed outside the house into the temple compound more likely to be infected with *Giardia* when compare with dogs confined indoors (Table 5, 6). This result could indicate that the transmission of *Giardia* within dogs in this community was primarily direct via dog to dog contact and were more important than other sources of infection such as contamination via water or food.

The majority of *Giardia* positive individuals in this study, regardless of the method of detection, were clinically asymptomatic. Previous studies have shown that most of humans positive for *Giardia* were suffering from chronic malabsorption (Jahidi, 2003). Malabsorption syndrome is especially significant in children with low nutritional status. In another study, *Giardia* infection in children was demonstrated to not necessarily be accompanied by diarrhoea (Read *et al.*, 2002). Also this study the majority of participants were adult male, therefore they may already have immunity against *Giardia* that why they not shown to be any clinical symptoms. However when looking at the microscopy result, all *Giardia* positive individuals were children below

age of 6 years of age, therefore intensity of this parasite in children were high and could detected by microscopy.

For prevent and control of giardiasis in this study. All of participants were clinically asymptomatic, so antiparasitic treatment for people in this communities is not necessarily advocated, however the following advice for preventing further transmission is recommended.

Humans

- Health education such as prevention of zoonotic disease from dogs such as rabies, hookworm, *Toxocara* and *Giardia*.
- Sanitation Hygiene such as wear footwear all the time when go out, wash your hand every time after contact with dog or after toilet, boiled water all the time before drink, disposed of the faeces daily in the rubbish bin (not in river) etc.

Dogs

- Population control.
- Sterilization.
- Vaccination.
- Community awareness-responsible pet ownership.

In the future, the number of samples must be increase to obtain more significant epidemiological results and stronger risk factors to be compiled. IF test for screening of both positive and negative *Giardia* by PCR to support PCR result or screening by other method such as CELISA is required. PCR products of 18S gene from microscopy negative samples will be sequenced to role out contamination or cross-amplification of other faecal organisms. Samples that positive from PCR screening will be genotyped and characterized to get more results that could be correlated to zoonotic transmission in these communities. Also, Development of highly sensitive and specific PCR using other loci that is semi variable such as Internal Transcribed Spacer gene will prove highly useful for unraveling zoonotic relationships associated with *G. duodenalis* among dogs and humans.

CHAPTER IV
CONCLUSION

In conclusion:

1. This is a first study to reveal the highly endemic prevalence of *Giardia* in humans and dogs in a developing communities using PCR technique.
2. This is a first study to show dog population has 2 cycles of *Giardia* transmission, both zoonotic cycle and dog-specific cycle in the same community.
3. Dogs in temple communities posing moderate zoonotic risk to humans with regards to transmission of *Giardia,* especially Assemblage A.
4. The main risk factor for transmission of *Giardia* among dogs is via direct contact. Dogs belonging to temples are significantly more likely to be infected with *Giardia* than household dogs and also dogs allowed outside the house into the temple compound are more likely to be infected with *Giardia* when compare with dogs confined indoors.
5. The prevalence of *Giardia* increases significantly among both dogs and humans following onset of the monsoon season.
6. Majority of individuals in this study were clinically asymptomatic because majority of participants in this study were adult male and they may have immunity for *Giardia* already.
7. Future study will be needed more highly variable loci to provide more definitive evidence for zoonotic transmission in these and other communities.

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APPENDIX

APPENDIX A

INFORM CONSENT FORM

Project Title: The epidemiology of canine gastrointestinal parasitic zoonoses in different community settings in Thailand

Murdoch University in Western Australia is collaborating with Mahidol University and the Phramongkutklao College of Medicine to investigating the epidemiology of canine gastrointestinal parasitic zoonoses in different community settings in Thailand. The purpose of this study is to find out the risks associated with humans acquiring parasites from their pet dogs as well as stray dogs.

You can help in this study by consenting to complete a survey about yourself and your pet dog where appropriate. It is anticipated that the time to complete the survey will be no more than 20 minutes. You will also be requested to donating a stool sample and allow a stool and blood sample to be collected from your dog/s. Sample collection in your dog will be conducted by a qualified Veterinary Surgeon or technician and will be done in a fashion that provides minimal distress to your dog. Contained in the survey are questions about level of education, income, health and other questions which may be seen as personal and private. Participants can decide to withdraw their consent at any time. All information given during the survey is confidential and no names or other information that might identify you will be used in any publication arising from the research.

If you are willing to participate in this study, could you please complete the details below. If you have any questions about this project please feel free to contact Dr Yaowalark Sukthana on 09-117-0955, Dr Mathirut Mungthin on 01-7353737 or Dr Rebecca Traub +61-8-93606954. We will be happy to discuss with you any concerns you may have on how this study has been conducted.

****

I (the participant) have read the information above. Any questions I have asked have been answered to my satisfaction. I agree to take part in this activity, however, I know that I may change my mind and stop at any time.

I understand that all information provided is treated as confidential and will not be released by the investigator unless required to do so by law.

I agree that research data gathered for this study may be published provided my name or other information which might identify me is not used.

Participant/Authorised Representative:

Date:

Investigator (Murdoch University):

Date:

Investigator's Name: Dr Rebecca Traub

APPENDIX B

QUESTIONNAIRE FORM 1

QUESTIONNAIRE PART ONE – GENERAL INFORMATION FOR TEMPLE SETTING

(circle appropriate answer, tick a box or fill in the blanks)

☐ (Code)

Date: _____

Temple Name: _____

Name of interviewer: _____

1. Size of the temple.

1. Small	
2. Medium	
3. Big	

2. Is the temple located within 200 meters from the banks of the Chao Praya River? 1. Yes 2. No

3. Number of Monks residing in temple _____

4. Number of Houses within the confines of the temple

_____

5. What animals are kept at this temple and approximately how many?

ANIMAL	APPROXIMATE NUMBER
1. Dogs	
2. Cats	
3. Chickens	
4. Ducks	
5. Pigs	
6. Other _____	
7. Other _____	

APPENDIX C
QUESTIONNAIRE FORM 2

QUESTIONNAIRE TEMPLE PART TWO – INDIVIDUAL DATA

(Circle appropriate answer, tick a box or fill in the blanks)

(Code sticker)

Name /Code Number of individual: _____

Temple Name /Code Number: _____

Address / House number: _____

Date: _____

Name of interviewer: _____

If individual is a child less than 12 years of age, please ask parents to answer questions on behalf of the child where questions are applicable

1. Age of individual: _____Years

2. Gender 1. Male 2. Female

3. Religion 1. Buddhist 2. Hindu 3. Christian 4. Muslim 5. OTHER, Please specify, _____

4. Level of education attained (If child under the age of 12 years then note mother's level of education.

1. None	
2. Primary	
3. Secondary	
4. Tertiary	

5. Occupation (If child < 12 years, father's occupation):

_____

6. Monthly household income (Baht)

1. < 5000	
2. 5,000-10,000	
3. 10,000-20,000	
4. > 20,000	

7. How many people reside permanently in your household?

_____

8. What lavatory (toilet) facilities do you utilize?

1. Indoor toilet attached to a septic tank / sewage	
2. Indoor toilet that disposed or flushed into a river / pond	
3. Outdoor pit latrine	

9. What is your main source of water for drinking?

1. Buy bottled water	
2. Piped	
3. Collect from rain water tank	
4. Collect from river	

10. What is your main source of water for household use?

1. Piped	
2. Collect from rain water tank	
3. Collect from river	

11. How often do you boil or filter your drinking water?

	Boil	Filter
1. All the time		
2. Sometimes		
3. Rarely		
4. Never		

12. Do you bath or swim in the Chao Praya River? 1. Yes 2. No

13. Have you suffered from any of the following symptoms in the past one week?

1. Upper abdominal pain or discomfort	
2. Gurgling abdomen	
3. Diarrhea	
4. Nausea	
5. Indigestion	
6. Malaise or general weakness	
7. Anorexia	
8. Weight loss	

14. How would you categorize your general health status?

1. Very Healthy	
2. Healthy	
3. Sick	
4. Very sick	

15. How often do you take de-worming medication?

Frequency		**Anthelmintic name, if known**
1. At least every 6 months		
2. At least once a year		
3. Rarely		
4. Never		
5. Unsure		

16. Do you own or feed a dog on a regular basis?

1. Yes No

17. How often do you come in close contact with dogs (petting, playing or feeding)?

1. Daily	
2. 2-3 times a week	
3. Once a week	
4. Sometimes (1-3 times a month)	
5. Rarely	
6. Never	

18. If you have contact with dogs, how often do you wash your hands after touching the dog?

1. At all times	
2. Sometimes	
3. Rarely	
4. Never	
5. Not applicable	

19. Are you aware that internal parasites can be transmitted between dogs and humans?

1. Yes (If yes, go to Q 20) 2. No

20. If you are aware that parasites can be caught from dogs, how can people become infected?

Patting or cuddling the dog	
Letting the dog lick you	
From cleaning the dog's faeces	
Contacting the soil or grassed area where the dog has been roaming	
Inhaling the dog's breath	
Other, please specify, _____	

APPENDIX D

QUESTIONNAIRE FORM 3

QUESTIONNAIRE TEMPLE PART THREE – DOG DATA

(Circle appropriate answer, tick a box or fill in the blanks)

			(Code)

Address or code number of temple: _____

Owner's Name (if applicable): _____

House code number (if applicable): _____

Dog code number(s): _____

Date: _____

Name of Veterinarian / Interviewer: _____

1. The dog's name or markings

Dog 1	Dog 2	Dog 3

2. Approximate age of dog

Age	Dog 1	Dog 2	Dog 3
1. Puppy: ≤ 16 weeks			
2. Young adult: 16 wk - 1 year			
3. Adult: 1 -7 yrs			
4. Elderly: > 7 years			

3. Gender of dog

Sex	Dog 1	Dog 2	Dog 3
1.Male entire			
2.Male castrated (Neutered)			
3.Female entire			
4.Female sprayed (Neutered)			
5.Female pregnant			
6.Female lactating			
7.Female unknown			

4. Purebred or crossbred

	Dog 1	Dog 2	Dog 3
1. Purebred			
2. Crossbred			

5. How many dogs are fed on a regular basis in this temple or household?

_____

6. Where is the dog allowed access?

	Dog 1	Dog 2	Dog 3
1. Allowed to roam freely including outside the temple complex			
2. Supervised at all times when outside the temple			
3. In the backyard (Inside the temple only)			
4. Inside the house			

7. Where does the dog/s most commonly defaecate?

	Dog 1	Dog 2	Dog 3
1. Indoors			
2. Within the temple grounds			
3. Outside temple			

8. If the dog/s defaecate indoors or in the temple complex, how often do you pick up or clean the faeces?

1. Daily	
2. 2-3 times a week	
3. Once a week	
4. Once in 2 weeks	
5. Never	
6. Other	

9. If you pick up the faeces, where do you dispose of them?

_____

10. What type of food do you feed the dogs?

1. Canned or packaged pet food	
2. Prepared food cooked especially for dog	
3. Same as your food – left-over	
4. Nothing – leave dog to scavenge for itself	
5. Other, please specify _____	

11. Do the dogs receive veterinary attention?

If yes, Name and contact details of Veterinarian

	Dog 1	Dog 2	Dog 3
1. Yes			
2. No			

Name and Address of Vet:

_____

_____

12. Has your dog ever been vaccinated against rabies?

	Dog 1	Dog 2	Dog 3
1. Yes			
2. No			
3. Not sure			

13. Has your dog even been treated for worms?

	Dog 1	Dog 2	Dog 3
1. Yes			
2. No			
3. Not sure			

14. If yes, do you know what the name of the medication is? Please, specify

_____

BIOGRAPHY

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