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THESIS

PREVALENCE OF *GIARDIA DUODENALIS* AND FACTORS ASSOCIATED WITH ITS INFECTION OF WATER BUFFALOES IN NORTHEAST THAILAND

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A Thesis Submitted in Partial Fulfillment of
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Benjamas Khurajog 2014: Prevalence of *Giardia duodenalis* and Factors Associated with Its Infection of Water Buffaloes in Northeast Thailand. Master of Science (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Sathaporn Jittapalapong, Ph.D. 61 pages.

Giardia duodenalis is the intestinal protozoa causing giardiasis in animals and humans. Transmission of the disease occurs via the fecal-oral route between animals to animals and animals to humans. The objectives of this study were to determine the prevalence of *G. duodenalis*, to identify the assemblage, and to analyze factors associated with *G. duodenalis* infections of water buffaloes in Northeast Thailand. A total of 567 buffalo fecal samples were collected from northeast provinces including Sakon Nakhon, Buri Ram, Ubon Ratchathani, Roi Et, Si Sa Ket and Surin. Fecal samples were tested by zinc sulphate floatation and nested PCR (nPCR) based on the SSU-rRNA gene. The overall prevalence of *G. duodenalis* infections by nPCR was 0.4% (2/567). The prevalence of *G. duodenalis* infections in water buffaloes among six provinces was ranged between 0 and 1.6%. Buri Ram had the highest individual infections (1.6%, 1/62) and the herd prevalence was 0.7% (2/276). The positive DNAs were sequenced and compared to reference nucleotide sequences from GenBank. A buffalo from Buri Ram was infected by *G. duodenalis* assemblage D and the other buffalo from Ubon Ratchathani was infected by the assemblage E. Regarding the factors associated with *G. duodenalis* infections, sex, age, herd size, and geographical landscape (basin) were analysed, but they were not statistically significant.

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Benjamas Khurajog

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LIST OF ABBREVIATIONS

°C	=	Degree of Celsius
µg	=	microgram
µl	=	microliter
µm	=	micrometer
bp.	=	base pair
cm	=	centimeter
DW	=	Distilled Water
et al.	=	et. alii (and others)
g	=	gram
L (l)	=	liter
mg	=	milligram
mm	=	millimeter
No.	=	number
PCR	=	Polymerase Chain Reaction
pH	=	negative logarithm of hydrogen ion activity

PREVALENCE OF *GIARDIA DUODENALIS* AND FACTORS ASSOCIATED WITH ITS INFECTION OF WATER BUFFALOES IN NORTHEAST THAILAND

INTRODUCTION

Giardia is intestinal protozoa parasites with a simple life cycle. Their cysts and trophozoites are excreted in feces. When cysts are ingested, trophozoites are excysted in the duodenum, divided by binary fission, and attached to the surface of the intestinal microvilli via a ventral adhesive disc. Transmission is occured by the faecal-oral route. *Giardia* cysts are ingested directly, as animal to animal transmission, or indirectly, such as contaminated water or food. Infection of this parasite may cause diarrhea , abdominal discomfort, dehydration, and weight loss and sometimes symptoms can be present clinical or subclinical (Geurden *et al*., 2010). *Giardia* has been classified into 6 species; *G. agilis* in amphibians, *G. ardeae* and *G. psittaci* in birds, *G. muris* in mice, *G. microti* in voles and *G. duodenalis* in mammals (Plutzer *et al*., 2010). *G. duodenalis* has been classified into 8 assemblages, assemblages A and B in humans and wide range of mammals, assemblages C and D in canids, assemblages E in livestock, assemblages F in cats, assemblages G in rodents and assemblages H in marine mammals (Feng and Xiao., 2011; Ryan and Caccio, 2013).

Giardia infection in animals have been reported in Central Europe with the high rate in companion animals, including dogs (0.5-18%) and cats (2-5%), and in livestock, including calves (27%) and lamps (30%) (Calum, 2005). In Italy, the prevalence of *G. duodenalis* in water buffaloes was 19.8 % and the herd prevalence was 39.5% (Rinaldi *et al*., 2007a). In other studies, 17.6 % in sheep (Giangaspero *et al.*, 2005), 19-21.3 % in dogs (Capelli *et al.*, 2003; Bianciardi *et al.*, 2004), 4.2 % in cats (Bianciardi *et al.*, 2004) and 25.4 in rodents (Fernández-Álvarez *et al*., 2014)

were reported. The close association among domestic animals provides the high risk for transmission of *Giardia* infection. *G. duodenalis* assemblage A and B was identified, as zoonotic potential pathogen. Giangaspero *et al*. (2005) and Aloisio *et al.* (2006) have demonstrated the zoonotic assemblages A and B of *G. duodenalis* in sheep so that human might get infected by this *Giardia*. Rodents have been reported as reservoirs for many zoonoses including giardiasis. Fernández-Álvarez *et al*. (2014) has reported that rodents were infected with *G. duodenalis* assemblage G and B.

A few studies of *Giardia* infections in animals was reported in Thailand such as dairy cattle (Inpankaew *et al*., 2010) and dog (Traub *et al*., 2009). Thailand is an agricultural country that contain both modern and conservative agricultural practices. Water buffaloes (*Bubalus bubalis*) in Thailand are used as draft animals in agricultural practice and as food animals for meat product. Currently, the population of buffaloes are decreasing due to the modern planting and consuming buffalo meat. Recently, Thailand have campaigned to conserve water buffaloes breed. The health conditions of buffaloes have an impact on fertility and production. Limited information of Thai's buffaloes' health is difficult to create strategies to increase buffalo production. Many pathogens were existed in buffaloes and were the obstacles in livestock development. *Giardia* is one of the common parasitic protozoa that cause clinical signs in animals and humans. Nowadays, epidemiological information of *Giardia* spp. infections of water buffaloes have not been revealed in Thailand. Giardiasis in buffaloes have been worldwide studies such as in Italy by using ELISA (Rinaldi *et al.*, 2007a, b, c) and molecular analysis of *Giardia β-giardin* gene (Caccio *et al*., 2007). In Pakistan, the prevalence and shedding intensity of giardiasis in buffaloes have been evaluated (Goraya *et al*., 2004).

OBJECTIVES

1. To detect of *Giardia* spp. infections in water buffaloes of Northeast Thailand.

2. To identify species and assemblage of *Giardia*.

3. To analyse factors associated with *Giardia* infections in water buffaloes of Northeast Thailand.

LITERATURE REVIEW

1. Taxonomy and Morphology

The first discovery of *Giardia* parasite in 1681 have been observed by Antonie van Leeuwenhoek and described morphologically later by Lambl in 1859 (Thompson and Monis, 2004). According to the new systematic based on genetic, structural and biochemical data, *Giardia* is belong to Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplozoa, Order Giardiida and Family Giardiidae (Plutzer *et al*.,2010). Species of *Giardia* have been classified based on the morphological and molecular identification. *Giardia* is consisted of 6 species including *G. agilis* in amphibians, *G. ardeae* and *G. psittaci* in birds, *G. microti* and *G. muris* in rodents, and *G. duodenalis* (syn. *G. intestinalis*, *G. lamblia*) in mammals (Ryan and Caccio, 2013). Moreover, the molecular data based on protein and DNA polymorphisms are shown that *G. duodenalis* has eight distinct genetic groups or assemblages (A to H), including Assemblage A and B in various of mammals included in humans, Assemblage C and D in canis, Assemblage E in livestock, Assemblage F in cats, Assemblage G in rodents, and Assemblage H in marine mammals (Ryan and Caccio, 2013).

Table 1 *Giardia* species, hosts, morphological features and size.

Species	Hosts	Morphological characteristics	Trophozoite dimensions	
			Length (mm)	Width (mm)
G. duodenalis	Wide range of domestic and wild mammals, including humans	Pear-shaped trophozoites with claw-shaped median bodies	12–15	6–8
G. agilis	Amphibians	Long, narrow trophozoites with club-shaped median bodies	20–30	4–5
G. muris	Rodents	Rounded trophozoites with small round median bodies	9–12	5–7
G. ardeae	Birds	Rounded trophozoites, with prominent notch in ventral disc and rudimentary caudal flagellum. Median bodies round-oval to claw shaped.	~ 10	~ 6.5
G. psittaci	Birds	Pear-shaped trophozoites, with no ventro-lateral flange. Claw-shaped median bodies.	~ 14	~ 6
G. microti	Rodents	Trophozoites similar to *G. duodenalis*. Mature cysts contain fully differentiated trophozoites.	12–15	6–8

Source: Monis *et al.* (2009)

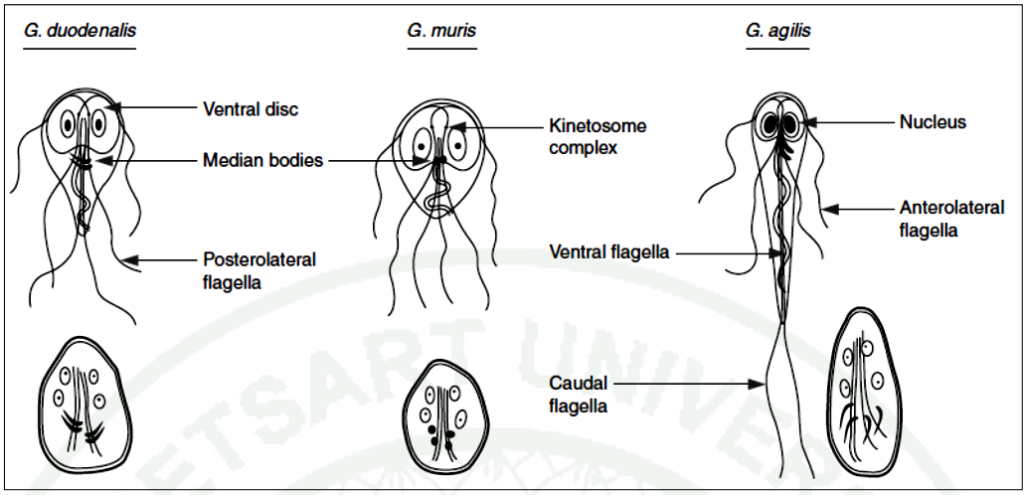

Figure 1 Morphological differentiation based on the shape of median bodies, body shape, and length trophozoite and cyst of *G. duodenalis*, *G. muris* and *G. agilis*.

Source: Thompson and Monis (2004)

Table 2 Genotypic groups (assemblages) of *G. duodenalis*.

Assemblage	Host distribution
A	Humans and other primates, livestock, dogs, cats and some species of wild mammals
B	Humans and other primates, dogs, cats and some species of wild mammals
C	Dogs and other canids
D	Dogs and other canids
E	Livestock
F	Cats
G	Rodent
H	Marine mammals (pinnipeds)

Source: Ryan and Caccio (2013)

Giardia are encountered in two forms such as trophozoites and cysts. Trophozoites are pear-shaped, 12 to 15 µm long and 5 to 9 µm wide, binucleated with a median bodies, four pairs of flagella (anterior, posterior, caudal, and ventral), and a large ventral adhesive (sucking) disc (Adam, 2000). A cyst is oval-shaped with diameter 6-10 µm. The cyst wall varies between 0.3-0.5 µm in thickness and is formed by an outer filamentous layer and an inner membranous layer including two membranes that enclose the periplasmic space. The cyst has two or four nuclei depending on stage of maturation, the contracted flagella, and fragmented portions of the ventral disk (Carranza and Lujan, 2010).

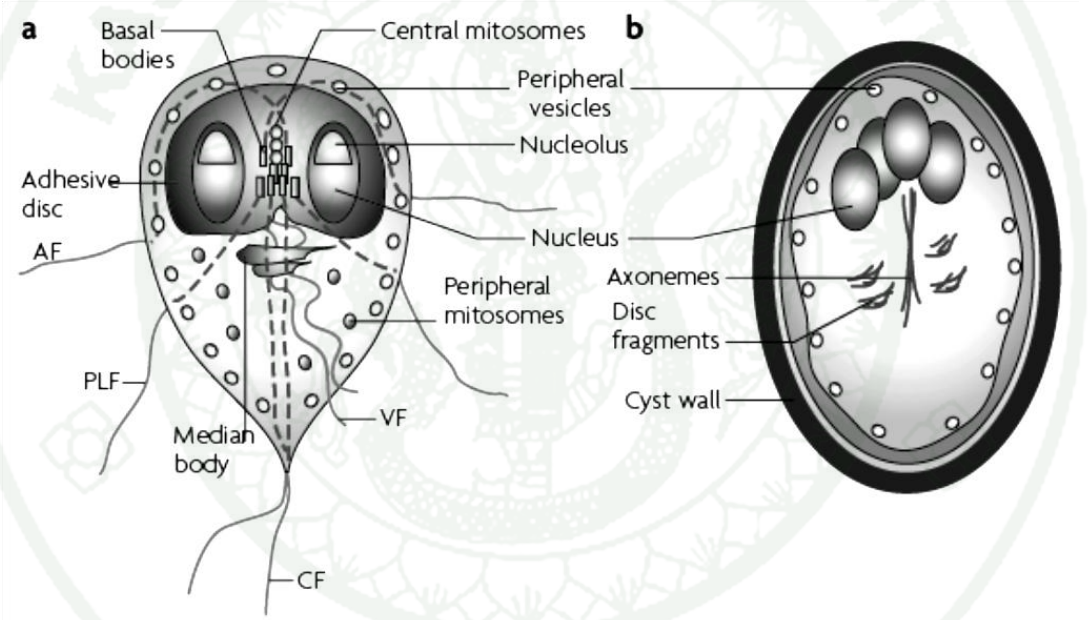

Figure 2 a) *Giardia* trophozoite or the vegetative stage of the life cycle including two nuclei, four pairs of flagella, a ventral adhesive disc, and various organelles. b) The dormant cyst, a typical feature of four nuclei, centrally located axonemes, and scattered ventral disc fragments.

Source: Ankarklev *et al.* (2010)

2. Life cycle

Giardia, a flagellated protozoa, inhabits in the small intestine of host with a direct life cycle. *Giardia*'s cyst is an infective stage. In host's intestine, cysts are exposed to gastric acid and pancreatic enzymes, excysted in the duodenum, released the trophozoites, and attached to the small intestine mucosa. Trophozoites have longitudinal binary fission on the brush border of villous epithelium of the mucosal surface. Under inhospitality environment, trophozoites will encyst to survive and be released through host feces. Cyst can survive outside the host for 3 month in water at 4˚C (Faubert, 2000) and are transmitted to a new host through contaminated water or food or contact. The prepatent period of *Giardia* spp. is 5-16 days in dogs and cats (Leib and Zajec, 1999), 7-8 days in calves (Taminelli *et al*., 1989), 6-10 days in goats (Koudela and Vitovec, 1998) and 10-21 days in sheep (Taminelli *et al*., 1989).

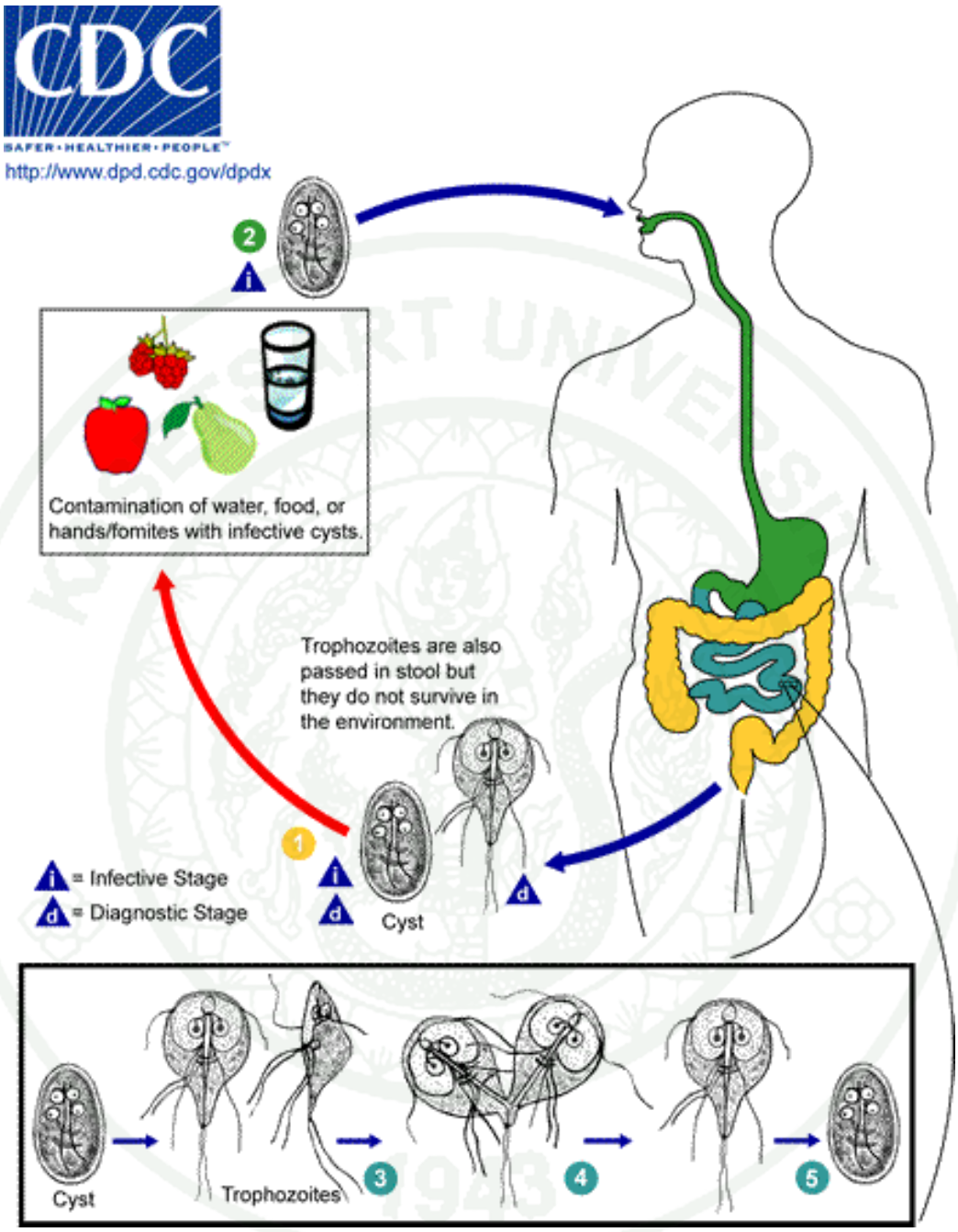

Figure 3 Life cycle of *Giardia* infection in human.

Source: Centers for Disease Control and Prevention (2010)

3. Clinical signs

Factors associated with the severity of clinical signs are depend on the virulence of the *Giardia* strain, the number of ingested cysts, the age of the host, and the state of the host immunity at the time of infection (Faubert, 2000). The clinical diagnosis of giardiasis is difficult, because of no specific symptoms. *Giardia* infections in ruminants is often asymptomatic, but they might be associated with the occurrence of diarrhea caused by more than one pathogen detected (O'Handley and Olson, 2006). Studies of humans and animals indicated that giardiasis essentially led to a decreased crypt to villus ratio and brush border enzyme deficiencies (Geurden *et al*., 2010) that have been associated with impaired intake of water, electrolytes and nutrients, and lower weight gain (Buret, 2007). The increased production of mucin by goblet cells might correlated with the mucous diarrhea in *Giardia* infected host (Moncada *et al*., 2003).

4. Economic impact

Giardiasis is characterised as a wide-spectrum illness, although the majority (60-80%) of infected individuals is asymptomatic (Silva *et al*., 2009). The main clinical symptom is diarrhea, but abdominal pain and weight loss are also reported (Silva *et al*., 2009). Children with clinical giardiasis have lower weight and height per age, and the higher risk of growth deficits than non infected children (Silva *et al*., 2009). Giardiasis in animals is often asymptomatic associated with diarrhea, poor growth, and even death in farm animals. A few studies have been conducted to assess the effect of giardiasis on the production or growth rates in livestock, such as in sheep farms in Central Italy (Aloisio *et al*., 2006). *Giardia* infection in lamps (30 to 90 days of age) had shown malabsorption syndrome, decreased weight gain, and impairment in feed efficiency. Diarrhea of dairy calves has potentially reduced the growth of ruminants (Rinaldi *et al*., 2007b). Prolonged infections can be persisted for several months in calves resulting in numerous episodes of diarrhea associated with lower production (Thompson, 2000). Significant damages were due to diarrhea, including

treatment, diagnosis, veterinary intervention and decreased number of herd replacements (Izzo *et al*., 2011).

Giardia cysts are insensitive to disinfectants at the concentration used in water treatment procedures, although it has higher concentrated chlorine and ozone (Caccio *et al*., 2003). The reports of contamination of surface water with *Giardia* and *Cryptosporidium* ranged from 60-96% in the USA and from 20-64% in Canada (Guy *et al*., 2003). Water treatment is required to get rid of protozoan cysts and oocysts by using filtration with the aid of coagulation and flocculation followed by increasing disinfectant contact times (WHO, 2011). More complex the process in terms of water treatment are also resulting with increasing costs (WHO, 2011).

5. Medical and Veterinary Impact

Parasites in the genera *Giardia* is responsible for mild gastrointestinal signs and reported worldwide with a serious impact on human and animal health (Savioli *et al*.,2006). Giardiasis is recognized in the 'Neglected Diseases Initiative' which all diseases in this group have a common link with poverty and lack of access to services influence outcomes (Savioli *et al*.,2006). Giardiasis associated with diarrhea is the most important factor association with nutrient malabsorption (Feng and Xiao, 2011). The World Health Organization (WHO) has reported about 200 million people with the symptomatic giardiasis annually in Asia, Africa, and Latin America and giardiasis is responsible for 2.5 million of diarrhea and nutritional deficiencies in children in developing countries (WHO, 1988). The high prevalence of the infection are negative effects on growth and development of infected children, livestock, and companion animals (Feng and Xiao, 2011).

Giardia spp. is recognized as emerging pathogens of humans and many other species of mammals. *Giardia* use a broad host range including livestock, companion animals, and wildlife and is comprises of different species and genotypes.

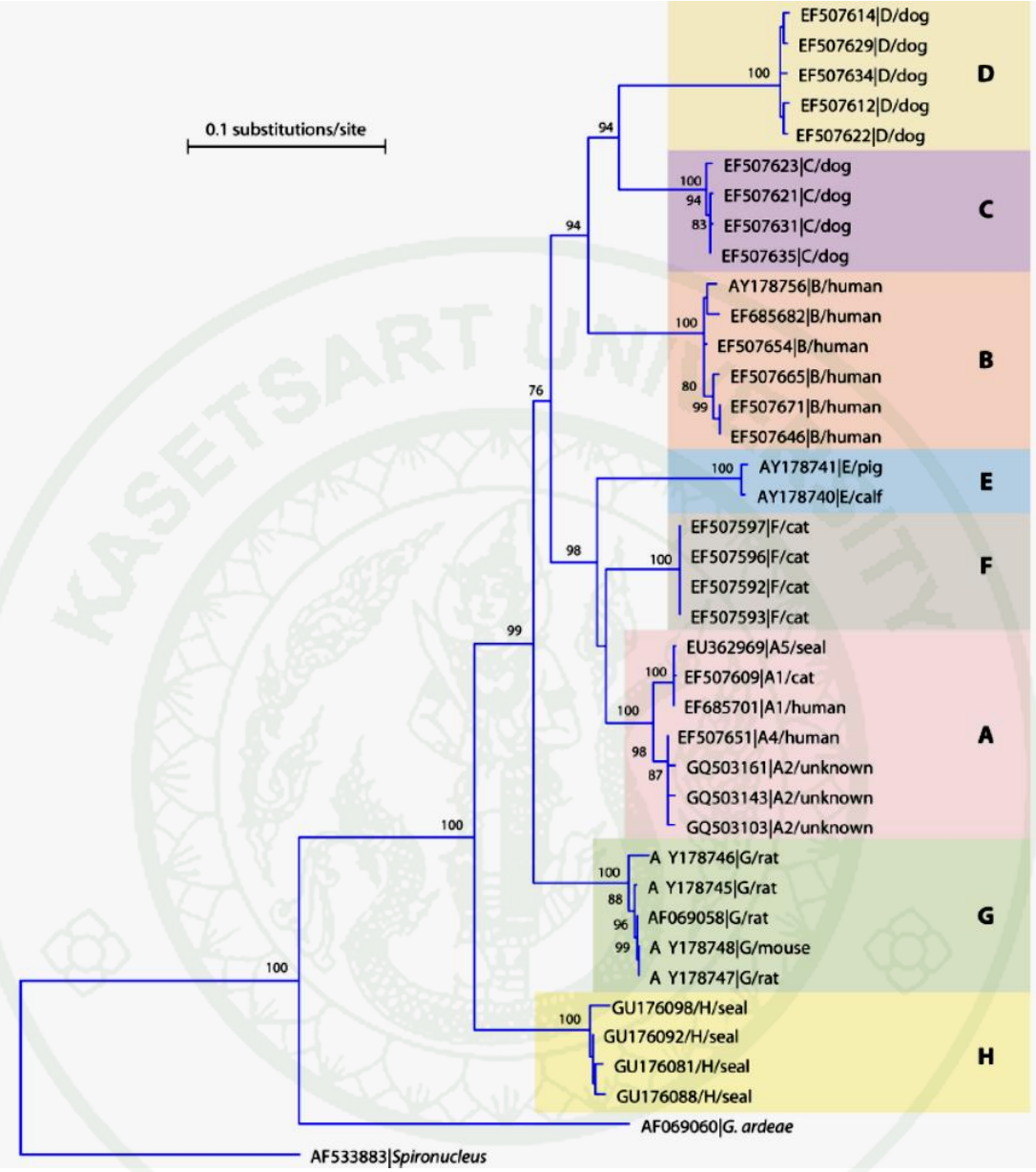

Figure 4 Phylogenetic relationships among assemblages of *G. duodenalis* at the *gdh* locus as assessed by a neighbor-joining analysis of the nucleotide sequence covering a 709-bp region (positions 256 to 964 of GenBank accession number AY178740) of the gene, using distance calculated by the Kimura two-parameter model.

Source: Feng and Xiao (2011)

Assemblages A and B can be transmitted among humans, wildlifes, domestic, and farm animals. Assemblages C and D contain only isolates from dogs, and Assemblage E contains isolates from cattle, sheep and goats. Assemblage F contains isolates from cats, and Assemblage G contains isolates from rat. Thompson and Monis (2004) have described *G. muris* (mice), *G. simondi* (rats) and *G. microti* (microtine rodents), which are all genetically distinct, but little is known about their host range, prevalence of infections, and geographical distribution. However, mice, rats, microtine rodents, and bandicoots are also susceptible to infection with zoonotic strains/species of *Giardia*. Additional subgroups are also reported in assemblage A that is belong to 2 subgroups such as AI and AII. Subgroup AI consists of closely related animal and human isolates. Subgroup AII consists only of human isolates. Assemblage B includes the subgroups III and IV and subgroup IV is human specific.

The experiment of cross infections were shown that both host-adapted and wide range species were occurred. This indicated that dogs could be infected with *Giardia* from assemblage AI and beavers might be susceptible to human *Giardia* strains (Ivanov, 2010). In Australia, zoonotic genotypes were found in mice on the Macquarie Island and Boullanger Island (Moro *et al*., 2003).

Community-wide giardiasis outbreaks might come from water-borne and food-borne transmission. The consumption of raw surface water and food contaminated with feces containing *Giardia* cysts from infected human, farm animals, and wildlife was the conventional route of transmission (Feng and Xiao, 2011). Untreated and recreational waters (e.g. swimming pools, water parks, fountains that use recirculating water, lakes and rivers) are also the other major routes of exposure (Smith *et al*., 2006). The contamination of fruit, vegetables and shellfish with *Giardia* cysts is an important public health concern because these raw products are frequently consumed without thermal processing (Smith *et al*., 2006).

Table 3 *Giardia* assemblages A and B subtypes identified in wildlife and domestic animals.

Host	Assemblage	Loci tested	Method used
Wildlife			
Beaver	B	*tpi, bg*	Sequencing
Beaver, rabbit, muskrat	B (various subtypes)	*tpi*	Sequencing
Deer	A1 and A[a]	*tpi, bg*	Sequencing
Fallow deer	A[b]	*tpi, bg*	Sequencing
Ferret	A1	*gdh, bg*	Sequencing
Fox	A1, A[a], B3	*bg*	Sequencing
Monkey	B4	*gdh*	Sequencing
Moose and reindeer	A[a]	*gdh, bg*	Sequencing, RFLP
Water buffalo	A1	*bg*	Sequencing
Domestic animals			
Cattle	B	*ssu-rRNA*	Sequencing
Cattle	A2, A3	*bg*	Sequencing
Cattle	A	*bg*	Sequencing
Cattle and pig	A[a]	*gdh*	Sequencing
Cattle	A2 and B	*gdh, bg*	Sequencing
Cattle	A1	*gdh*	Sequencing
Calf	A1–A4 and B3	*bg*	Sequencing
Horse	A1, A2, B4	*tpi*	Sequencing
Lamb	B4	*tpi*	Sequencing
Sheep	B	*gdh, bg*	Sequencing
Sheep	A1	*bg*	Sequencing, RFLP

[a] Sequences similar but not identical to A1, A2 and A3 were identified.

[b] A new assemblage A subgroup.

Source: Caccio *et al.* (2008)

Table 4 Prevalence of *G. duodenalis* assemblages A and B in humans reported worldwide.

Origin	Nature of the sample (no. of isolates)	Loci tested	Assemblage A (%)	Assemblage B (%)	A+ B (%)
Italy	Sporadic (120)	*ssu-rRNA*, *bg*	65(54%)	39(32.5%)	16(13.5%)
UK	Nursery outbreak (21)	*tpi*	-	21(100%)	-
The Netherlands	Population survey (18)	*gdh*	9(50%)	9(50%)	-
The Netherlands	Sporadic (98)	*ssu-rRNA*, *gdh*	34(35%)	64(%)	-
France	Sporadic (25)	*tpi*	9(36%)	16(64%)	-
Spain	Case control study (108)	*tpi*	43(39.8%)	61(56.5%)	4(3.7%)
USA	Sporadic (14)	*ssu-rRNA*	14(100%)	-	-
Mexico	Sporadic, children (9)	*bg*	9(100%)	-	-
Australia	Population survey (23)	*ssu-rRNA*	7(30%)	16(70%)	-
Australia	Sporadic (12)	*ssu-rRNA*	-	11(92%)	1(8%)
India	Sporadic (10)	*tpi*	-	10(100%)	-
China	Sporadic (8)	*ssu-rRNA*	4(50%)	4(50%)	-
Korea	Sporadic (5)	*ssu-rRNA*	5(100%)	-	-

Source: Caccio *et al.* (2008)

6. Epidemiology

Table 5 Prevalence of *Giardia* infections in livestock worldwide

Country	Diagnosis	No. of animals	No. of farms	The individual prevalence	The farm prevalence	References
Water buffalo						
Italy	cELISA	177	43	19.8	39.5	Rinaldi *et al.* (2007a)
	cELISA	347	90	18	30	Rinaldi *et al.* (2007b)
Pakistan	ME	300	-	33	-	Goraya *et al.* (2004)
Dairy cattle						
Belgium	IFAT	499	100	22	48	Geurden *et al.* (2008b)
Canada	IFAT	386	20	73	100	Olson *et al.* (1997)
Denmark	IFAT	518	50	43	100	Maddox-Hyttel *et al.* (2006)
	IFAT	377	50	24	82	Maddox-Hyttel *et al.* (2006)
New Zealand	IFAT	-	10	-	31	Winkworth *et al.* (2008)
Norway	IFAT	1386	136	49	93	Hamnes *et al.* (2006)
Spain	IFAT	379	60	27	97	Castro-Hermida *et al.* (2007)

Table 5 (Continued)

Country	Diagnosis	No. of animals	No. of farms	The individual prevalence	The farm prevalence	References
Thailand	ME	400	-	2.5	-	Inpankaew *et al.* (2010)
	PCR	400	-	1.0	-	
USA	PCR	571	14	36	100	Trout *et al.* (2006)
	PCR	407	14	40	100	Trout *et al.* (2004)
Vietnam	IFAT	68	8	50	88	Geurden *et al.* (2008c)
Beef						
Belgium	IFAT	333	50	45	64	Geurden *et al.* (2008b)
Canada	IFAT	605	100	23	48	Gow and Waldner (2006)
Sheep						
Australia	ME	1647	-	9	-	Ryan *et al.* (2005)
Belgium	IFAT	137	10	36	100	Geurden *et al.* (2008a)
Canada	IFAT	89	6	38	100	Olson *et al.* (1997)
Italy	ME	325	20	1.5	10	Giangaspero *et al.* (2005)

Table 5 (Continued)

Country	Diagnosis	No. of animals	No. of farms	The individual Prevalence	The farm prevalence	References
Goats						
Belgium	IFAT	148	10	53	80	Geurden *et al.* (2008a)
Brazil	ME	105	6	14	66	Bomfim *et al.* (2005)
Spain	ME/ELISA	315	40	42	95	Ruiz *et al.* (2008)
Pigs						
Canada	IFAT	236	6	9	66	Olson *et al.* (1997)
Denmark	IFAT	504	50	38	84	Hamnes *et al.* (2007)
	IFAT	488	50	3	22	Hamnes *et al.* (2007)
Norway	IFAT	684	100	1.5	10	Hamnes *et al.* (2007)
Croatia	cELISA	-	38	-	66	Bilic *et al.* (2006)

IFAT = indirect fluorescent antibody test, ME = microscopic examination, ELISA = enzyme-linked immunosorbent assay, cELISA = copro-antigen enzyme-linked immunosorbent assay, PCR = polymerase chain reaction

Table 6 The prevalence of human giardiasis In Thailand ranged from 0.36% to 37.7%

Location(s)	Infection rate (%)	References
North		
Phitsanulok	0.86% (5/584)	Waree *et al.* (2001)
Nan	5.3% (54/1,010)	Waikagul *et al.* (2002)
Chiang Mai	14.9% (60/403)	Piangjai *et al*. (2003)
Chiang Mai	2.21% (12/542)	Saksirisampant *et al.* (2004)
Chiang Mai	5.2% (40/765)	Saksirisampant *et al*. (2012)
Central		
Bangkok	10.5% (39/371)	Termmathurapoj *et al.* (2000)
Bangkok	0.36% (22/6,018)	Tungtrongchitr *et al*. (2010)
Samut Sakhon	6.5% (43/656)	Wongjindanon *et al.* (2005)
Pathum Thani	37.7%(40/106)	Saksirisampant *et al*. (2003)
Pathum Thani	3%(6/202)	Kitvatanachai *et al*. (2013)
Northeast		
Surin	2.2%(75/3,358)	Wongjindanon *et al*. (2005)
Khon Kaen	1.92%(1/52)	Rhongbutsri *et al*. (2010)
Ubon Ratchathani	1.2%(6/479)	Tungtrongchitr *et al*. (2007)
East		
Chachoengsao	6.2%(33/531)	Ratanapo *et al*. (2008)
Chachoengsao	5.8%(11/189)	Boontanom *et al.* (2011)
West		
Ratchaburi	4.1%(39/949)	Tungtrongchitr *et al*. (2010)
Kanchanaburi	13.1%(77/587)	Pothipak *et al*. (2005)

7. Diagnosis

7.1 Microscopic examination

Giardiasis is usually diagnosed from fecal specimens by microscopic examination as the gold standard. The most practical approach for diagnosis of *Giardia* from feces is zinc sulphate centrifugation for concentration of cysts (Zajac *et al*., 2002). The sensitivity of this method is fairly moderate ranged from 50 to 70% (Barazesh *et al*., 2011) because a few yielded fecal specimen was used for test and cysts excretion is sporadic and low number of cyst shedding in feces. Repeating examination ones or twice and consecutive fecal sample collection could improve the sensitivity of the test (Chakarova, 2010). Moreover, this technique is time-consuming and requires experienced personnel for interpretation. In the other methods, some chemical materials such as formalin, iodine, mercury, and ethers were used for the detection or isolation of protozoan cysts but these could affect physical and biological properties of cysts (Barazesh *et al*., 2011).

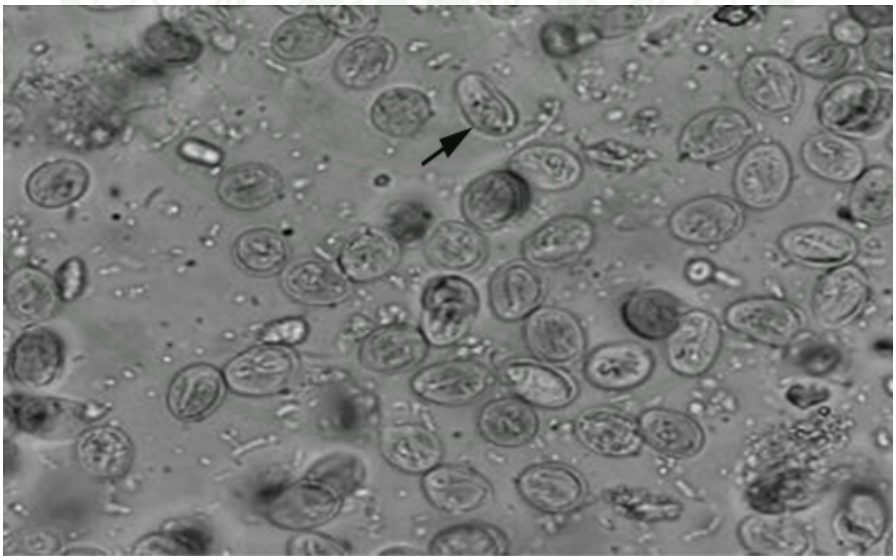

Figure 5 *Giardia* cysts (arrow) stained with Lugol's iodine in floatation.

Source: Dryden *et al.* (2006)

7.2 Immunological test

Immunological methods (IFAT and ELISA) based on the determination of anti-parasite antibody in fecal samples are relatively high sensitivity and specificity. However, the detection of *G. duodenalis* by ELISA of feces is of limited (Ivanov, 2010). The development of immunofluorescence microscopy has improved the sensitivity for detection and quantitation of cysts shed (Ivanov, 2010). However this test can not identity the assemblage of *Giardia* species that is important for zoonotic aspects.

7.3 Molecular method

A molecular technique, polymerase chain reaction (PCR), has highly sensitive and specific for *Giardia* determination and epidemiological studies (Thompson, 2008). PCR has higher specificity and sensitivity than microscopic examination or IFA (Verweij *et al*., 2004, Trout *et al*., 2005). However, PCR on stool specimens could be insensitive because of some inhibitors and the difficulty of cyst disruption. To increase the sensitivity of PCR, an effective DNA extraction method is needed. Commercial DNA extraction kits such as the QIAamp stool mini kit (QIAGEN, Hilden, Germany), FTA/FTA Elute Sample Collection Cards and Kits: FTA filter paper (Whatman Bioscience, Cambridge, UK) and conventional phenol/chloroform method have been used for isolation of *Giardia* DNA. The comparison of efficiency among three technique showed that FTA filter paper was the most efficient DNA extraction method which could detect the cysts quantity in stool dilution at168 cysts/ml, while both QIAamp stool mini kit and phenol/chloroform extraction method could detect the cysts quantity at 674 cysts/ml (Nantavisai *et al*., 2007).

PCR for detection of *G. duodenalis* are based on small subunit ribosomal RNA (ssrRNA), β-giardin (*bg*), glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef-1*), triose phosphate isomerase (*tpi*), GLORF-C4 (*C4*) gene and recently,

inter genomic rRNA spacer region (Caccio and Ryan, 2008). Approximately 60-130 copies of ssrRNA gene per nucleus of *G. duodenalis* arranged in 135 tandem repeat have been reported (Nantavisai *et al*., 2007). Moreover, ssrRNA sequence is more conserved than the other regions so that they might be used as templates. Because the genetic loci of *Giardia* differ in substitution rates, the resolution for parasite gene typing is different among loci. For example, substitution rates for the partial ssrRNA, *bg*, *gdh*, and *tpi* genes were reported to be 0.01, 0.03, 0.06, and 0.12 substitutions per nucleotide, respectively (Feng and Xiao, 2011). Thus, the conserved ssrRNA gene is traditionally used for species and assemblage differentiation (mostly genotyping), whereas the most variable locus, *tpi*, is frequently used for subtyping such as subunit within assemblages A (AI and AII) and B (BIII and BIV) (Lebbad *et al.*, 2010).

MATERIALS AND METHODS

1. Fecal samples collection

A total of 567 fecal samples were collected from buffaloes of 6 northeast provinces with the highest population consensus (Table 7) including Sakon Nakhon, Buri Ram, Ubon Ratchathani, Roi Et, Si Sa Ket and Surin (Figure 5). Each fecal sample (approximately 10 g) was directly collected from rectum of an individual buffalo by using disposable gloves. Fecal samples were stored in icebox, transported to Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. Small portions of fecal sample were separately preserved in 10% formalin and 70% alcohol and left at room temperature while the rest was stored at -20˚C before processing for PCR.

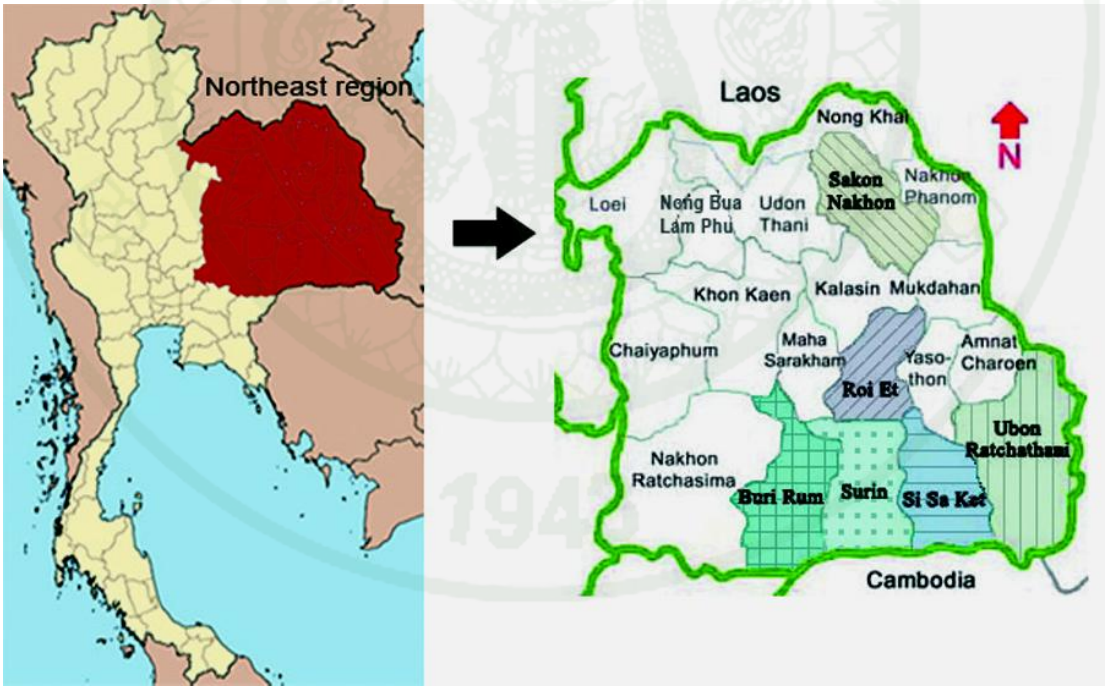

Figure 6 The location of sample collection from northeast Thailand

Table 7 Study areas with the number of buffaloes and sampling number

No.	Provinces	Total Number of Buffaloes[a]	Sample collections
1	Ubon Ratchathani	121,169	98
2	Surin	105,533	70
3	Buri Ram	89,226	62
4	Si Sa Ket	87,999	55
5	Sakon Nakhon	78,105	202
6	Roi Et	50,118	80
	Total	**453,151**	**567**

[a] Thai buffalo conservation & development center (2011)

Figure 7 Fecal sample collection.

2. Zinc sulphate floatation technique

Fecal samples in 10% formalin were mixed with 10 ml of zinc sulphate solution which had specific gravity of 1.18 to 1.20 for lifting *Giardia* cyst specific gravity of 1.05. The tube was later on filled up with zinc sulphate so that a slight positive meniscus would be formed. A cover slip was placed on top of the tube for 15 min, removed, and placed on a glass slide. The entire area under the cover slip was thoroughly examined by light microscopy for the presence of *Giardia* cysts or trophozoites.

3. DNA Extraction

Fresh feces at -20˚C and preserved feces in 70% alcohol were extracted for DNA. Briefly, 200 µl of the fecal suspension was aliquotted, washed three times with distilled water, and centrifuged at 2000 G for 10 min. The DNA was isolated from the pellet using the Qiagen stool mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

4. PCR amplification

The SSU-rRNA gene was amplified and yielded 130 bp using primers RH11, RH4 and GiarF and GiarR as previously described by Hopkins *et al*. (1997) and Read *et al*. (2002). All PCR products were separated on a 1.5% agarose gel. The amplified bands were cut out, purified using the UltraClean™ GelSpin DNA purification Kit (MO BIO LABORATORIES INC., Solana Beach, CA, USA) according to the recommendations of the manufacturer and consequently submitted for DNA sequencing.

5. *G. duodenalis* assemblage identification

Nucleotide sequences were compared with known reference sequences from the National Center for Biotechnology Information (NCBI) by using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/ BLAST). This program compares nucleotide sequence to sequence databases and calculates the statistical significance of matches.

6. Statistical Analysis

The age of animal was considered as one of the high risk for *Giardia* infection. In addition, herd size and sex factor were also included in the statistical analysis for predicting their effects on disease distribution. Association between the prevalence of *Giardia* infection and factors such as age, sex, province, herd, and herd size was analyzed by using Number Cruncher Statistical System (NCSS) version 2000 (Kaysville, UT) programs and Chi-square (χ^2).

RESULTS AND DISCUSSION

Results

A total of 567 fecal samples from 276 buffalo farms were tested for *Giardia* infection using zinc sulphate floatation technique and nested PCR (nPCR). There were no *Giardia* cysts nor trophozoites in all fecal samples subjected to microscopic examination. Two *G. duodenalis* positive samples (0.4%, 2/567) were found by nPCR based on SSU-rRNA gene and DNA bands of *G. duodenalis* at 130 bp were shown in Figure 8. By the province, the highest infection was found in Buri Ram (1.6%, 1/62), followed by Ubon Ratchathani (1.0%, 1/98). For the herd prevalence, only 2 out of 276 herds (overall prevalence of 0.7 %) were infected with *G. duodenalis.* Factors including age, sex, herd, herd size, and provinces were brought to analysis for the correlation with the infection (Table 8). The result showed that higher infections were found in buffaloes aged between 1 and 5 years (0.6%, 2/327), males (1.3%, 1/78), and in small herd size (≤5 buffalo per herd) (0.7%, 2/268). The geographical landscape of northeast areas was divided into Khorat and Sakon Nakhon basin (Potapon, 2013). The Khorat basin is located in the South covering Buri Ram, Ubon Ratchathani, Roi Et, Si Sa Ket and Surin province. The Sakon Nakhon basin is located in the North covering Sakon Nakhon province. The higher prevalence was found in Khorat basin (0.5%, 2/365). However, the overall results were not statistically significant.

Two positive samples for *G. duodenalis* were sequenced and compared to reference nucleotide sequences from GenBank using BLAST. For assemblage identification, one positive sequence from the buffalo of Buri Ram was 100% identical to assemblage D of *G. duodenalis*. The other sequence from Ubon Ratchathani was 100% identical to assemblage E.

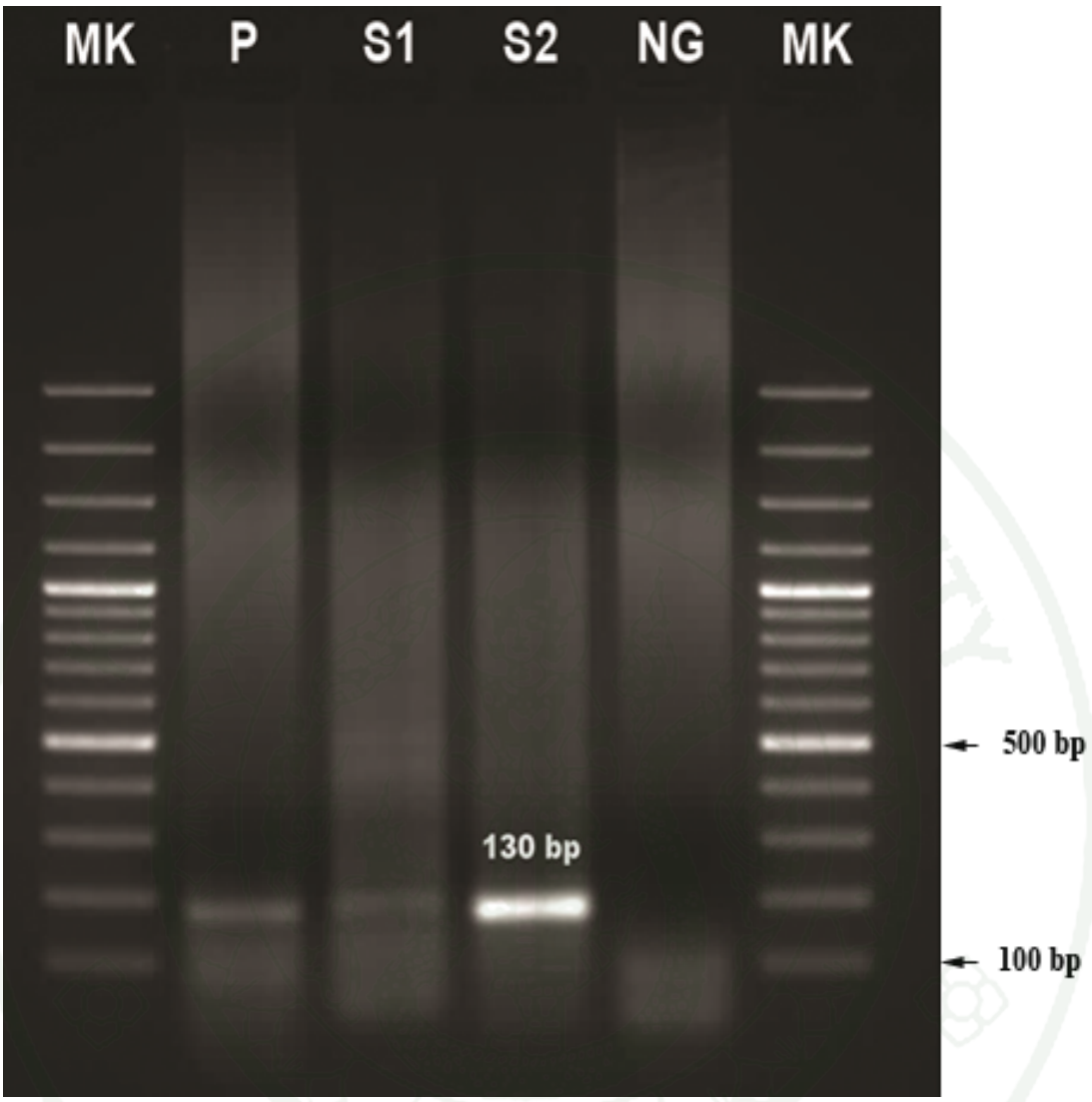

Figure 8 DNA bands of *G. duodenalis* at approximately 130 bp. (MK = DNA marker 100 bp; P = *Giardia* positive control; S1-S2 = positive samples; NG = negative control)

Table 8 Prevalence and factors associated with *Giardia* infections of water buffaloes from six provinces of Northeast Thailand

Parameter	***Giardia* positive (%)**	**Chi-square statistic**
Individual prevalence		
Province		$\chi^2 = 5.48$, df = 5, $p = 0.359$
Buri Ram	1/62 (1.6%)	
Ubon Ratchathani	1/98 (1.0%)	
Roi Et	0/80 (0.0%)	
Si Sa Ket	0/55 (0.0%)	
Surin	0/70 (0.0%)	
Sakon Nakhon	0/202 (0.0%)	
Total	2/567 (0.4%)	
Basin		$\chi^2 = 1.11$, df = 1, $p = 0.292$
Sakon Nakhon	0/202 (0.0%)	
Khorat	2/365 (0.5%)	
Age group (year)		$\chi^2 = 1.47$, df = 2, $p = 0.479$
<1	0/4 (0.0%)	
1-5	2/327 (0.6%)	
>5	0/236 (0.0%)	
Sex		$\chi^2 = 2.22$, df = 1, $p = 0.136$
Male	1/78 (1.3%)	
Female	1/489 (0.2%)	

Table 8 (Continued)

Parameter	***Giardia* positive (%)**	**Chi-square statistic**
Herd prevalence		
Province		$\chi^2 = 7.20$, df = 5, $p = 0.206$
Buri Ram	1/22 (4.5%)	
Ubon Ratchathani	1/48 (2.1%)	
Roi Et	0/58 (0.0%)	
Si Sa Ket	0/22 (0.0%)	
Surin	0/28 (0.0%)	
Sakon Nakhon	0/98 (0.0%)	
Total	2/276 (0.7%)	
Herd size		$\chi^2 = 0.06$, df = 1, $p = 0.806$
≤5 animals	2/268 (0.7%)	
>5 animals	0/8 (0.0%)	

Discussion

This study is the first report of *Giardia duodenalis* infections of water buffaloes in Northeast Thailand. The overall prevalence of *Giardia* spp. infection in water buffaloes in this study was 0.4% (2/567) compared to 0.7% (2/297) *Giardia* infection in river buffaloes in Sri Lanka (Abeywardena *et al*.,2014). In addition, the prevalence in this study was relatively low when compared to 13% (62/476) and 14% (8/57) *Giardia* infection in water buffaloes in Australia (Abeywardena *et al*., 2013) and in Italy (Caccio *et al*., 2007), respectively. For the other studies in Thailand, the prevalence of *Giardia* infection have been reported in animals including dairy cattle (1%, 4/400) (Inpankaew *et al*., 2010) and dogs (70.7%, 162/229) (Traub *et al*., 2009). Nevertheless, the prevalence of giardiasis in Thai people was ranged from 0.9% to 14.9% in the North (Waree *et al*., 2001; Piangjai *et al*., 2003), 0.4% - 37.7% in the Central (Saksirisampant *et al*., 2003; Wongjindanon *et al*., 2005; Tungtrongchitr *et al*., 2010), 1.2% - 2.2% in the Northeast (Wongjindanon *et al*., 2005; Tungtrongchitr *et al*., 2007), 5.8% - 6.2% in East (Ratanapo *et al*., 2008; Boontanom *et al*., 2011) and 4.1% - 13.1% in the West (Pothipak *et al*., 2005; Tungtrongchitr *et al*., 2010).

The gold standard of diagnosis *Giardia* for infections is microscopic examination (Schuurman *et al*., 2007; Chakarova, 2010). Nevertheless, this method is time-consuming, labour-intensive, and lacks sensitivity (Schuurman *et al*., 2007). Due to the intermittent of *Giardia* cysts excretion, detection of a single fecal specimen collection may not found *Giardia* cysts. Therefore, consecutively collected fecal specimens over 3-5 days is adequate to improve its accuracy of diagnosis for example, a single fecal stool detection is 46% sensitivity compared to 94% sensitivity when using consecutive collecting fecal samples (Chakarova, 2010). For fecal examination, zinc sulphate floatation method is an accepted technique to detect *Giardia* cysts because the high concentration of this solution is not distorting or destroying *Giardia* cysts (Dryden *et al*., 2006; Broussard *et al*., 2003). Using specific gravity of zinc sulphate solution ranged 1.18-1.20 for lifting the *Giardia* cysts which had 1.05 specific gravity, *Giardia* cysts are separated from debris floating to the surface of

solution (Dryden *et al*., 2006; Broussard *et al*., 2003). By zinc sulphate floatation examination, *Giardia* cysts were not found. However, cestode eggs, (*Moniezia* sp.), were found in 2 buffaloes fecal samples.

A diversity of genetic markers for genotyping *G. duodenalis* was based on small subunit ribosomal RNA (SSU-rRNA), β-giardin (*bg*), glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef1α*), triose phosphate isomerase (*tpi*), GLORF-C4 (*C4*), and inter genomic rRNA spacer region (*IGS*) (Caccio and Ryan, 2008). In this region, the SSU-rRNA sequence is more conserved and sensitive for the detection of different assemblages; however, intra-assemblage variation of this gene is limited. The *bg*, *gdh*, and *tpi* genes have shown more intra-assemblage variation that were used to discriminate assemblages A (AI and AII) and B (BIII and BIV) (Caccio *et al*., 2008). The SSU-rRNA gene contains the variable at 5' and 3' ends that can be used to identify *G. duodenalis* assemblage, and the specific conserved regions on this gene would be used to identify *Giardia* species (Feng and Xiao, 2011). However, the short sequence of PCR product (140-280 bp) might not be sufficient to differentiate *G. duodenalis* assemblages (Feng and Xiao, 2011).

G. duodenalis assemblage A and B are considered potential zoonotic pathogens. Assemblages A and B are existed and transmitted among humans, wildlifes, domestic and farm animals (Thompson *et al*., 2004). The high risk of giardiasis transmission might come from pets such as dogs and cats that have a close relationship to humans (Thompson *et al*., 2004). However, the *Giardia* infected dairy cattle were also an important reservoir for giardiasis to humans (Ryan and Caccio, 2013). The transmission of zoonotic assemblages from cattle to humans had been associated with water-borne outbreaks in USA (Ryan and Caccio, 2013). A previously studies of molecular characterization of *Giardia* assemblages in water buffaloes have been reported as assemblage E and A (Caccio *et al*., 2007; Caccio *et al*., 2008; Abeywardena *et al*., 2013; Abeywardena *et al*., 2014). In Italy and Sri Lanka, Assemblage E and A were found in water buffaloes which also had more than one assemblage (Caccio *et al*., 2007; Abeywardena *et al*., 2014) while water buffaloes in

Australia were infected with only assemblage A (Abeywardena *et al*., 2013). In this study, *G. duodenalis* infection of buffaloes in Thailand, based on SSU-rRNA gene, were identified as Assemblage D and E. The assemblage D, which specific infected in dog, was also reported in cattle in England (Minetti *et al*., 2014).The assemblage D infection in buffalo was not affect to buffalo's health. However, assemblage D might be transmitted to dog by directly ingested buffalo's feces or indirect via contaminated water or food. The assemblage E, which specific infected in livestock, could be transmitted to other animals such as cattle, sheep, and pigs and this assemblage was predominantly distributed among cattle in North America, Europe and Australia (Feng and Xiao, 2011).

Since the modern agricultural practice was growing, while a number of buffaloes was dramatic decreasing. Many buffaloes were slaughtered for meat product and female buffaloes had low reproductive efficiency due to delay puberty which are associated with the age at first calving (Chaikhun *et al*., 2012) and common age range of buffaloes were found older than younger buffaloes. Thus, the representative buffalo samples collection were mostly older than 1 year of age. For the age groups of buffaloes associated with *Giardia* infection, the age of buffaloes 1-5 years old were found higher *Giardia* infection than other age groups (Table 8). *G. duodenalis* infections were normally found in young animals (less than 1 year) particularly animals between 2–10 weeks with significantly high (Olson *et al*., 2004; Xiao and Fayer, 2008). The higher prevalence of *Giardia* in pre-weaned (40%) and post-weaned (52%) calves compared with adult cattle (27%) has been reported (Trout *et al.*, 2005; 2007). Accordance to Minetti *et al*. (2014), this had been reported that the highest prevalence of *Giardia* infection was found in pre-weaned calves (43.6%), followed by neonatal (31.8), post-weaned calves (26.1%) and adult (23%). In a study of Goraya *et al*. (2004), the prevalence of *Giardia* infection in buffaloes were mostly reported in buffaloes less than 1 year of age (43%), followed by 28.7% in 1-3 years of age and 27.5% in more than 3 years of age.

The provinces with higher infected *Giardia* were located in the Khorat basin where geographical consist of hilly terrain with small mountainous or highland areas and presented the rivers and their branches. Livestock and rainfall also contributed to disease outbreaks but less frequency occurred (Plutzer *et al*., 2010) and there is a significant association between survivals of *Giardia* cysts and the percent moisture increasing in soil (Gow and Waldner, 2006). Therefore, it is possible that the buffaloes were exposure to *Giardia* cyst contaminated in environment such water, soil and grass when they were grazing nearby a water source. However, there were not found in *Giardia* infection of Sakon Nakhon basin. The sample collection in Sakon Nakhon basin was located in only Sakon Nakhon province that might not be equally distributed sampling among provinces over Sakon Nakhon basin, so that *Giardia* prevalence in this basin might be underestimated.

For the herd prevalence, only 2 out of 276 herds (the overall prevalence of 0.7 %) were infected with *G. duodenalis* that was the low prevalence compared with 30% to 39.5% of the herd prevalence in water buffaloes in Italy (Rinaldi *et al*., 2007a; 2007b). Conventionally, buffalo farms in Thailand are a small farm (the average 4.82 buffaloes per household). The animal density per household was classified in 2 groups such as ≤ 5 buffaloes per household and > 5 buffaloes per household. The highest prevalence infection was found in farm with ≤ 5 buffaloes. For sex factors associated with infection, males had slightly higher prevalence than females. However, the other studies were reported that female calves had more prevalence than male calves (Ayaz, 2009).

For Thai community in the rural area, buffaloes were raised by roaming in the public pasture such as rice and corn fields, and their behaviors such as submerging in mud or swimming at the public canal so that these areas might be polluted by the animals' feces leading to the transmission of *Giardia* to humans and other domestic animals, such as dog cat pig cow, under poor sanitary and improper hygiene. During the agricultural practice in the rice or corn field, farmer might also defecate at the field and contaminated the environment. *Giardia* cysts are capable of survive for

months in surface water and in soil (Feng and Xiao, 2011) so that humans and animals in nearby environment might be at high risks of ingestion contaminated *Giardia* cysts in their water or food.

CONCLUSION

The prevalence of giardiasis (0.4%, 2/567) was found in two water buffaloes at Buri Ram and Ubon Ratchathani provinces of Northeast Thailand. *Giardia* discrimination based on molecular genotyping by SSU-rRNA gene, a buffalo from Buri Ram infected *G. duodenalis* Assemblage D and the other buffalo from Ubon Ratchathani infected with Assemblage E. All factors, including age, sex, herd, and provinces (locations of the fecal samples) associated with *Giardia* infection were not statistically significant.

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APPENDIX

The figure of Northeast provinces of Thailand area collecting fecal buffalo specimens

Appendix Figure 1 The habitat of rice field of Buri Ram province

Appendix Figure 2 The buffaloes pen in nearby house

Appendix Figure 3 The buffaloes were restrained for sample collection

Appendix Figure 4 The buffaloes were restrained for sample collection

Appendix Figure 5 The restraining stall for buffaloes in the farm

Appendix Figure 6 The buffaloes were waited for sample collection

Appendix Figure 7 The buffaloes graze in rice field

Appendix Figure 8 The farmer and their buffaloes

Appendix Figure 9 The buffalo restraining stall

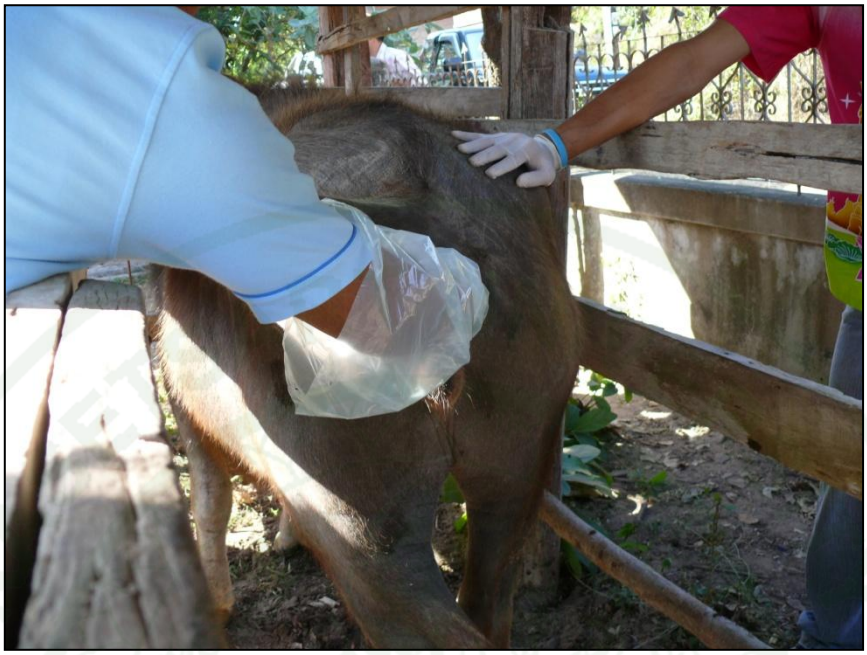
Appendix Figure 10 The habitat in Surin province

Appendix Figure 11 The buffalo pen in Surin province

Appendix Figure 12 The habitat of farm of Surin province

Appendix Figure 13 The buffalo were restrained for sample collection

Appendix Figure 14 The buffalo was restrained for sample collection



Appendix Figure 15 The fecal sample collection

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2014 : **Khurajog, B.**, Masakul, A., Inpankaew, T., Kamyingkird, K., Wongpanit, K. and Jittapalapong S. 2014. Prevalence of *Giardia duodenalis* and factors associated with its infection in water buffaloes in Northeast Thailand**.** J.Trop.Med.Parasitol. 37: 35-41