COMPARISON OF NESTED-POLYMERASE CHAIN REACTION AND LOOP-MEDIATED ISOTHERMAL AMPLIFICATION IN DETECTION OF *CRYPTOSPORIDIUM* SPP AND *GIARDIA DUODENALIS* FROM WATER SOURCES IN PHAYAO PROVINCE, THAILAND

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Abstract. *Cryptosporidium* spp and *Giardia duodenalis* are common intestinal protozoa, causes of water-borne outbreaks of gastroenteritis throughout the world. Nested-PCR and loop-mediated isothermal amplification (LAMP) assays were used to detect *Cryptosporidium* spp and *G. duodenalis* cysts in 170 water samples from four dams and two lakes in Phayao Province, Thailand during May to July, 2017. LAMP was superior to nested-PCR in detecting both cyst species: 36% compared to 22% for *Cryptosporidium* spp and 12% compared to 21% for *G. duodenalis*. Kwan Phayao and Nong Leng Sai Lakes had the highest prevalence: 60% and >30% for *Cryptosporidium* spp and *G. duodenalis*, respectively. Thus, LAMP provided a sensitive and convenient assay for surveillance of these zoonotic pathogenic protozoa, which were highly prevalent in lakes in Phayao Province. These results provide information useful for development of appropriate public health measures to control and eliminate diarrheal diseases caused by *Cryptosporidium* spp and *G. duodenalis* in Phayao Province and other regions of Thailand with high risk of infection.

Keywords: *Cryptosporidium* spp, *Giardia duodenalis*, catchment area, LAMP assay, nested-PCR assay

INTRODUCTION

Diarrheal disease is the second leading cause of death in children under the age of five years worldwide with 500,000 deaths annually (WHO, 2017). Ingested microorganisms, such as viruses, bacteria and parasites, can cause infectious diarrhea, among which *Cryptosporidium* spp and *Giardia duodenalis* are often causes of public health concern (Haque, 2007). Infections by *Cryptosporidium* spp and *G. duodenalis* are considered the most common among food- and water-borne protozoa, which results in abdominal pain and diarrhea, respectively of infected individuals (Chalmer, 2012; Ryan *et al*, 2014). Worldwide, 936 outbreaks of water-borne protozoa infections were

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recorded between 1946 and 2016, of which *Cryptosporidium* spp and *G. duodenalis* is responsible for about 58% and 38% of the cases, respectively (Baldursson and Karanis, 2011; Efstratiou *et al,* 2017).

Many types of water sources, especially natural and constructed catchments, are suitable for survival and transmission of *Cryptosporidium* spp and *G. duodenalis* (Tiyo *et al,* 2015). Community waste water provides parasites with a convenient niche for fecal-oral transmission, and in addition *Cryptosporidium* spp oocysts and *G. duodenalis* cysts have become resistant to disinfectants, such as chlorine or chloramine, used in treatment of tap water (Baldursson and Karanis, 2011). Studies used for identification of cysts in environmental specimens mainly rely on immunofluorescence assay (IFA) (ISO, 2006; US EPA, 2012); however, IFA identification requires a skillful microscopist to avoid false-positive or -negative results.

Less subjective methods, such as PCR (of various kinds) have been employed for identification and analysis of *Cryptosporidium* spp and *G. duodenalis* in environmental (water) samples (Gallas-Lindemann *et al,* 2016). More recently, loop-mediated isothermal amplification (LAMP) assay, which obviates the need of a thermal cycler, making it less expensive and more suitable for on-site application, has become the method of choice, especially in laboratories with limited resources (Notomi *et al*, 2000). An additional advantage is LAMP products can be detected by the naked eye (as a turbid solution), a simple spectrophotometer or gel-electrophoresis (Gallas-Lindemann *et al,* 2016).LAMP assay has successfully been applied in the diagnosis of *Cryptosporidium* spp (Karanis *et al,* 2007; Bakheit *et al,* 2008) and *G. duodenalis* (Plutzer and Karanis, 2009; Plutzer *et al,* 2010).

Identification of *Cryptosporidium* spp and *G. duodenalis* in developed countries has focused mainly on host infestations rather than from environmental sources (Dib *et al,* 2008; Lim *et al,* 2010). Previous studies in Thailand showed aquatic environments contain both *Cryptosporidium* spp and *G. duodenalis* (Anceno *et al,* 2007; Diallo *et al,* 2008; Srisuphanunt *et al,* 2010; Koompapong and Sukthana, *et al,* 2012; Kumar *et al,* 2014; Chuah *et al,* 2016). Hence, a comparison between nested-PCR and LAMP techniques in the detection of *Cryptosporidium* spp and *G. duodenalis* present in the same samples was undertaken using water samples from catchment areas in Phayao Province, Thailand. The study should prove instructive for future studies involving use of a PCR- or LAMP-based assay for detection of multiple microorganisms present in the same sample.

MATERIALS AND METHODS

Sampling sites and water samples

Catchment collection sites were Kwan Phayao Lake (KPY), Nong Leng Sai Lake (NLS), Mae Puem Reservoir (RMP), Mae Jun Reservoir (RMJ), Mae Rong Suk Reservoir (RMS), and Mae Tam Reservoir (RMT), Phayao Province (Fig 1). A cross-sectional study was undertaken of 170 water samples collected from the six sites (two natural and four manmade surface water bodies) during May - July 2017. Each sample consisted of 5 liters of water collected directly from the water surface, stored in a polystyrene plastic bottle at 37°C and transported to the Faculty of Medical Sciences Laboratory, University of Phayao, Phayao within 2 hours.

Collection of cysts

Each 5 liters sample was centrifuged at 2,300*g* for 5 minutes at 25°C, pellet

Fig 1-Catchment areas in Phayao Province, Thailand investigated for *Cryptosporidium* spp and *Giardia duodenalis* cysts.

suspended in 1 ml of distilled water containing 50 µg/ml penicillin/streptomycin and 1 µg/ml amphotericin B and stored at -20°C until used.

Nested-PCR assay

DNA was extracted using QIAamp DNA Stool Minikit (QIAGEN, Hilden, Germany) subsequent to a 6-cycle freeze-thaw procedure (immersion in liquid nitrogen for 5 minutes and at 65°C for 15 minutes) (Plutzer *et al,* 2010). Nested-PCR was conducted in a 50-µl mixture containing 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 0.2 µM of each primer (Table 1), 10 mM dNTPs, 20 µg of bovine serum albumin (Himedia, Mumbai, India), 0.02 U/ml *Taq* DNA polymerase (New England BioLabs, Ipswich, MA), and 2 µl of DNA (1st round amplification) or 1 µl of completed 1st round PCR reaction mixture (2nd round amplification). Thermocycling was performed in MyCycler (Bio-Rad Labs, Hercules, CA) as described in Table 1. Amplicons were separated using 1.5% agarose gel-electrophoresis and stained with GelRed (Biotium, Fremont, CA).

LAMP assay

LAMP reaction mixture, containing 2 µl of DNA template, 16 U *Bst* DNA polymerase (Lucigen, Middleton, WI), primer mixture [40 pmol each of FIP and BIP primers (Table 2), 20 pmol of LF and LB primers, 5 pmol each of F3 and B3 primers], 21 µmol of $MgSO_4$, 87.5 µmol of dNTPs, 2.5 µl of 10X DNA polymerase Buffer B (Lucigen, Middleton, WI), and

Table 1
Primers used in nested-PCR assay.

Primer	Nucleotide sequence (5′→3′)	PCR cycling conditions	Amplicon size (bp)	Reference
Cryptosporidium spp 18S rDNA				
18SiCF2 (1st)	GACATATCATTCAAGTTTCTGACC	95°C 15 min/35 cycles of 95°C 30 s, 55°C 1 min, 72°C 1 min/ 72°C 5 min	763	Chuah *et al* (2016)
18SiCR2	CTGAAGGAGTAAGGAACAACC			
18SiCF1 (2nd)	CCTATCAGCTTTAGACGGTAGG	95°C 15 min/25 cycle of 95°C 30 s, 55°C 1 min, 72°C 1 min/72°C 5 min	587	
18SiCR2	TCTAAGAATTTCACCTCTGACTG			
Giardia duodenalis 18S rDNA				
Gia2029 (1st)	AAGTGTGGTGCAGACGACTC	95°C 15 min/35 cycles of 95°C 30 s, 55°C 1 min, 72°C 1 min/72°C 5 min	497	Appelbee *et al* (2003)
Gia2150c	CTGCTGCTGCCGTCCTTGGATGT			
RH11 (2nd)	CATCCGGTCGATCCTGCC	95°C 15 min/25 cycles of 95°C 30 s, 55°C 1 min, 72°C 1 min/72°C 5 min	292	
RH14	AGTCGAACCCTGATTCTCCGCCAGG			

min, minute.

Table 2
Oligonucleotide primers used in LAMP assay.

Primer	Nucleotide sequence (5→3′)
Cryptosporidium spp (SAM-1)[a]	
SAMCF3	ATTTGATRGACAAAGAAACTAG
SAMCB3	CGATTGACTTTGCAACAAG
SAMCLF	CTGCTGGCCCMCCAATTG
SAMCLB	CATGGRGGTGGTGCATTTAG
SAMCFIP	TTGCGCCCTGTTAATCCAGCATTAATTAATCCATCTGGCAGRTTT
SAMCBIP	TTGTAGATACATACGGAGGATGGGTCTACTTTAGTTGCATCTTTCC
Giardia duodenalis (EF1A)[b]	
GL8F3	ATGGACGACGGCCAGG
GL8B3	CCCTCGTACCAGGGCATC
GL8FIP	AGCCGATGTTCTTGAGCTGTCTGTACTCGAAGGAGCGCTACG
GL8BIP	GGAAGAAGGCCGAGGAGTTCGTTGTCGGACCTCTCCATGA
GL8LB	TCATCTCGCCCTTGATCTCG
GL8LF	CTGGACCGGGACAACA

[a]Karanis *et al* (2007); Bakheit *et al* (2008). [b]Plutzer and Karanis (2009); Plutzer *et al* (2010).
SAM-1, S-adenosylmethionine synthase 1; EF1A, Elongation factor 1-alpha; M = A or C; R = A or G.

distilled water to make up 50 µl, was incubated at 63°C for 90 minutes for *Cryptosporidium* spp or 120 minutes for *G. duodenalis* detection. LAMP products were analyzed by 2% agarose gel-electrophoresis and staining with Gel red dye (Biotium).

Statistical analysis

Sensitivity, specificity (Altman and Bland, 1994), likelihood ratio (Jaeschke *et al*, 1994), derived tables of Altman (1991) and kappa-coefficient of Cohen (1960) were employed to evaluate concordance of comparison between nested-PCR and LAMP assays. Kappa coefficients 0.41-0.60 are considered to be moderate agreement and 0.61-0.80 good agreement.

RESULTS

Of the 170 water samples tested, nested-PCR assay detected *Cryptosporidium* spp in 37 (22%) samples and *G. duodenalis* in 20 (12%) samples (Fig 2A shows representative results), while LAMP assay detected *Cryptosporidium* spp in 62 (36%) samples (Fig 2B) and *G. duodenalis* in 35 (21%) samples (Fig 2C). *Cryptosporidium* spp and *G. duodenalis* were detected only at KYP (highest frequency for former), NLS (highest frequencies for both), RMP and RMT sites (Table 3). In total, 67 (39%) samples showed positive results for *Cryptosporidium* spp and 39 (23%) for *G. duodenalis* by at least one of the two detection methods, with 7% and 8% negative by both methods for *Cryptosporidium* spp and *G. duodenalis*, respectively. LAMP assay was more efficient than nested-PCR for detection of *Cryptosporidium* spp (36% *vs* 22%) and *G. duodenalis* 20% *vs* 13%).

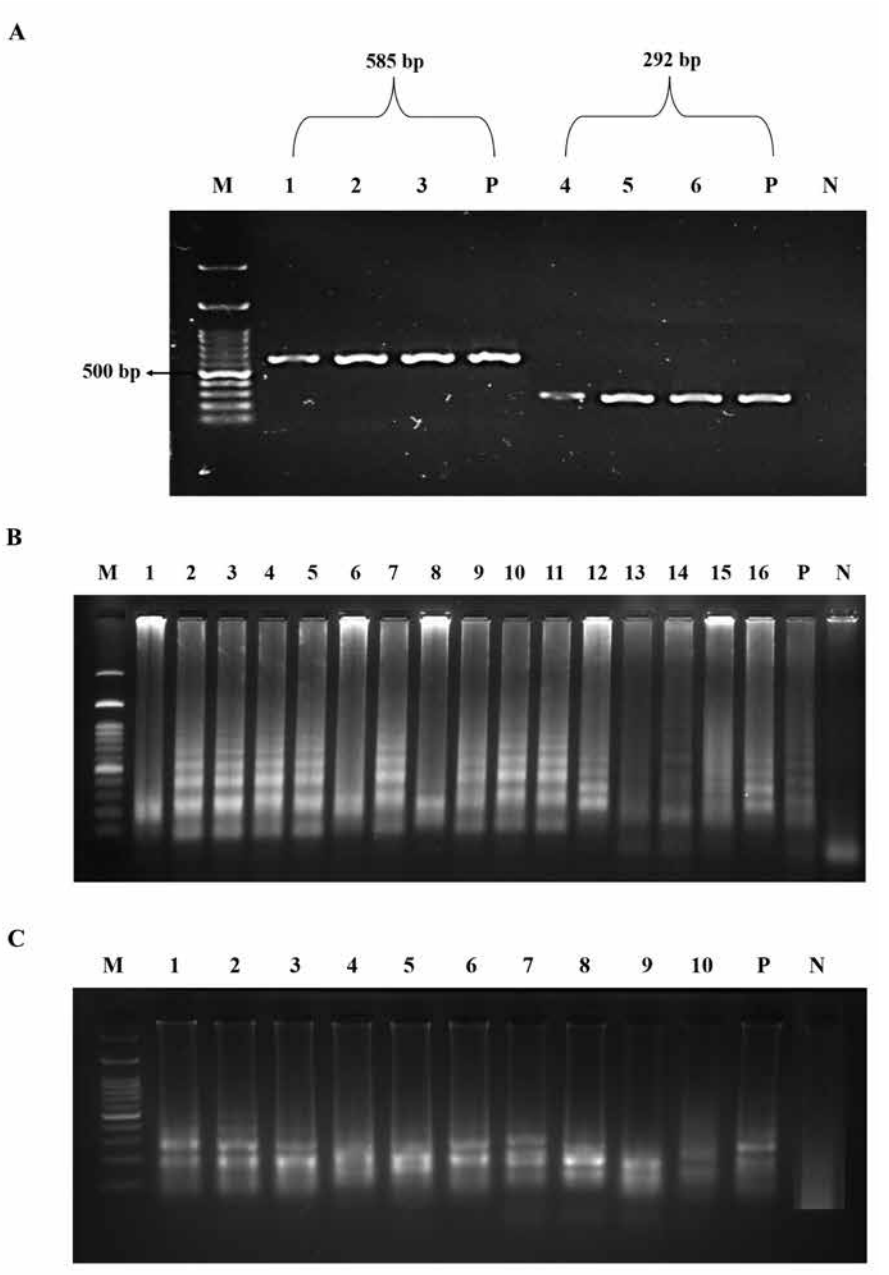

Fig 2-Agarose gel-electrophoresis of amplicons from nested-PCR (A) and LAMP of *Cryptosporidium* spp. (B) and *Giardia duodenalis* (C). DNA was extracted from cysts in water samples collected from catchment areas in Phayao Province, Thailand. Nested-PCR amplified a fragment of 18S rDNA, and LAMP that of *Cryptosporidium* spp SAM-1 and *G. duodenalis* EF1A gene. A. Lanes 1-3, *Cryptosporidium* spp; lanes 4-6, *G. duodenalis*. B. Lanes 1-16: *Cryptosporidium* spp. C. Lanes 1-10: *G. duodenalis*. Lane M, 100 bp DNA size markers; lane N, negative control (distilled water); lane P, *C. pavum* or *G. duodenalis* field isolate confirmed by 18S rDNA fragment sequence analysis (unpublished).

Table 3
Nested- PCR and LAMP detection of *Cryptosporidium* spp and *Giardia duodenalis* in water samples from catchments in Phayao Province, Thailand, May - July 2017.

Sampling site (Number of samples)	*Cryptosporidium* spp		*G. duodenalis*	
	Nested-PCR	LAMP	Nested-PCR	LAMP
	Positive *n* (%)	Positive *n* (%)	Positive *n* (%)	Positive *n* (%)
KPY (70)	28 (40)	42 (60)	16 (23)	22 (31)
NLS (20)	5 (25)	12 (60)	2 (10)	9 (45)
RMP (20)	3 (15)	5 (25)	1 (5)	2 (10)
RMJ (20)	0 (0)	0 (0)	0 (0)	0 (0)
RMS (20)	0 (0)	0 (0)	0 (0)	0 (0)
RMT (20)	1 (5)	3 (15)	1 (5)	2 (10)
Total (170)	37 (22)	62 (36)	20 (12)	35 (21)

KPY, Kwan Phayao Lake; NLS, Nong Leng Sai Lake; RMJ, Mae Jun Reservoir; RMP, Mae Puem Reservoir; RMS, Mae Rong Suk Reservoir; RMT, Mae Tam Reservoir; *n*, number.

There was concordance (total number of samples showing positive and negative results from both assays) for 133 (78%) samples, and 32 (19%) nested-PCR negative and LAMP positive for *Cryptosporidium* spp, and 14 (8%) nested-PCR negative and LAMP positive for *G. duodenalis* (Table 4). Sensitivity of detection for *Cryptosporidium* spp and *G. duodenalis* in all samples was 48% and 61%, respectively; and specificity was 95% and 97%, respectively (Table 4). Nested-PCR *vs* LAMP result of Kappa coefficient tests was 0.482 for *Cryptosporidium* spp (moderate agreement) and 0.663 for *G. duodenalis* (good agreement).

DISCUSSION

Comparison of nested-PCR and LAMP assays to detect *Cryptosporidium* spp and *G. duodenalis* cysts in water samples from six catchment areas in Phayao Province, Thailand revealed superior sensitivity of LAMP over nested-PCR but equal specificity for detection of both types of protozoan cysts.

Detection of *Cryptosporidium* spp and *G. duodenalis* in environmental water samples is influenced by many factors, such as timing, frequency and site of sampling (Wilkes *et al*, 2013). Hot and rainy seasons are peak periods for occurrences of water-borne cryptosporidiosis and giardiasis (Xiao *et al*, 2013; Efstratiou *et al*, 2017; Perkins and Trimmier, 2017; Rosado-Garcia *et al*, 2017). The current surveillance covered the end of the hot season and the beginning of the rainy season in Thailand. Highest frequency of detection of both kinds of cysts was at KPY, a lake located within the city of Phayao, and NLS, Mae Jai District bordered by communities, recreational areas and animal farms. Sturdee *et al* (2007) reported 32% of positive results correlate with areas where livestock and wild mammal feces contaminated water sources. In Australia,

Table 4
Statistical analysis of concordance of methods used for *Cryptosporidium* spp and *Giardia duodenalis* detection in water samples from catchments in Phayao Province, Thailand, May - July 2017.

	Cryptosporidium spp Nested-PCR *vs* LAMP			*G. duodenalis* Nested-PCR *vs* LAMP		
	Positive	Negative	Total	Positive	Negative	Total (95% CI)
Positive	30	5	35	22	3	25
Negative	32	103	135	14	131	145
Total	62	108	170	36	134	170
Concordance of results (pos + neg/170 samples x 100)		78% (n = 133)			90% (n = 153)	
Kappa coefficient		0.48 (0.35 - 0.62)* SE = 0.069			0.66 (0.52 - 0.81)* SE = 0.075	
Sensitivity		48% (35-61%)*			61% (43-77%)*	
Specificity		95% (89-98%)*			98% (94-99%)*	
Positive likelihood ratio		10.45 (4.28-25.55)*			27.30 (8.65-86.10)*	
Negative likelihood ratio		0.54 (0.42-0.69)*			0.40 (0.26-0.60)*	

*95% confidence interval. n, number; neg, negative sample; pos, positive sample; SE, standard error.

positive correlations of *Cryptosporidium* spp and *G. duodenalis* presence turbidity and slow water flow were observed (Swaffer *et al*, 2014). These factors might have played roles in determining the differences in the positivity rates of the two protozoa of interest sampled from different catchment areas in Phayao Province.

The gold standard for detection of *Cryptosporidium* spp and *G. duodenalis* cysts is microscopic examination (ISO, 2006; US EPA, 2012). However, this method is labor intensive, requiring an expert microscopist and at least one independent confirmation of the results. Moreover, debris can obscure the microscopic observation of cysts in environmental water samples (Nichols *et al*, 2003; Utzinger *et al*, 2010). Although PCR-based methods are more objective and sensitive (in theory the presence of one organism in the assay solution is sufficient) PCR technique are prone to DNA polymerase inhibitors contamination of extracted DNA, such as environmental water samples containing fecal matter (Xiao *et al*, 2000; Monis and Saint, 2001), humic substances or DNA of other (similar) microorganisms (Lowery *et al*, 2001; Loge *et al*, 2002). Addition of bovine serum albumin significantly increases the efficiency of PCR amplification (Farell and Alexandre, 2012). In our study, the nested-PCR assay employed primers capable of amplifying more than the three human *Cryptosporidium* spp (*C. hominis*, *C. meleagridis* and *C. parvum)* (Bakheit *et al, 2008)*, and more than the two human Assemblages (A and B) of *G. duodenalis* (Plutzer and Karanis, 2009); thus, nested-PCR could also detect non-human protozoa (false positives) not related to the objectives of public health surveillances.

Whereas in the LAMP assay *Bst* DNA polymerase is not subject to inhibitory effects (Notomi *et al*, 2000), and the primers used are highly specific for the three human *Cryptosporidium* spp. (Karanis *et al*, 2007; Bakheit *et al*, 2008) and *G. duodenalis* human Assemblage A and B (Plutzer and Karanis, 2009; Plutzer *et al*, 2010). LAMP detection of *Cryptosporidium* spp requires a minimum number of cysts equivalent to a single oocyst and for *G. duodenalis* 4-6 cysts of Assemblages A and B (Karanis *et al*, 2007; Plutzer and Karanis, 2009; Plutzer *et al*, 2010).

The ability of both assays to produce positive results for *Cryptosporidium* spp and *G. duodenalis* DNA present in the same sample indicates that the primers were specific for each protozoan DNA and statistical analysis confirmed both nested-PCR and LAMP assays were effective tools for DNA investigation of *Cryptosporidium* spp and *G. duodenalis* isolated from the same water samples. Furthermore, the LAMP assay detected *Cryptosporidium* spp and *G. duodenalis* in samples negative by nested-PCR, suggesting the LAMP assay elicits a higher level of sensitivity than that of nested-PCR; however, as no internal controls for the latter assay were performed, this apparent superior sensitivity of the LAMP might be due in part to inhibition of *Taq* polymerase. Other weaknesses in the current study were the unequal and small number of samples collected from each site, the time period for collection might not be optimal, and no confirmation of the limit of detection of *Cryptosporidium* spp and *G. duodenalis*, alone and in the presence of each other.

In conclusion, this study demonstrates the possible adoption of LAMP assay in surveillance of *Cryptosporidium* spp and *G. duodenalis* present alone or together in environmental water sources not only in Phayao Province but also in other geographical locations suspected of harboring these zoonotic protozoa. The

higher prevalence of *Cryptosporidium* spp and *G. duodenalis* in Kwan Phayao and Nong Leng Sai Lakes, located in urban communities, are causes of concern and prompts an urgent need of relevant authorities to prevent and control the spread of these pathogens among city and town residents. This also applies to catchment areas where communities, recreational areas and animal farms are located near these bodies of water. Environmental and human factors affecting zoonotic pathogenic protozoa breeding pattern, population density fluctuation and transmission need to be studied more closely at each catchment site to assist in formulating a public health policy for management and elimination of these pathogens suitable for each site at risk of infection.

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