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Prevalence of *Cryptosporidium* and *Giardia* in the water resources of the Kuang River catchment, Northern Thailand

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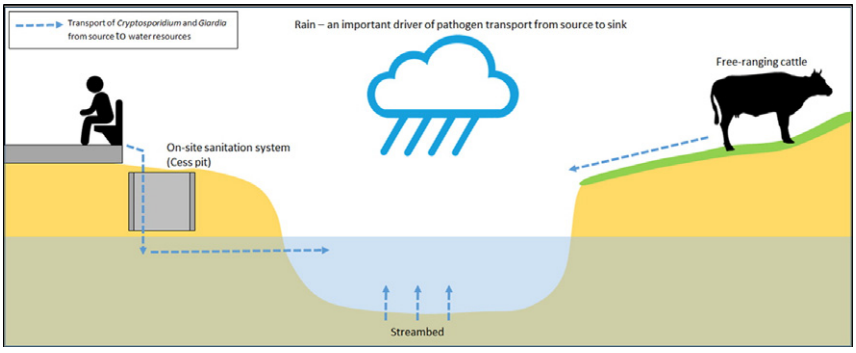
HIGHLIGHTS

- Cryptosporidium and Giardia are ubiquitous in the aquatic environment of N. Thailand.
- Rain is an important driver of pathogen transport.
- Streambed may be an important repository of Cryptosporidium and Giardia.
- Cattle farming management strategies may influence parasitic infection.
- Rural communities are especially at risk to giardiasis and cryptosporidiosis.

GRAPHICAL ABSTRACT



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ABSTRACT

A catchment-scale investigation of the prevalence of *Cryptosporidium* and *Giardia* in the Kuang River Basin was carried out during the dry and rainy seasons. Water samples were collected from the Kuang River and its tributaries as well as a major irrigation canal at the study site. We also investigated the prevalence of gastrointestinal parasitic infection among dairy and beef cattle hosts. *Cryptosporidium* and/or *Giardia* were detected in all the rivers considered for this study, reflecting their ubiquity within the Kuang River Basin. The high prevalence of *Cryptosporidium*/*Giardia* in the upper Kuang River and Lai River is of a particular concern as both drain into the Mae Kuang Reservoir, a vital source of drinking-water to many local towns and villages at the research area. We did not, however, detected neither *Cryptosporidium* nor *Giardia* were in the irrigation canal. The frequency of *Cryptosporidium*/*Giardia* detection nearly doubled during the rainy season compared to the dry season, highlighting the importance of water as an agent of transport. In addition to the overland transport of these protozoa from their land sources (e.g. cattle manure, cess pits), *Cryptosporidium*/*Giardia* may also be re-suspended from the streambeds (a potentially important repository) into the water column of rivers during storm events. Faecal samples from dairy and beef cattle showed high infection rates from various intestinal parasites — 97% and 94%, respectively. However, *Cryptosporidium* and *Giardia* were only detected in beef cattle. The difference in management style between beef (freeranging) and dairy cattle (confined) may account for this disparity. Finally, phylogenetic analyses revealed that the *Cryptosporidium*/*Giardia*-positive samples contained C. ryanae (non-zoonotic) as well as *Giardia intestinalis*

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assemblages B (zoonotic) and E (non-zoonotic). With only basic water treatment facilities afforded to them, the communities of the rural area relying on these water supplies are highly at risk to *Cryptosporidium*/*Giardia* infections.

1. Introduction

Diarrhoea claims more lives of children than AIDS, malaria and measles combined and is ranked as the second most common cause of death for children under five years of age worldwide after pneumonia (UNICEF and WHO, 2009). Nearly one in five children die each year from diarrhoea (UNICEF and WHO, 2009). Diarrhoea is caused by a wide range of pathogens including viruses, bacteria and protozoa. Of these, *Cryptosporidium* and *Giardia* are two of the most globally dominant and dangerous parasitic protozoa that infect not only humans, but also domestic animals and wildlife, (Caccio et al., 2005; Haque, 2007; Hunter and Thompson, 2005).

Cryptosporidium and *Giardia* are monoxenous: they complete their life-cycles within a single host, which excretes large numbers of infective stages (*Cryptosporidium* oocysts and *Giardia* cysts) in faeces. A gram of faeces from an infected host may contain as many as 1×10^7 and 2×10^6 *Cryptosporidium* and *Giardia* cysts, respectively (Smith et al., 2006). While there may be several modes of transmission, infection typically occurs following ingestion of water contaminated with *Cryptosporidium* and *Giardia* cysts — even in small doses. Infections in humans have been reported occur in doses as low as 9 and 10 cysts for cryptosporidiosis and giardiasis, respectively (Smith et al., 2006). The cysts are environmentally robust, allowing them to persist for long periods of time outside the host. Their small size allows them to penetrate the physical barriers of conventional water treatment systems. They are also insensitive or resistant to many disinfectants used in the water industry (e.g. chlorine). *Cryptosporidium* and *Giardia* therefore constitute a significant health hazard, even in developed countries.

Between World War I and 2003, a total of 325 recorded water-associated outbreaks of parasitic protozoan diseases occurred (Karanis et al., 2007). North America (Canada and the United States) and Europe (primarily the United Kingdom) accounted for 93% of the reported outbreak (Karanis et al., 2007) — most likely due to reporting bias (Baldursson and Karanis, 2011). Cryptosporidiosis and giardiasis make up nearly all of the reported cases: 51% and 41%, respectively. More recently, at least 199 outbreaks occurred between January 2004 and December 2010 (Baldursson and Karanis, 2011). Again, *Cryptosporidium* (60%) and *Giardia* (35%) were the main etiological agents of these waterborne parasitic outbreaks. Documented cases were mainly reported from North America and Europe, along with 'newcomers', Australia and New Zealand. Reports from these countries/continents make up approximately 96% of the documented outbreaks (Baldursson and Karanis, 2011).

Consideration of these two reviews discloses several important findings. Firstly, *Cryptosporidium* and *Giardia* are dominant causative agents of waterborne disease outbreaks, compared with other protozoan parasites. Secondly, even first world nations with reliable and modern water treatment systems and technology are susceptible to parasitic outbreaks. Thirdly, marked progress has been made in the detection and diagnostic methods, which in turn has resulted in the improvement in surveillance and reporting systems. Finally, there is a lack of research and monitoring in developing countries of Asia, Africa and Latin America. This latter issue is ironic, yet important, because the poorer communities from these regions without reliable water and sanitation facilities are likely more vulnerable to these diseases than those in the developed world where most cases are reported (Hotez et al., 2009; Prüss-Üstün et al., 2008; WHO, 2008).

Similarly, in the developing nations of Southeast Asia, *Cryptosporidium*- and *Giardia*-related studies are relatively rare compared with their first world counterparts. Wherever available, studies on *Cryptosporidium* and *Giardia* almost always pertain to their prevalence in hosts rather than the environment (Dib et al., 2008; Lim et al., 2010 and the references therein). In Thailand, for example, only five such studies have been published (Anceno et al., 2007; Diallo et al., 2008; Koompapong and Sukthana, 2012; Kumar et al., 2014; Srisuphanunt et al., 2010). These studies typically only investigated the occurrence of *Cryptosporidium* and *Giardia* in the aquatic environment and water resources. Investigations on the factors contributing to their distribution are rare.

Herein, this research void is addressed by investigating the prevalence of *Cryptosporidium* and *Giardia* in the surface water resources in a rural study area in northern Thailand. The role of seasonality (i.e. dry weather vs. wet weather) is also explored by investigating the association of hydroclimatological factors with the distribution of *Cryptosporidium* and *Giardia* in the environment. In addition, faecal samples from cattle, an important host for *Cryptosporidium* and *Giardia*, were also screened for both protozoa as well as other intestinal parasites. Finally, isolates of both organisms in faecal samples from cattle were molecularly characterised.

2. Study area

The area investigated for this study is centred on the Kuang River Basin, which is located on the eastern bank of the Ping River in Northern Thailand. The catchment area is 1661 km^2 and has a population of 291,000, of which, half are classified as rural (Ganjanapan and Lebel, 2014). The area spans across the districts of San Sai, San Kamphaeng, Mae On and Doi Saket within the Chiang Mai Province, as well as the districts of Ban Thi, Pa Sang and the capital district (*Amphoe Mueang*) of the Lamphun Province. Forests, mostly deciduous and dry dipterocarp, cover just over half of the drainage basin and are mostly restricted to higher elevations. Approximately one third of the area is devoted to agriculture and about 7–8% to residential use (Ganjanapan and Lebel, 2014).

The Kuang River is an important tributary to the Ping River, which drains into the Chao Phraya River in Central Thailand (Fig. 1). The Ping River basin is the largest (catchment area of over 35,000 km^2) in the Chao Phraya River basin. It is a vital source of water not only in the northern region, but also to the nation's capital, Bangkok, as well as many parts of Central Thailand for domestic, agricultural and industrial uses (Thomas, 2005).

The Lai River, Pong River and San River are major tributaries to the Kuang River. The former, together with the upper reach of the Kuang River, form the primary inflows to the Mae Kuang Reservoir. With a water storage capacity of approximately 260 million m^3 and a catchment area of over 550 km^2, the Mae Kuang Reservoir is a major source of irrigation, domestic and drinking-water supply for many locations in the provinces of Chiang Mai and Lamphun (Chansribut, 2002; Nutniyom, 2003).

A complex network of canals also distributes surface water across the study site. Importantly, the Mae Taeng-San Sai Canal, constructed and managed by the Royal Irrigation Department of Thailand, is a 40-km long, trapezoidal concrete canal that receives water from the Ping River, immediately downstream of the Mae Ngat Reservoir, and conveys water to the San Sai District from the Mae Taeng District, to the north. The water from this canal is typically used from irrigation purposes. The canal can also be used for recreational purposes where we observed villagers bathing in the waters particularly during the dry season.

Chiang Mai and Lamphun have a tropical wet and dry climate (Köppen *Aw*), typical of the northern region of Thailand. Annual rainfall

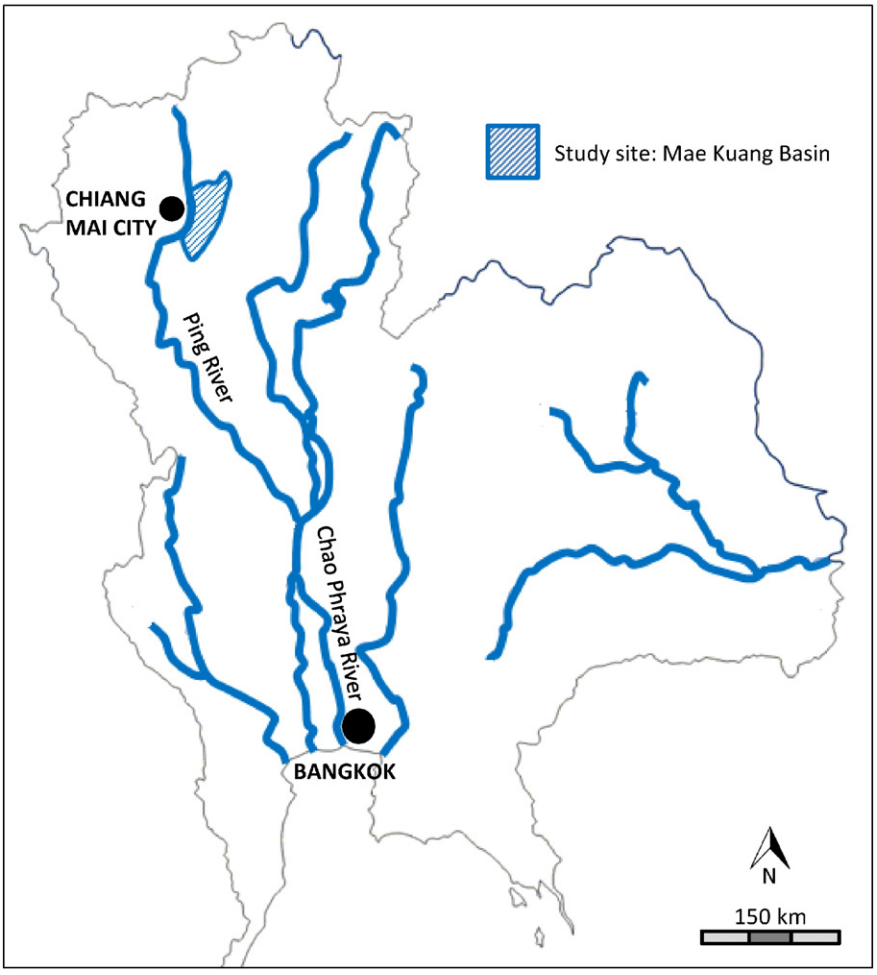


Fig. 1. The location map of the study area, the Mae Kuang Basin.

in Northern Thailand is approximately 1200 mm with seasonal rainfall, between May and October, accounting for almost 92% of the annual total (Wood and Ziegler, 2008; Lim et al., 2012).

The livestock sector contributes approximately 2.3% to Thailand's gross domestic product (GDP) (FAO, 2005). The cattle farming industry has been growing steadily over the years with an increase of nearly 46% in the number of cattle between 1961 and 2013 (FAO, 2015). Correspondingly, milk as well as beef production had also increased during this period. Milk production increased nearly 550 fold from 2000 tonnes in 1961 to 1,095,000 tonnes in 2013 (FAO, 2015). Growth in the beef production was lower (56%) with 70,000 tonnes of beef produced in 1961 compared to 160,000 tonnes in 2013 (FAO, 2015). Approximately 26% of the total number of cattle in Thailand is found in the northern region whereby over 70% are from small-scale farms with less than 10 cows (FAO and APHCA, 2002).

3. Materials and methods

3.1. Water samples

3.1.1. Sample collection

A total of 120 water samples were collected from 60 sampling sites from natural (52) and manmade (8) surface water bodies at the study area which include the following: the Kuang River (K1–K18), the Upper Kuang River (uK1–uK4), the Lai River (L1–L7), a Lai River tributary (tL1–tL3), the Pong River (P1–P9), the Upper Pong River (uP1–uP3); a Pong River Tributary (tP1 – tP3), the San River (S1–S5) and the Mae Taeng-San Sai Canal (C1–C8). Sampling was carried out twice at each sampling site: (i) near the end of dry season (April–May 2014) when water levels are at the lowest; and (ii) during the peak of the rainy season (July–August 2014). For each sampling occasion, 40 L of river/canal water were collected directly from water bodies with a bucket and stored in two 20-L plastic bottles before transferring to the laboratory for analyses. For the dry weather samples, no rain events were recorded at least one week preceding the days of collection. For the wet weather samples, collection was carried out on five separate occasions — 15th July, 16th July, 14th August, 16th August and 18th August. The total rainfall three days preceding the days of collection were 258 mm, 63 mm, 99 mm, 124 mm and 11 mm, respectively. Rainfall data were obtained from our weather station in San Sai District.

3.1.2. Sample analyses

The procedures employed for detection and enumeration of *Cryptosporidium* and *Giardia* processes followed those developed and validated by the United States Environmental Protection Agency (USEPA, 2012): (i) filtration; (ii) elution (wash); (iii) concentration; (iv) purification (immunomagnetic separation); (v) staining; and (vi) immunofluorescence assay microscopy. Briefly, 40 L of water sampled from each site were transported immediately to a private laboratory in Chiang Mai. The samples were filtered on the same day of collection using Filta-Max® foam cartridge filters (IDEXX Laboratories, Inc., Westbrook, ME, USA), which retain *Cryptosporidium* or *Giardia* cysts. Filtration was assisted by a motorised pump located on the inlet side (upflow) of the filters.

Following filtration, the filters were transferred to a Filta-Max® manual wash system (IDEXX Laboratories, Inc., Westbrook, ME, USA)

to elute all cysts retained. To do so, the filters were washed with 600 mL of phosphate buffered saline (PBS) (10 mM) with 0.01% Tween® 20 (PBST). Following washing, the concentrator tube containing the eluate was transferred onto a magnetic stirring plate. While stirring (to ensure that any cysts present stay afloat), the tube was drained from its base to concentrate the samples to approximately 20 mL. The filters were washed for the second time with 600 mL of PBST. The concentrates from the first wash were pooled with those from the second wash and then concentrated to a final volume of approximately 20 mL. Any cysts retained on the filter membranes from the base of the concentrator tube were washed off with PBST by transferring the filter membranes into a small sealable plastic bag with approximately 5 mL of PBST and then manually kneading the membranes to remove cysts retained. The wash products from the membranes were pooled together with the primary concentrated eluates in centrifuge tubes (final volume of approximately 25 mL) and stored in the dark at 4 °C before transferring to the laboratory at the Department of Parasitology, University of Malaya, Kuala Lumpur (Malaysia), for the subsequent procedures.

Before immunomagnetic separation (IMS), the samples were centrifuged at 1500 g for 15 min. The supernatants were then carefully aspirated to 5 mL above the pellets. The samples were *re*-suspended vigorously to ensure complete homogenisation before transferring to Leighton tubes for IMS. For each sample, 1 mL of 10× SL-buffers A and B and 100 μL of *Cryptosporidium* and *Giardia* IMS beads (Dynabeads® GC-Combo, Invitrogen Dynal AS, Oslo, Norway) were added then mixed with a rotating mixer at approximately 18 rpm for 1 h at room temperature (~25 °C). The Leighton tubes were then placed in a magnetic particle concentrator and gently rocked at an angle of 90° for 2 min at 1 tilt/s. The supernatants were decanted before removing the tubes from the magnetic particle concentrator. The samples were gently rocked to re-suspend the bead-cyst complexes with 1 mL of 1× SL-buffer A before transferring to labelled 1.5-mL polypropylene centrifuge tubes. The tubes were then placed in a second magnetic particle concentrator and rocked for 1 min to aspirate the supernatants before removing the magnet.

For the disassociation of the bead-cyst complexes, 50 mL of 0.1 N HCl were added to each sample, which was then vortexed for 50 s then allowed to stand for at least 10 min in an upright position. The samples were vortexed for a further 10 s, replaced in the magnetic particle concentrator and left undisturbed for at least 10 s. At this point, the beads were collected at the back of the tube and the acidified suspensions were transferred to the wells microscope slides, each containing 5 μL of 1.0 N NaOH.

Before staining, the samples were allowed to dry at 37 °C (max. 1 h) and then fixed with methanol. Then, 50 μL of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Co., Ontario, Canada) solution (2 μg/mL in PBS) were added to each well. After 2 min, the excess DAPI was removed and 50 μL of distilled water were added to wash the wells. After 1 min, the excess water was removed. Fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* sp. and anti-*Giardia* sp. monoclonal antibodies (FITC-MAb) (EasyStain™, BTF Pty. Ltd., NSW, Australia) were added (50 μL) and the mixture was incubated at room temperature. After 30 min, the excess FITC-MAb was removed and 300 μL of the fixing buffer from the EasyStain™ kit were added to the wells. The fixing buffer was drained after 2 min and 5 μL of EasyStain™ mounting medium were added before sealing the slides with cover slips for subsequent examination.

The slides were scanned at a magnification of 400× by epifluorescence microscopy (Olympus BX51, Tokyo, Japan). *Cryptosporidium* spp. and *Giardia* spp. cysts were first identified and enumerated by immunofluorescence reaction and then confirmed by DAPI fluorescence on the basis of their sizes, morphological features and the presence of nuclei as described in Method 1623.1 (USEPA, 2012).

3.1.3. Protozoan cyst recovery efficiency

To establish the ability to demonstrate control over the analytical system as described in the preceding section and to generate acceptable precision recovery, protozoan cyst recovery efficiency tests were conducted. EasySeed™ (BTF Pty. Ltd., NSW, Australia), which contains 100 inactivated *Cryptosporidium* oocysts and 100 inactivated *Giardia* cysts, was spiked into 10 L of deionised water. The samples were then processed in accordance to the procedures described above for the detection and enumeration of *Cryptosporidium* and *Giardia* cysts.

The cyst recovery efficiency test was replicated 6 times. The mean recovery for *Cryptosporidium* oocysts and *Giardia* cysts was 39% and 45%, respectively. The acceptance criteria for the mean recovery of *Cryptosporidium* and *Giardia* as specified by the USEPA are 38% and 27% (minimum), respectively (USEPA, 2012). The mean recovery of cysts is comparable to those from other recent studies: 41–55% for *Cryptosporidium* and 31–41% for *Giardia* (Budu-Amoako et al., 2012; Castro-Hermida et al., 2015; Sato et al., 2013; Xiao et al., 2012). The precision (as relative standard deviation) of the recovery was 36% and 37% for *Cryptosporidium* oocysts and *Giardia* cysts, respectively. The acceptance criteria for the precision of *Cryptosporidium* and *Giardia* recovery system as specified by the USEPA are 37% and 39% (maximum), respectively (USEPA, 2012). With a recovery efficiency of less than 100%, it is important to consider that the cysts concentrations in water samples reported in this study are likely underestimated.

3.2. Faecal samples

3.2.1. Sample collection

A total of 126 faecal samples were collected from Brahman beef cattle ($n = 64$) and Holstein-Friesian dairy cattle ($n = 62$) between May and July 2014. All samples were collected from Chiang Mai Province. Dairy cattle samples were collected directly from four farms in the San Sai District while samples from beef cattle were collected from free-ranging herds in the grazing fields along the Mae Taeng-San Sai canal. Approximately 10 g of faeces were collected using disposable plastic spoons and stored in sterile plastic containers. Although collection of faeces directly from the rectum of individual animals would have minimised the potential for cross-contamination, the lack of a trained animal handler prevented us from doing so. However, to minimise the contamination of samples and to ensure that repeat samples did not occur, only fresh and wet samples were collected — often immediately following defecation. Collected samples were preserved in 2.5% potassium dichromate solution before being transported to the Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia where they were stored at 4 °C until required for subsequent analyses.

3.2.2. Identification and molecular analysis

All samples were first concentrated using solvent-free faecal parasite concentrators (Mini Parasep® SF, Apacor, Berkshire, United Kingdom) as per the manufacturer instructions. Faecal samples were screened for the presence of *Giardia* and other protozoa (except *Cryptosporidium*) and helminths by smearing the concentrated products onto microscope slides. The samples were stained with Lugol's iodine before viewing under a light microscope at 100× and 400× magnification. Samples were not screened for *Cryptosporidium* by light microscopy because even with staining, the threshold of detection is typically low (Weber et al., 1991).

Molecular techniques were carried out to determine the assemblage of *Giardia intestinalis* detected in the positive samples via microscopy as well as to determine the presence and species of *Cryptosporidium* in all the faecal samples. Genomic DNA was extracted from all samples using the NucleoSpin® Soil Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), according to the manufacturer's protocol.

For molecular typing of *G. intestinalis*, a two-step nested PCR and partial sequencing of the triosephosphate isomerase (TPI) gene were performed based on the work of Sulaiman et al. (2003). In the primary reaction, a 605 base-pair (bp) fragment was amplified with the forward primer AL3543 5′-AAATIATGCCTGCTCGTCG-3′ and reverse primer AL3546 5′-CAAACCTTITCCGCAAACC-3′. The PCR reaction consisted of

1.0 μL of DNA, 12.5 μL (2×) of ExPrime Taq Premix (containing ExPrime TaqTM DNA Polymerase 1 unit/10 μL, 20 mM Tris-HCl, 80 mM KCl, 4 mM $MgCl_2$, and 0.5 mM of each dNTP) (GeNet Bio Inc., Daejeon, S. Korea) and 0.25 μM of both the forward and reverse primers with a final volume of 20 μL. The PCR was performed with an initial denaturation step of 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s; and a final extension cycle of 72 °C for 10 min. For the nested PCR reaction, a PCR product of 530 bp was amplified by using the forward primer AL3544 5′-CCCTTCAT CGGIGGTAACTT-3′ and the reverse primer AL3545 5′-GTGGCCAC CACICCCGTGCC-3′. The nested PCR mixture consisted of 1.0 μL of the first PCR product, 25.0 μL (2×) of ExPrime Taq Premix, 0.2 μM of both forward and reverse primers with a final volume of 50 μL. The conditions for the secondary PCR were identical to the primary PCR.

Cryptosporidium species and genotyping were also determined by a two-step nested PCR protocol and sequencing of the partial 18S rDNA gene based on the work by Ryan et al. (2003). For the primary PCR, a PCR product of 763 bp was amplified using the forward primer 18SiCF2 5′-GAC ATA TCA TTC AAG TTT CTG ACC-3′ and reverse primer 18SiCR2 5′-CTG AAG GAG TAA GGA ACA ACC-3′. The PCR mixture consisted of 2.5 μL of the purified DNA, 15.0 μL (2×) of ExPrime Taq Premix, and 10 μM of the forward and reverse primers. The PCR was performed with an initial denaturation of 94 °C for 5 min followed by 45 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 10 min. For the secondary PCR, a fragment of ~587 bp was amplified using the forward primer 18SiCF1 5′-CCT ATC AGC TTT AGA CGG TAG G-3′ and the reverse primer 18SiCR1 5′-TCT AAG AAT TTC ACC TCT GAC TG-3′. The secondary PCR mixture consisted of 2.0 μL of the first PCR product, 25.0 μL (2×) of ExPrime Taq Premix, and 10 μM for both the forward and reverse primers. The conditions for the nested PCR were identical to those for the first PCR. Amplicons were analysed by electrophoreses on 1.5% agarose gel and visualised under UV lamp after staining with GelRed (Biotium, Hayward, CA, USA). Amplicons of the expected size were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen, Germantown, MD, USA). Purified PCR products were sent to Axil Scientific for sequencing in forward and reverse directions.

Sequences were initially examined using BLAST (Altschul et al., 1990). Reference *Cryptosporidium* and *Giardia* sequences were downloaded and aligned using MAFFT in Geneious 7.1.6 (Kearse et al., 2012) and then manually curated. FastTree was run to create an initial phylogeny after which redundant sequences were removed (Price et al., 2010). Ambiguous regions were removed for the final *Cryptosporidium* alignment. Model testing and Maximum Likelihood tree inference was performed with IQTREE v 1.3.8 (Nguyen et al., 2015). Based on the best Bayesian Information Critera (BIC) score, a TN + G4 nucleotide substitution model was selected for *Giardia* sequences and a K3Pu + I + G4 model was selected for *Cryptosporidium* sequences. Branch support values were provided through 10,000 bootstrap repetitions (Minh et al., 2013). The *Cryptosporidium* tree was midpoint rooted, while the *Giardia* tree was rooted to a *Giardia muris* sequence. Outgroups were removed in the final tree.

Table 1Please check if the intended presentation/layout of Table 1 has been achieved
Concentration of *Cryptosporidium* and *Giardia* cysts at all river sampling sites for both the dry and wet seasons.

River	Sampling site	Dry season		Rain season	
		Cryptosporidium (oocyst/10 L)	*Giardia* (cyst/10 L)	*Cryptosporidium* (oocyst/10 L)	*Giardia* (cyst/10 L)
Kuang River	uK1	–	–	–	–
	uK2	–	–	–	–
	uK3	–	–	–	0.50
	uK4	–	–	–	0.28
	K1	–	–	–	–
	K2	–	–	–	–
	K3	–	–	–	–
	K4	–	–	–	–
	K5	–	–	–	4.17
	K6	–	–	–	–
	K7	–	–	–	–
	K8	–	–	0.40	0.40
	K9	–	–	–	–
	K10	–	–	–	–
	K11	–	0.50	0.37	0.74
	K12	–	–	–	–
	K13	–	–	–	–
	K14	–	–	–	–
	K15	0.25	0.25	–	–
	K16	–	–	–	0.48
	K17	0.50	0.25	–	–
	K18	–	–	–	–
Lai River	tL1	–	–	–	–
	tL2	–	–	4.00	1.50
	tL3	–	–	–	–
	L1	–	–	1.41	2.12
	L2	–	–	–	3.23
	L3	–	–	1.82	4.85
	L4	0.25	–	0.65	10.32
	L5	–	–	–	4.28
	L6	–	–	–	5.71
	L7	–	–	–	13.89
Pong River	uP1	–	–	–	–
	uP2	–	–	–	–
	uP3	–	–	–	0.50
	tP1	0.74	2.94	–	–
	tP2	–	0.80	–	–
	tP3	–	1.19	–	–
	P1	–	–	–	0.50
	P2	6.50	1.00	–	1.00
	P3	0.50	–	–	–
	P4	–	0.50	0.75	1.25
	P5	–	–	–	–
	P6	–	–	–	1.99
	P7	–	–	–	–
	P8	0.50	2.50	0.57	–
	P9	–	–	–	–
San River	S1	–	–	–	2.24
	S2	–	–	–	–
	S3	–	–	–	–
	S4	–	–	–	–
	S5	–	–	–	–

4. Results

4.1. Water samples

All four rivers at the study area were contaminated with varying levels of *Cryptosporidium* and/or *Giardia* cysts (Table 1 and Fig. 2). More than half of the 52 river sampling sites (27/52) tested positive for *Cryptosporidium* and/or *Giardia*. *Giardia* was detected in half (26/52) of the river sampling sites while 25% (13/52) of these sites contained *Cryptosporidium* (Fig. 3a). *Cryptosporidium-Giardia* co-contamination occurred in nearly a quarter (12/52) of the monitored river sampling sites. The highest concentration of *Cryptosporidium* (6.50 oocysts/10 L) was detected in P2 at the Pong River during the dry season while the highest concentration of *Giardia* (13.85 cysts/10 L) was detected during the wet season in L7 at the Lai River. Neither *Cryptosporidium* nor *Giardia* was detected in the water samples collected from any of the eight sampling sites of the Mae Taeng-San Sai Canal in either the dry or wet season.

During the dry season, *Cryptosporidium* or *Giardia* were detected in 21% (11/52) of the river sampling sites while the samples containing either protozoa nearly doubled (40%; 21/52) during the wet season (Table 1; Fig. 2). *Giardia* cysts were detected more frequently than *Cryptosporidium* oocysts for both dry and wet seasons (Fig. 3b). For the dry season samples, 13% contained *Cryptosporidium* (0.25–6.50 oocysts/10 L). In comparison, 18% of the tested samples contained *Giardia* (0.25–2.94 cysts/10 L). Meanwhile, for the wet season samples, 15% tested positive for *Cryptosporidium* (0.37–4.00 oocysts/10 L) and 38% tested positive for *Giardia* (0.28–13.89 cysts/10 L).

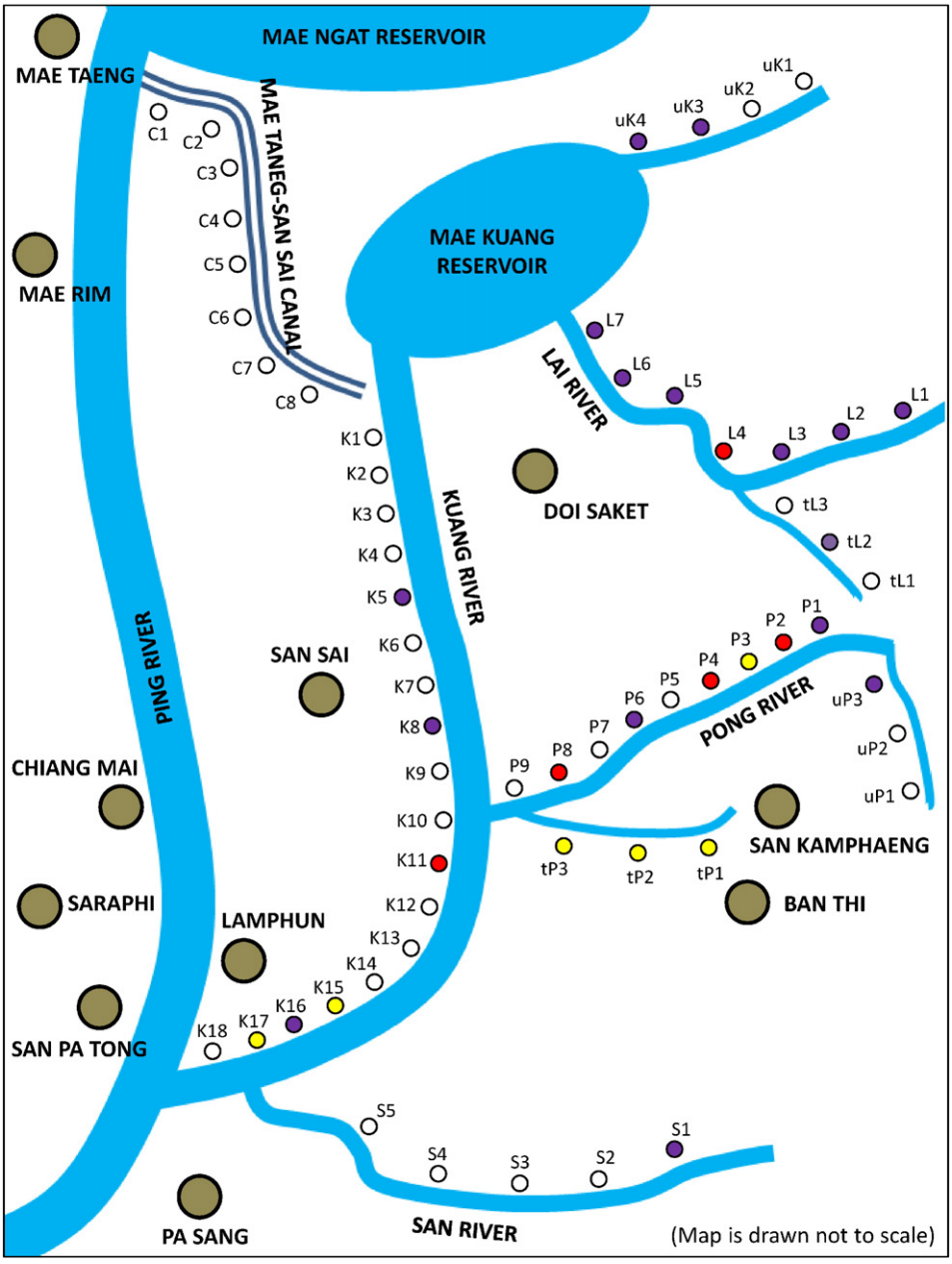


Fig. 2. Locations of sampling sites denoting absence/presence of *Cryptosporidium*/*Giardia* during dry and wet seasons (Red: Dry and wet season; Yellow: Dry season only; Violet: Wet season only; White: non-detection).

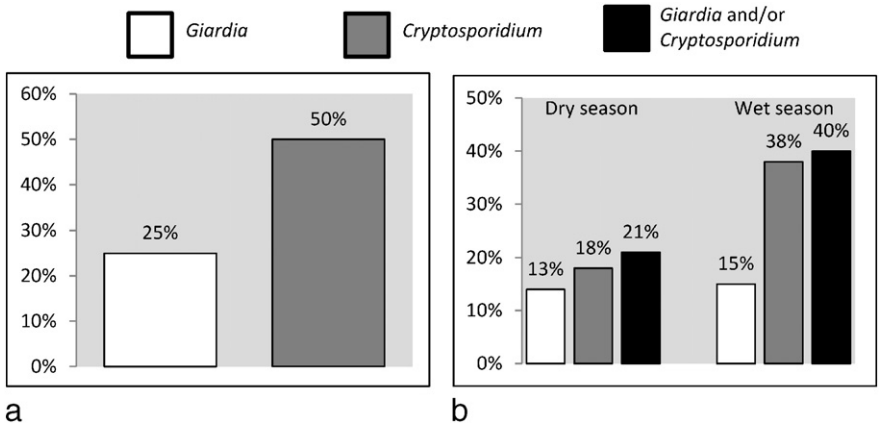


Fig. 3. a Prevalence of *Cryptosporidium* and *Giardia* in the sampling sites of the rivers at the study area. b Seasonal variation of *Cryptosporidium* and *Giardia* in the rivers of the study area.

4.2. Faecal samples

Both dairy and beef cattle had high parasitic infection rates with 97% of the faecal samples from dairy cattle testing positive for at least one intestinal parasite compared to the 94% in faecal samples from beef cattle. *Entamoeba* (53%) was the most prevalent of the gastrointestinal parasites detected in beef cattle followed by *Eimeria* (42%), *Paramphistomum* (33%), strongyle (25%), *Buxtonella sulcata* (23%), *Giardia* (13%), *Fasciola* (8%), *Cryptosporidium* (3%) and *Dicrocoelium* (2%) (Fig. 4). Some of the parasites found in beef cattle were also detected in dairy cattle: *Entamoeba* (98%), *Eimeria* (18%), *Paramphistomum* (13%), *Buxtonella sulcata* (5%) and strongyle (3%) (Fig. 4). *Dicrocoelium*, *Fasciola*, *Giardia* and *Cryptosporidium* were not detected in any dairy cattle samples. Co-infections were observed in 56% ($n = 36$) of the meat cattle samples and 38% ($n = 24$) of dairy cattle. Beef cattle showed noticeably higher infection for all parasites except *Entamoeba* where infection in dairy cattle was almost double that in beef cattle.

Giardia cysts were detected in eight of the 64 faecal samples from beef cattle (~13%) through microscopy. PCR was conducted on the *Giardia*-positive samples, but only three were successfully amplified. Three PCR-positive *Giardia* samples were sequenced and phylogenetic analysis revealed two were from the non-zoonotic assemblage E that only infects hoofed livestock, while the third was identified as assemblage B, which is known to infect humans (Fig. 5a). Only two (~3%) of the samples from beef cattle tested positive for *Cryptosporidium*. Sequence analysis determined both were *Cryptosporidium rynae*, *a non*-zoonotic species that infects cattle (Fig. 5b).

5. Discussion

5.1. Environmental prevalence and population vulnerability

Both *Cryptosporidium* and *Giardia* were detected in varying levels in all the rivers at the study area, reflecting their ubiquity, and correspondingly, the associated risk of cryptosporidiosis and giardiasis within the predominantly rural landscape. The findings reflect the water quality in this headwater region draining to the Ping River, and ultimately the Chao Phraya River. These streams and rivers are not only vital drinking-water resources for the local regional communities but also for those downstream in the more densely populated areas, including Bangkok, which rely on the northern region for much of its municipal water. Prior studies have largely focused on the highly polluted surface waters in Bangkok and its vicinities in Central Thailand (Anceno et al., 2007; Diallo et al., 2008; Koompapong and Sukthana, 2012). Anceno et al. (2007) and Diallo et al. (2008), for example, surveyed many canals, some of which function as open conveyance systems for wastewater.

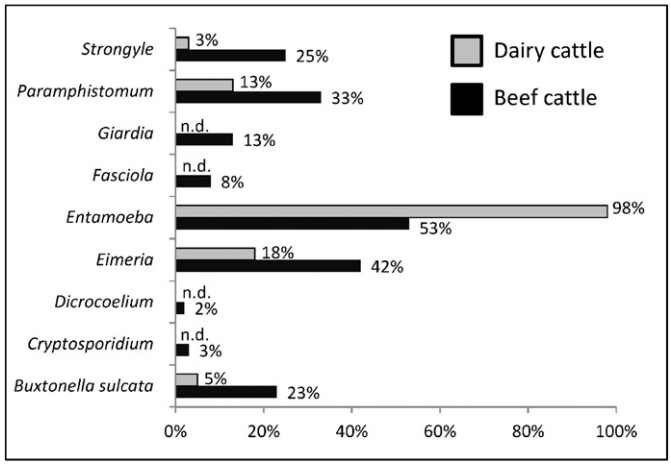


Fig. 4. Prevalence of *Cryptosporidium*, *Giardia* and other parasites in the faecal samples of beef and dairy cattle.

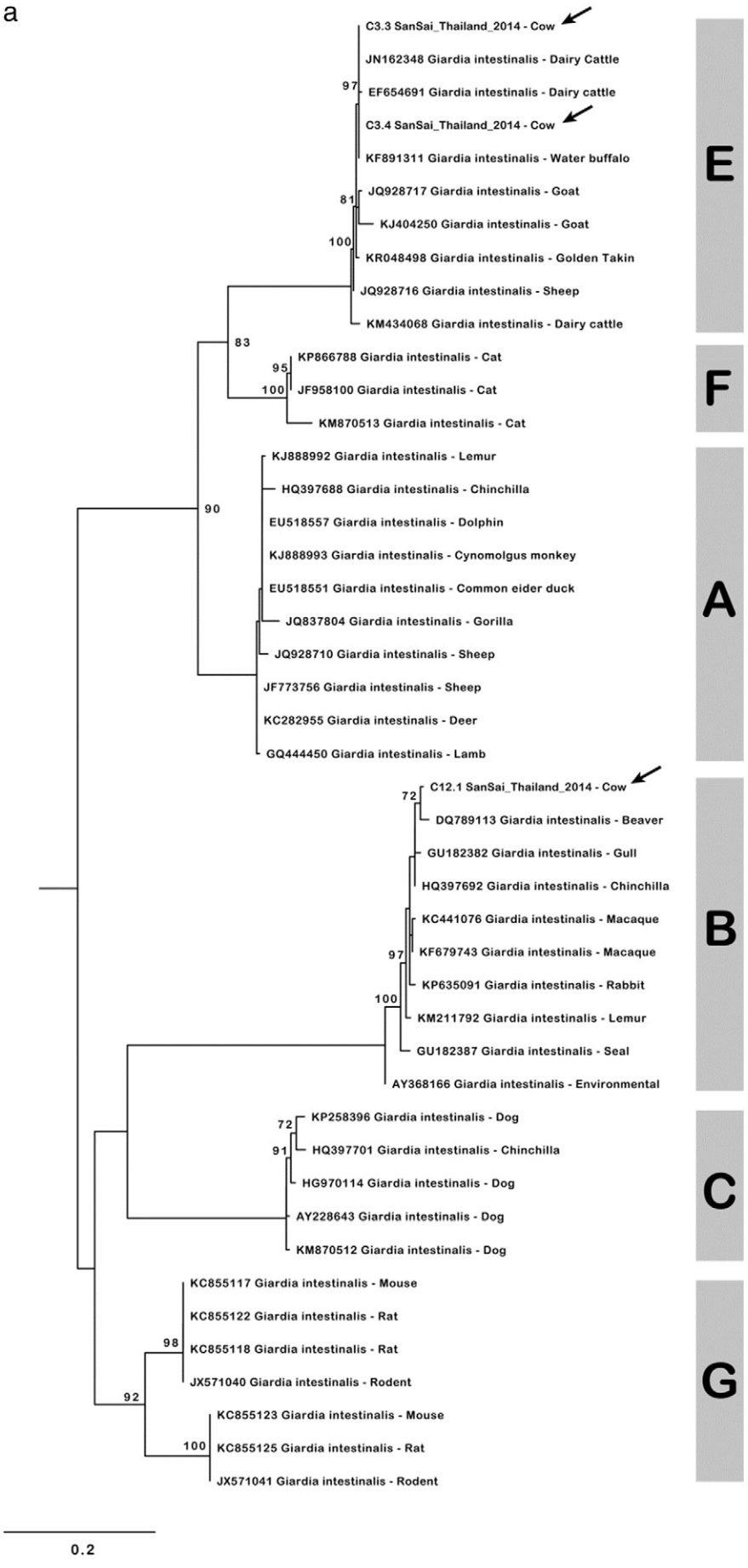
Not surprisingly, they frequently detected *Cryptosporidium* and *Giardia* cysts in high concentrations. In another Central Thai study, Koompapong and Sukthana (2012) reported the presence of the zoonotic *Cryptosporidium parvum*, as well as the non-zoonotic *Cryptosporidium meleagridis* and *Cryptosporidium serpentis*, in the surface waters at the mouth of the Chao Phraya.

We sampled the river/stream/canal network systematically at the catchment scale to assess the spatial variability of *Cryptosporidium* and *Giardia* contamination. All the previous studies in Thailand (i.e. Anceno et al., 2007; Diallo et al., 2008; Koompapong and Sukthana, 2012; Kumar et al., 2014; Srisuphanunt et al., 2010) have not been able to assess this aspect. These studies only reported the presence or absence of *Cryptosporidium* and *Giardia* in various water resources without providing the origins of the water samples and other informative details that may be useful for management. Studies conducted in southern Thailand, for example those by Srisuphanunt et al. (2010) and Kumar et al. (2014), tested for *Cryptosporidium* and *Giardia* in numerous samples ranging from raw to processed water. Detailed information regarding the source (e.g. river, aquifer etc.) and the type of treatment (e.g. filtration, disinfection etc.) for processed water like tap and bottled water was however not reported, preventing the tracking of contamination sources and the understanding of processes driving the microbial dynamics (Kay et al., 2007). Elsewhere, the European Union Water Framework Directive and its USA counterpart, the Clean Water Act, require catchment-scale investigations for microbial contamination studies such that programmes of measures can be designed by water resource managers and policy makers to preserve, protect and improve the quality of drinking water resources (Council of the European Communities, 2000; Davison et al., 2005; Horn et al., 2004).

This catchment-scale study provided new and invaluable insight to the spatial prevalence of *Cryptosporidium* and *Giardia* in the surface water bodies of the study area. These findings make it possible to identify and prioritise the next steps for research in order to track the source of contamination and understand the processes that underpin microbial transport. In particular, the detection of *Cryptosporidium* and *Giardia* cysts in the upper reaches of the Kuang River (sites uK3 and uK4), and especially their high prevalence and intensity in the Lai River (sites L1 to L7, tL2) is of concern because these rivers are the two primary inflows to the Mae Kuang Reservoir, the principal source of drinking water to several districts in the provinces of Chiang Mai and Lamphun. In the highly publicised 1993 Milwaukee (USA) cryptosporidiosis outbreak, where over 400,000 people were infected, the concentration of *Cryptosporidium* oocysts in the public water supply ranged between 0.03 and 1.32 per 10 L water (MacKenzie et al., 1994). In the Kuang River Basin, significantly higher levels of *Cryptosporidium* oocysts were recorded in the Lai River. For example, between 0.65 and 4.00 *Cryptosporidium* oocysts per 10 L of water were detected, while the concentrations of *Giardia* cysts were even higher, ranging from 1.50 to 13.89 per 10 L of water.

The drinking water resources in the district centres and major towns/cities of Northern Thailand, including those in the study area, are managed by the Provincial Waterworks Authority (PWA). Water supplies are typically treated using the conventional method of coagulation–sedimentation–filtration–disinfection via chlorination. However, studies have consistently shown that such drinking water treatment procedure are unable to remove these pathogens completely, especially when the raw (incoming) water contains high concentrations of *Cryptosporidium* and *Giardia* cysts (Ali et al., 2004; Castro-Hermida et al., 2008; Castro-Hermida et al., 2015; Hashimoto et al., 2002). With cysts having a generally high-resistance to chlorination, the threat of cryptosporidiosis and giardiasis from drinking-water treated through such means is far from eliminated.

Village water resources, in comparison, are usually managed by the local communities themselves. The financial capacities of these rural populations to operate and maintain their water resource management systems are usually limited. As such, water treatment, whenever


a
C3.3 SanSai_Thailand_2014 - Cow
JN162348 Giardia intestinalis - Dairy Cattle
EF654691 Giardia intestinalis - Dairy cattle
C3.4 SanSai_Thailand_2014 - Cow
KF891311 Giardia intestinalis - Water buffalo
JQ928717 Giardia intestinalis - Goat
KJ404250 Giardia intestinalis - Goat
KR048498 Giardia intestinalis - Golden Takin
JQ928716 Giardia intestinalis - Sheep
KM434068 Giardia intestinalis - Dairy cattle
KP866788 Giardia intestinalis - Cat
JF958100 Giardia intestinalis - Cat
KM870513 Giardia intestinalis - Cat
KJ888992 Giardia intestinalis - Lemur
HQ397688 Giardia intestinalis - Chinchilla
EU518557 Giardia intestinalis - Dolphin
KJ888993 Giardia intestinalis - Cynomolgus monkey
EU518551 Giardia intestinalis - Common eider duck
JQ837804 Giardia intestinalis - Gorilla
JQ928710 Giardia intestinalis - Sheep
JF773756 Giardia intestinalis - Sheep
KC282955 Giardia intestinalis - Deer
GQ444450 Giardia intestinalis - Lamb
C12.1 SanSai_Thailand_2014 - Cow
DQ789113 Giardia intestinalis - Beaver
GU182382 Giardia intestinalis - Gull
HQ397692 Giardia intestinalis - Chinchilla
KC441076 Giardia intestinalis - Macaque
KF679743 Giardia intestinalis - Macaque
KP635091 Giardia intestinalis - Rabbit
KM211792 Giardia intestinalis - Lemur
GU182387 Giardia intestinalis - Seal
AY368166 Giardia intestinalis - Environmental
KP258396 Giardia intestinalis - Dog
HQ397701 Giardia intestinalis - Chinchilla
HG970114 Giardia intestinalis - Dog
AY228643 Giardia intestinalis - Dog
KM870512 Giardia intestinalis - Dog
KC855117 Giardia intestinalis - Mouse
KC855122 Giardia intestinalis - Rat
KC855118 Giardia intestinalis - Rat
JX571040 Giardia intestinalis - Rodent
KC855123 Giardia intestinalis - Mouse
KC855125 Giardia intestinalis - Rat
JX571041 Giardia intestinalis - Rodent
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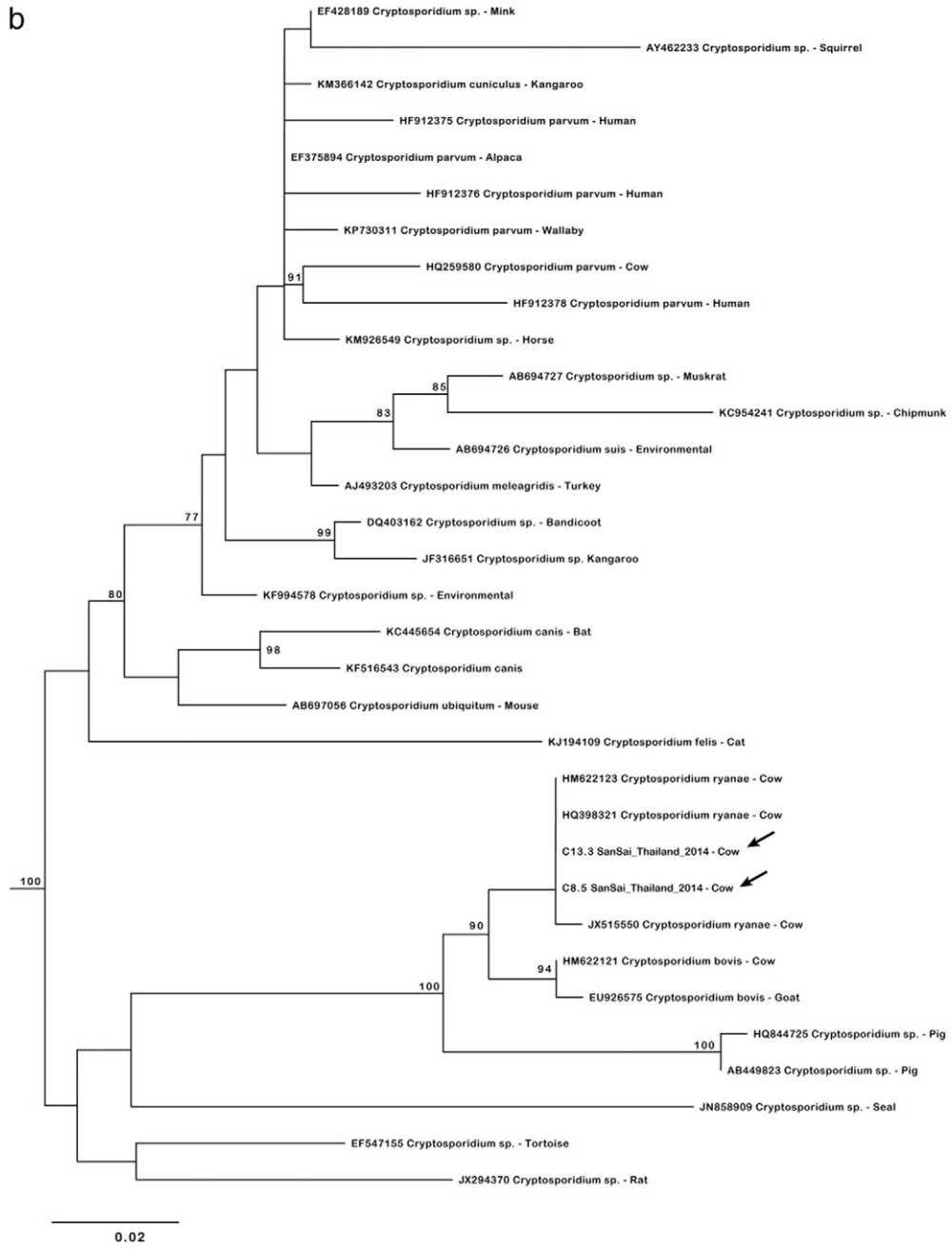

Fig. 5. Phylogenetic analysis of the partial sequences of the *Giardia* triosephosphate isomerase gene (5a) and the *Cryptosporidium* 18S ribosomal RNA gene (5b). Nucleotide sequences were retrieved from GenBank, aligned using MAFFT in Geneious 7.1.6 and phylogenetic tree reconstruction was done using a Maximum Likelihood approach in IQ-Tree. Node labels are estimates of branch robustness obtained using an ultrafast bootstrap. Only node labels greater than 70 were shown. The scale bar is nucleotide substitutions per site.

available, are generally limited to sand filtration only. Given the limited treatment of drinking-water in these rural communities, the risk of cryptosporidiosis and giardiasis is therefore amplified over the urban communities with better access to treatments facilities.

5.2. Seasonal factors and transport processes

The frequency of detection of *Cryptosporidium*/*Giardia* nearly doubled during the wet season compared to the dry season. Thus, the hydroclimatological factors play a crucial role in the prevalence of *Cryptosporidium* and *Giardia* in surface waters in the study area. Unfortunately, direct comparisons cannot be made with most other temporal-based studies, as they were largely conducted in temperate regions where the seasons (e.g. spring, summer, autumn and winter) do not affect water resources in the same manner as in the tropics (Caccio et al., 2003; Castro-Hermida et al., 2008, 2009a, 2009b; Hansen and Ongerth, 1991; Wilkes et al., 2009). Nevertheless, most of these studies inferred that *Cryptosporidium* and *Giardia* are generally more prevalent during the wetter periods of the year. Xiao et al. (2013) compared the occurrence of *Cryptosporidium* and *Giardia* between the flood period (rainy season) and the impounding period (dry season) in the Three Gorges Reservoir, China. Their results revealed the reservoir was more prone to *Cryptosporidium* contamination during the flood period. However, the opposite was observed for *Giardia*. Epidemiological studies conducted in the tropics also reported positive correlations between infections and the rainy season. For example, Siwila et al. (2011) reported that both cryptosporidiosis and giardiasis infections in 100 preschool children aged 3 to 6 years in Kafue, Zambia were significantly more prevalent in the rainy season than the dry season. Wongstitwilairoong et al. (2007) also reported a similar result in their

survey of intestinal parasitic infections among 472 pre-school children in Sangkhlaburi, western Thailand.

Several reasons may contribute to the significant increase of *Cryptosporidium* and *Giardia* in the surface waters during the wet season. Firstly, domestic wastewater is an important source of faecal and microbial contaminants in surface water. In Thailand, only a little over 20% of the domestic wastewater generated is directed to central wastewater treatment facilities for treatment; the rest is managed using on-site sanitation systems (Simachaya, 2009; World Bank, 2008). In rural and peri-urban areas of Thailand, open-bottomed, non-watertight cess pits are still commonly used for domestic waste. During the wet season, local water tables may rise and mix with the waste in these pits. Sequestered cysts in these cess pits may then be transported laterally through the soil matrix via groundwater flow, eventually reaching surface waters (Corapcioglu and Haridas, 1984; Abu-Ashour et al., 1994; Torkzaban et al., 2008). Many studies have identified wastewater as an important source of *Cryptosporidium* and *Giardia*, whereby high concentrations of cysts can be detected even in the treated effluents of wastewater treatment plants (Castro-Hermida et al., 2011; Cheng et al., 2009; Kitajima et al., 2014). The untreated, raw wastewater from the prevalent on-site sanitation systems in Thailand therefore likely contribute to the presence of *Cryptosporidium* and *Giardia* in surface waters, especially during wet periods (Fig. 6).

In addition, during storm events, surface runoff facilitates the transport of animal manure and *Cryptosporidium* and *Giardia* cysts (if the host is infected) from land to surface waters (Ferguson et al., 2003; Pachepsky et al., 2006; Tyrrel and Quinton, 2003). When the hydrologic connectivity between sources and receiving bodies is high, cysts may enter the stream network efficiently (Fig. 6). For example, the presence of rills, gullies, storm drains and canals will accelerate the transport processes while increased surface roughness associated with vegetated land plots yields an opposite effect (Bracken and Croke, 2007; Darboux et al., 2001; Jencso et al., 2009; Penuela et al., 2015). In grazing pastures, we observed the compaction of soil along animal paths (trails) due to the trampling of cattle herds. The compacted soil of these cattle trails can increase hydrologic connectivity and surface runoff (Batey, 2009; Trimble and Mendel, 1995), which in turn, can lead to an increase of *Giardia* and *Cryptosporidium* contamination in surface waters. Furthermore, soil compaction can also result in the reduction of storm runoff infiltration (Batey, 2009; Trimble and Mendel, 1995). Percolating storm water facilitates the vertical (downward) transport of *Giardia* and *Cryptosporidium* whereby the cysts can be strained by the underlying soil matrix. The decrease of the infiltration capacity therefore reduces the efficiency of this natural filtration system provided by the soil layers that is important for the protection for water resources against contamination.

Streambeds are also potentially important repositories of *Cryptosporidium* and *Giardia* (Jamieson et al., 2005; McDonald et al., 1982; Nagels et al., 2002). In the dry season, cysts may be deposited into surface waters by direct defecation or transported from source areas either by surface or sub-surface flow. Under stream baseflow conditions (i.e. low velocity, low discharge rate), cysts in the water column settle to the streambed as both *Cryptosporidium* (specific gravity: 1.009–1.080; settling velocity: 0.35–1.31 μm s^{-1}) and *Giardia* (specific gravity: 1.013–1.117; settling velocity: 0.84–1.40 μm s^{-1}) have natural propensities to sink in water (Dumetre et al., 2011). Settling rate is further enhanced when cysts in the water column are associated with (adhered to) suspended particles, altering their physical properties. Dai et al. (2004) revealed that hydrophobicity and surface charges of the cysts are important characteristics that are responsible for their adhesion to solid surfaces. Settling column experiments conducted on *Cryptosporidium* oocysts by Searcy et al. (2005) demonstrated that oocysts were removed from suspension at a much higher rate when associated with sediments, whereby the settling rate depended primarily on the type of sediment present in the water.

The rivers in the Kuang River Basin are highly managed. For example, many weirs have been built to impound water during the dry season when water levels are low. Sediments are known to accumulate behind dams and weirs (Lai and Shen, 1996). Thus, in areas where *Cryptosporidium* and *Giardia* are prevalent, cysts may be stored along with river sediments behind (or upstream of) these retention structures. During storm events, turbulent waters may *re*-suspend and entrain the cysts (Fig. 7). Jamieson et al. (2005) demonstrated this phenomenon using tracer bacteria in a small alluvial stream, where the increase of the tracer bacteria concentration occurred during the rising limb of storm hydrographs. Nagels et al. (2002) also investigated this process by creating an artificial flood in a stream by releasing water from a supply reservoir during dry weather conditions (i.e. no wash-in from the upper catchment was allowed). Increases and decreases of the faecal indicator organism concentration corresponded with the rising and falling limbs of the hydrograph.

Nagels et al. (2002) suggested that cysts accumulated in the streambeds may be of similar or greater importance than the wash-in from land. This may explain the non-detection of both protozoa in the canal even though *Cryptosporidium* and *Giardia* cysts were detected in faecal samples of beef cattle grazing along it. The flow in the canal is mechanically-controlled at the source (Ping River) which maintains a constant flow rate in the dry season. The continuous flushing of water minimises the settling potential of the cysts into the channel bed. In addition, the low surface roughness of the concrete-lined canal in comparison to those of meandering rivers and streams increases the volumetric discharge rate and velocity of water which in turn also decreases the settling rate of the cysts (Rouse, 1965). However, it may also be that these protozoa were present in the waters of the canal but in much lower concentrations than those in the rivers monitored in this study. Given that the recovery efficiencies were only 39% and 45%

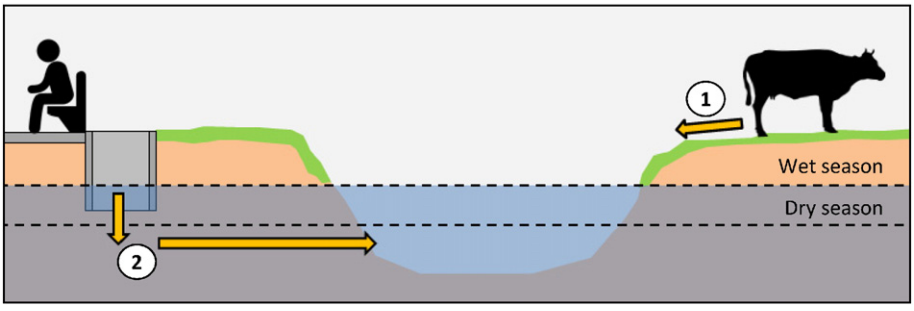

Fig. 6. Cross sectional profile of a stream and the various sources of *Cryptosporidium* and *Giardia* as well as the processes involved the transport of cysts to surface water. [1] During the dry season, manure from cattle and cysts enter surface waters by direct deposition while manure deposited on land can be washed into stream by surface runoff during the wet season. [2] During the wet season, groundwater may rise, and thereby increasing the proximity of the water table to cess pits. Sometimes, in low-lying areas, groundwater may even flood these on-site sanitation systems. Wastewater, potentially laden with cysts, will contaminate groundwater and transported horizontally via soil matrix to surface waters.

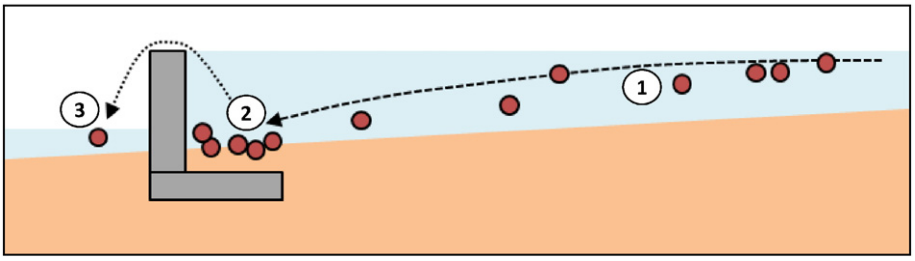

Fig. 7. Longitudinal profile of a fragmented stream and processes influencing the transport of cyst in surface waters. [1] *Cryptosporidium* and *Giardia* cysts settles from water column to streambed. Settling rate is enhanced when cysts are associated to sediments. [2] A store of cysts may exist behind weirs. [3] During storm events, stream velocity and volumetric discharge rate will increase which causes the resuspension of cysts from streambed to water column.

for *Cryptosporidium* and *Giardia* respectively (see Section 3.1.3), low concentrations of cysts may escape detection altogether thereby yielding the negative result.

5.3. Livestock management

The dairy cattle surveyed in this study were not infected with either *Cryptosporidium* or *Giardia*. However, a high prevalence of infection by other parasites, especially *Entamoeba* spp., reflects the potential risk of parasitic transmission and infection–particularly for the parasites that occur via the faecal-oral route (e.g. *Cryptosporidium* and *Giardia*). In contrast, cysts of both parasites were detected in the faecal samples from the beef cattle, for which, at least one of the samples tested positive for parasites with zoonotic potential (*G. intestinalis* assemblage B). Only one other study on the prevalence of *Cryptosporidium* and *Giardia* infection in beef cattle in Thailand was available for comparison. Kaewthamasorn and Wongsamee (2006) screened 207 faecal samples of beef cattle in Nan Province, Northern Thailand but did not detect either parasite in their samples. While we did not detect either *Cryptosporidium* or *Giardia* in faecal samples of dairy cattle at our study site, other Thai studies have (Inpankaew et al., 2010; Jittapalapong et al., 2006, 2011; Nuchjangreed et al., 2008). In Northern Thailand, the seroprevalence of *Cryptosporidium parvum* infection of 642 dairy cows were 3.3%, 5.1% and 3.0% in the provinces of Chiang Mai, Chiang Rai and Lampang, respectively (Inpankaew et al., 2009).

Several factors associated with cattle farm management may have contributed to the disparity of the *Cryptosporidium*/*Giardia* infection between dairy and beef cattle. The beef cattle from this study area are typically left to graze in pastures where drinking water is available. Transmission and infection may occur through direct ingestion of faecal matter containing *Cryptosporidium*/*Giardia* in the communal fields in which cattle from different herds graze and defecate. Alternatively, beef cattle may also be exposed to these parasites from surface waters (e.g. rivers, streams, canals, ditches etc.) contaminated with *Cryptosporidium* or *Giardia*.

In comparison, dairy cattle are typically housed in shelters and in some instances are separated by stalls. In contrast with free-ranging beef cattle, cattle feed such as cogon grass (*Imperata cylindrica*), stalks of rice and corn are brought to the shelters and placed in troughs or raised surfaces that minimise faecal contamination. The shelters of dairy cattle are cleaned daily, typically up to twice a day before milking. The cow manure is often collected and dried in separate areas to be sold as fertiliser. Furthermore, dairy cattle are usually provided water piped from the local village waterworks system. These water supplies are derived from protected sources such as deep aquifers or have undergone some form of treatment (typically sand-filtration) and therefore less prone to *Cryptosporidium* or *Giardia* contamination. Like the cattle-feed, drinking water for the dairy cattle is placed in a common trough or in individual concrete containers that decreases the chances of faecal contamination.

The management practices for both the beef and dairy cattle may result in the *Cryptosporidium* and *Giardia* contamination of water resources in the study area. For the beef cattle, the contamination process is straightforward. Free-ranging cattle are typically found near water bodies where parasites may be directly deposited into water bodies during defecation. Manure deposited on land may also be washed into surface waters during storm events. Even though most of the manure from dairy cattle was collected for the production of fertiliser, the residual faecal matter left behind after collection is a possible source of *Cryptosporidium* and *Giardia*. During the cleaning of the dairy cattle sheds, faecal residues are washed into ditches which may be ultimately drained into nearby surface waters thereby resulting in *Cryptosporidium* and *Giardia* contamination.

6. Conclusion

This novel study reveals for the first time the prevalence and distribution of *Cryptosporidium* and *Giardia* in the water resources of Northern Thailand. Both intestinal parasites were detected in varying levels in all the monitored rivers of the study area. With regards to public health, the detection of these protozoa in high concentrations in rivers upstream of the drinking-water reservoir is of great importance. Immediate precautionary measures must be taken to minimise future contamination of these raw water supplies while treated drinking-water must be thoroughly tested to ensure public safety. Additionally, the frequency of *Cryptosporidium* and *Giardia* detection was found to be higher during the wet season highlighting the importance of water as an agent of transport for *Cryptosporidium* and *Giardia* from sources to water bodies.

We also screened faecal samples from potential *Cryptosporidium* and *Giardia* hosts, i.e. beef and dairy cattle, and detected *G. intestinalis* assemblage B, known for its ability to infect humans. We also found that different cattle management strategies employed can influence the transmission of intestinal parasites among cattle and potentially to humans. Grazing pastures and water bodies from which free-ranging cattle drink from must be carefully managed to prevent *Cryptosporidium* and *Giardia* contamination of water resources.

The ubiquity of these pathogens in water resources of the study area highlights the potential risks of cryptosporidiosis and giardiasis not only to the local population but also the consumers in urban centres that rely on headwaters areas in Northern Thailand to provide drinking water. As such, monitoring plans for *Cryptosporidium* and *Giardia* are essential for developing programs to ensure clean and safe drinking-water supplies. Finally, as demonstrated in our study, these monitoring plans must take into account the spatial and temporal aspects to provide a better understanding of the contamination sources and important factors which may influence the prevalence and distribution of *Cryptosporidium* and *Giardia* in the local water resources.

Acknowledgements

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References

Abu-Ashour, J., Joy, D.M., Lee, H., Whiteley, H.R., Zelin, S., 1994. Transport of microorganisms through soil. Water Air Soil Pollut. 75 (1–2), 141–158.

Ali, M.A., Al-Herrawy, A.Z., El-Hewaary, S.E., 2004. Detection of enteric viruses, *Giardia* and *Cryptosporidium* in two different types of drinking water treatment facilities. Water Res. 38, 3931–3939.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.

Anceno, A.J., Ozaki, M., Dang, Y.N.D., Chuluun, B., Shipin, O.V., 2007. Canal networks as extended waste stabilization ponds: fate of pathogens in constructed waterways in Pathumthani Province, Thailand. Water Sci. Technol. 55 (11), 143–156.

Baldursson, S., Karanis, P., 2011. Waterborne transmission of protozoan parasites: review of worldwide outbreaks — an update 2004–2010. Water Res. 45, 6603–6614.

Batey, T., 2009. Soil compaction and soil management — a review. Soil Use Manag. 25, 335–345.

Bracken, L.J., Croke, J., 2007. The concept of hydrological connectivity and its contribution to understanding runoff-dominated geomorphic systems. Hydrol. Process. 21, 1749–1763.

Budu-Amoako, E., Greenwood, S., Dixon, B.R., Barkema, H.W., McClure, J.T., 2012. Occurrence of *Cryptosporidium* and *Giardia* on beef farms and water sources within the vicinity of the farms on Prince Edward Island, Canada. Vet. Parasitol. 184, 1–9.

Caccio, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E., 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69 (6), 3393–3398.

Caccio, S.M., Thompson, R.C.A., McLauchlin, J., Smith, H.V., 2005. Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol. 21 (9), 430–437.

Castro-Hermida, J.A., Garcia-Presedo, I., Almeida, A., Gonzalez-Warleta, M., Correia Da Costa, J.M., Mezo, M., 2008. Presence of *Cryptosporidium* spp. and *Giardia duodenalis* through drinking water. Sci. Total Environ. 405, 45–53.

Castro-Hermida, J.A., Garcia-Presedo, I., Almeida, A., Gonzalez-Warleta, M., Correia Da Costa, J.M., Mezo, M., 2009a. *Cryptosporidium* spp. and *Giardia duodenalis* in two areas of Galicia (NW Spain). Sci. Total Environ. 409, 2451–2459.

Castro-Hermida, J.A., Garcia-Presedo, I., Almeida, A., Gonzalez-Warleta, M., Correia Da Costa, J.M., Mezo, M., 2009b. Detection of Cryptosporidium spp. and Giardia duodenalis in surface water: a health risk for humans and animals. Water Research 43, 4133–4142.

Castro-Hermida, J.A., Garcia-Presedo, I., Almeida, A., Gonzalez-Warleta, M., Da Costa, J.M.C., Mezo, M., 2011. Cryptosporidium spp. and Giardia duodenalis in two areas of Galicia (NW Spain). Sci. Total Environ 409 (13), 2451–2459.

Castro-Hermida, J.A., Gonzalex-Warleta, M., Mezo, M., 2015. *Cryptosporidium* spp. and *Giardia duodenalis* as pathogenic contaminants of water in Galicia, Spain: the need for safe drinking water. Int. J. Hyg. Environ. Health 218 (1), 132–138.

Chansribut, W., 2002. Monitoring of Toxic Blue-green Algae Related to Water Quality in Mae Kuang Domtar Reservoir, Chiang Mai (Master of Science Thesis) Chiang Mai University.

Cheng, H.A., Lucy, F.E., Graczyk, T.K., Broaders, M.A., Tamang, L., Connolly, M., 2009. Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during secondary wastewater treatments. Parasitol. Res. 105, 689–696.

Corapcioglu, M.Y., Haridas, A., 1984. Transport and fate of microorganisms in porous media: a theoretical investigation. J. Hydrol. 72, 149–169.

Council of the European Communities, 2000. Directive 2000/60/EC of the European Parliament and of the Council of the 23 October 2000 establishing a framework for community action in the field of water policy. Off. J. Eur. Union (L327/1-L327/72).

Dai, X., Boll, J., Hayes, M.E., Aston, D.E., 2004. Adhesion of *Cryptosporidium parvum* and *Giardia lamblia* to solid surfaces: the role of surface charge and hydrophobicity. Colloids Surf. B: Biointerfaces 34 (4), 259–263.

Darboux, F., Davy, P., Gascuel-Odoux, C., Huang, C., 2001. Evolution of soil surface roughness and flowpath connectivity in overland flow experiments. Catena 46, 125–139.

Davison, A., Howard, G., Stevens, M., Callan, P., Fewtrell, L., Deere, D., Bartram, J., 2005. Water Safety Plans — Managing Drinking-water Quality from Catchment to Consumer. World Health Organization, Geneva, Switzerland.

Diallo, M.B.C., Anceno, A.J., Tawatsupa, B., Houpt, E.R., Wangsuphachart, V., Shipin, O.V., 2008. Infection risk assessment of diarrhea-related pathogens in a tropical canal network. Sci. Total Environ. 407, 223–232.

Dib, H.H., Lu, S.Q., Wen, S.F., 2008. Prevalence of *Giardia lamblia* with or without diarrhea in South East, South East Asia and the Far East. Parasitol. Res. 103, 239–251.

Dumetre, A., Dominique, A., Puech, P., Hohweyer, J., Azas, N., Villena, I., 2011. Interaction forces drive the environmental transmission of pathogenic protozoa. Appl. Environ. Microbiol. 78 (4), 905–912.

FAO, 2005. Livestock Sector Brief — Thailand. Food and Agriculture Organization, Rome, Italy.

FAO, 2015. FAOSTAT database. Retrieved from http://faostat3.fao.org/browse/Q/*/E (accessed 18 October 2015).

FAO and APHCA, 2002. The Livestock Industries of Thailand. Food and Agriculture Organisation and Animal Production and Health Commission for Asia and the Pacific, Rome, Italy.

Ferguson, C., de Roda Husman, A.M., Altavilla, N., Deere, D., Ashbolt, N., 2003. Fate and transport of surface water pathogens in watersheds. Crit. Rev. Environ. Sci. Technol. 33 (3), 299–361.

Ganjanapan, S., Lebel, L., 2014. Interplay between new basin organisations, pre-existing institutions and emerging environmental networks in the Mae Kuang watershed, Northern Thailand. In: Huitema, D., Meijerink, S. (Eds.), The Politics of River Basin Organisations Coalitions, Institutional Design Choices and Consequences. Edward Elgar, Cheltenham, UK, pp. 298–325.

Hansen, J.S., Ongerth, J.E., 1991. Effects of time and watershed characteristics on the concentration of *Cryptosporidium* oocysts in river water. Appl. Environ. Microbiol. 57 (10), 2790–2795.

Haque, R., 2007. Human intestinal parasite. J. Health Popul. Nutr. 25 (4), 387–391.

Hashimoto, A., Kunikane, S., Hirata, T., 2002. Prevalence of *Cryptosporidium* oocysts and *Giardia* cysts in the drinking water supply in Japan. Water Res. 36, 519–526.

Horn, A.L., Rueda, F.J., Hormann, G., Fohrer, N., 2004. Implementing river water quality modelling issues in mesoscale watershed models for water policy demands — an overview on current concepts, deficits, and future tasks. Phys. Chem. Earth 29 (11 — 12), 725–737.

Hotez, P.J., Fenwick, A., Savioli, L., Molyneux, D.H., 2009. Rescuing the bottom billion through control of neglected tropical diseases. Lancet 373 (9674), 1570–1575.

Hunter, P.R., Thompson, R.C., 2005. The zoonotic transmission of *Giardia* and *Cryptosporidium*. Int. J. Parasitol. 35, 1181–1190.

Inpankaew, T., Jittapalapong, S., Phasuk, J., Pinyopanuwut, N., Chimnoi, W., Kengradomkit, C., Sunanta, C., Zhang, G., Aboge, G., Nishikawa, Y., Igarashi, I., Xuan, X., 2009. Seroprevalence of *Cryptosporidium parvum* infection of dairy cows in three northern provinces of Thailand determined by enzyme-linked immunosorbent assay using recombinant antigen CpP23. Onderstepoort J. Vet. Res. 76 (2), 161–165.

Inpankaew, T., Jiyipong, T., Pinyopanuwat, N., Chimnoi, W., Thompson, R., Jittapalapong, S., 2010. Prevalence and genotyping of *Cryptosporidium* spp. from dairy cow fecal samples in Western Thailand. Southeast Asian J. Trop. Med. Public Health 41 (4), 770–775.

Jamieson, R.C., Joy, D.M., Lee, H., Kostaschuk, R., Gordon, R.J., 2005. Resuspension of sediment-associated *Escherichia coli* in a natural stream. J. Environ. Qual. 34 (2), 581–589.

Jencso, K.G., McGlynn, B.L., Gooseff, M.N., Wondzell, S.M., Bencala, K.E., Marshall, L.A., 2009. Hydrologic connectivity between landscapes and streams: transferring reach- and plot-scale understanding to the catchment scale. Water Resour. Res. 45, W04428.

Jittapalapong, S., Pinyopanuwat, N., Chimnoi, W., Siripanth, C., Stich, R., 2006. Prevalence of *Cryptosporidium* among dairy cows in Thailand. Ann. N. Y. Acad. Sci. 1081, 328–335.

Jittapalapong, S., Sangwaranond, A., Nimsuphan, B., Inpankaew, T., Phasuk, C., Pinyopanuwat, N., Chimnoi, W., Kengradomkij, C., Arunwipat, P., Anakewith, T., 2011. Prevalence of gastro-intestinal parasites of dairy cows in Thailand. Kasetsart J. (Nat. Sci.) 45, 40–45.

Kaewthamasorn, M., Wongsamee, S., 2006. A preliminary survey of gastrointestinal and haemoparasites of beef cattle in the tropical livestock farming system in Nan Province, Northern Thailand. Parasitol. Res. 99 (3), 306–308.

Karanis, P., Kourenti, C., Smith, H., 2007. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J. Water Health 5 (1), 1–38.

Kay, D., Edwards, A.C., Ferrier, R.C., Francis, C., Kay, C., Rushby, L., Watkins, J., McDonald, A.T., Wyer, M., Crowther, J., Wilkinson, J., 2007. Catchment microbial dynamics: the emergence of a research agenda. Prog. Phys. Geogr. 31 (1), 59–76.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A., 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649.

Kitajima, M., Haramoto, E., Iker, B.C., Gerba, C.P., 2014. Occurrence of *Cryptosporidium*, *Giardia*, and *Cyclospora* in influent and effluent water at wastewater treatment plants in Arizona. Sci. Total Environ. 484, 129–136.

Koompapong, K., Sukthana, Y., 2012. Seasonal variation and potential sources of Cryptosporidium contamination in surface waters of Chao Phraya River and Bang Pu Nature Reserve Pier, Thailand. Southeast Asian J. Trop. Med. Public Health 43 (4), 832–840.

Kumar, T., Onichandran, S., Lim, Y.A.L., Sawangjaroen, N., Ithoi, I., Andiappan, H., Salibay, C.C., Dungca, J.Z., Chye, T.T., Sulaiman, W.Y.W., Lau, Y.L., Nissapatorn, V., 2014. Comparative study on waterborne parasites in Malaysia and Thailand: a new insight. Am.J.Trop. Med. Hyg. 90 (4), 682–689.

Lai, J., Shen, H.W., 1996. Flushing sediment through reservoirs. J. Hydraul. Res. 34 (2), 237–355.

Lim, H.S., Boochaphun, K., Ziegler, A.D., 2012. Modifiers and amplifiers of high and low flows on the Ping River in Northern Thailand (1921–2009): The roles of climatic events and anthropogenic activity. Water Resour. Manag. 26 (14), 4203–4224.

Lim, Y.A.L., Jex, A.R., Smith, H.V., Gasser, R.B., 2010. Chapter 1 — *Cryptosporidiosis* in Southeast Asia: what's out there? Adv. Parasitol. 71, 1–31.

MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, S.M., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., Davis, J.P., 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. 331, 161–167.

McDonald, A., Kay, D., Jenkins, A., 1982. Generation of fecal and total coliform surges by stream flow manipulation in the absence of normal hydrometeorological stimuli. Appl. Environ. Microbiol. 44 (2), 292–300.

Minh, B.Q., Nguyen, M.A.T., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. 30, 1188–1195.

Nagels, J.W., Davies-Colley, R.J., Donnison, A.M., Muirhead, R.W., 2002. Faecal contamination over flood events in a pastoral agricultural stream in New Zealand. Water Sci. Technol. 45 (12), 45–52.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol. Biol. Evol. 32, 268–274.
Nuchjangreed, C., Boonrod, K., Ongerth, J., Karanis, P., 2008. Prevalence and molecular characterization of human and bovine *Cryptosporidium* isolates in Thailand. Parasitol. Res. 103 (6), 1347–1353.
Nutniyom, P., 2003. Optimization of Microcystins Extraction and Monitoring of Toxins in Water Samples from Mae Kuang Udomtara Reservoir, Chiang Mai Province (Master of Science Thesis) Chiang Mai University.
Pachepsky, Y.A., Sadeghi, A.M., Bradford, S.A., Shelton, D.R., Guber, A.K., Dao, T., 2006. Transport and fate of manure-borne pathogens: modeling perspective. Agric. Water Manag. 86 (1–2), 81–92.
Penuela, A., Javaux, M., Bielders, C.L., 2015. How do slope and surface roughness affect plot-scale overland flow connectivity? J. Hydrol. 528, 192–205.
Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS One 5 (3), e9490.
Prüss-Ûstün, A., Bos, R., Gore, F., Bartram, J., 2008. Safer Water, Better Health: Costs, Benefits and Sustainability of Interventions to Protect and Promote Health. World Health Organization, Geneva, Switzerland.
Rouse, H., 1965. Critical analysis of open-channel resistance. J. Hydraul. Div. 91, 1–25.
Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69 (7), 4302–4307.
Sato, M.I.Z., Galvani, A.T., Padula, J.A., Nardocci, A.C., de Souza Lauretto, M., Razzolini, M.T.P., Hachich, E.M., 2013. Assessing the infection risk of *Giardia* and *Cryptosporidium* in public drinking water delivered by surface water systems in Sao Paulo State, Brazil. Sci. Total Environ. 442, 389–396.
Searcy, K.E., Packman, A.I., Atwill, E.R., Harter, T., 2005. *Association of Cryptosporidium parvum* with suspended particles: impact on oocyst sedimentation. Appl. Environ. Microbiol. 71 (2), 1072–1078.
Simachaya, W., 2009. Wastewater tariffs in Thailand. Ocean Coast. Manag. 52, 378–382.
Siwila, J., Phiri, I.G.K., Enemark, H.L., Nchito, M., Olsen, A., 2011. Seasonal prevalence and incidence of *Cryptosporidium* spp. and *Giardia duodenalis* and associated diarrhoea in children attending pre-school in Kafue, Zambia. Trans. R. Soc. Trop. Med. Hyg. 105, 102–108.
Smith, H.V., Caccio, S.M., Tait, A., McLauchlin, J., Thompson, R.C., 2006. Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. Trends Parasitol. 22 (4), 160–167.
Srisuphanunt, M., Karanis, P., Charoenca, N., Boonkhao, N., Ongerth, J.E., 2010. *Cryptosporidium* and *Giardia* detection in environmental waters of southwest coastal areas of Thailand. Parasitol. Res. 106, 1299–1306.
Sulaiman, I., Fayer, R., Bern, C., Gilman, R., Trout, J., Schantz, P., Das, P., Lal, A., Xiao, L., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9 (11), 1444–1452.
Thomas, D.E., 2005. Developing Watershed Management Organisations in Pilot Sub-Basins of the Ping River Basin. Office of Natural Resources and Environmental Policy and Planning, Ministry of Natural Resources and Environment, Bangkok, Thailand.
Torkzaban, S., Tazehkand, S.S., Walker, S.L., Bradford, S.A., 2008. Transport and fate of bacteria in porous media: coupled effects of chemical conditions and pore space geometry. Water Resour. Res. 44, W04403.
Trimble, S.W., Mendel, A.C., 1995. The cow as a geomorphic agent — a critical review. Geomorphology 13, 233–253.
Tyrrel, S.F., Quinton, J.N., 2003. Overland flow transport of pathogens from agricultural land receiving faecal wastes. J. Appl. Microbiol. 94, 87–93.
UNICEF and WHO, 2009. Diarrhoea: Why Children are Still Dying and What Can be Done. World Health Organisation, Geneva, Switzerland.
USEPA, 2012. Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. United States Environmental Protection Agency, Washington, US.
Weber, R., Bryan, R.T., Bishop, H.S., Wahlquist, S.P., Sullivan, J.J., Juranek, D.D., 1991. Threshold of detection of Cryptosporidium oocysts in human stool specimens: evidence for low sensitivity of current diagnostic methods. J. Clin. Microbiol. 29 (7), 1323–1327.
WHO, 2008. Health in Asia and the Pacific. World Health Organisation, New Delhi, India.
Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Diane, M., Neumann, N., Ruecker, N., Topp, E., Lapen, D., 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Res. 43, 2209–2223.
Wongstitwilairoong, B., Srijan, A., Serichantalergs, O., Fukuda, C.D., McDaniel, P., Bodhidatta, L., Mason, C.J., 2007. Intestinal parasitic infections among pre-school children in Sangkhlaburi, Thailand. Am.J.Trop. Med. Hyg. 76 (2), 345–350.
Wood, S.H., Ziegler, A.D., 2008. Floodplain sediment from recent 50-year-recurrence floods of the Ping River in northern Thailand. Hydrol. Earth Syst. Sci. 12, 959–973.
World Bank, 2008. Thailand Infrastructure Annual Report 2008. World Bank, Washington, US.
Xiao, S., An, W., Chen, Z., Zhang, Q., Yu, J., Yang, M., 2012. Occurrences and genotypes of *Cryptosporidium* oocysts in river network of southern-eastern China. Parasitol. Res. 110, 1701–1709.
Xiao, G., Qiu, Z., Qi, J., Chen, J., Liu, F., Liu, W., Luo, J., Shu, W., 2013. Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in the Three Gorges Reservoir, China. Water Res. 47, 2431–2445.