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SHORT COMMUNICATIONS

Molecular detection of *Cryptosporidium* spp. infections in water buffaloes from northeast Thailand

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Abstract The objectives of this study were to determine the individual and herd-level prevalence and genotype of *Cryptosporidium* and to identify putative risk factors associated with *Cryptosporidium* spp. infections in water buffaloes in northeast Thailand. Fecal samples from 600 water buffaloes of 287 farms in six provinces were collected and tested using DMSO-modified acid-fast staining and polymerase chain reaction. The overall prevalence of *Cryptosporidium* infections in buffaloes was 5.7 and 8.7 % among individual animals and herds, respectively. The provinces with highest infected *Cryptosporidium* were located in the Sakon Nakhon Basin in the northern part of the region. In addition, higher herd prevalence was observed among farms with more than five buffaloes (30 %) than those with five or less animals (16.2 %). Thirty (88.2 %) of the 34 *Cryptosporidium*-positive samples were *Cryptosporidium parvum* and four (11.8 %) were *Cryptosporidium ryanae*.

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Introduction

Approximately, 1.2 million water buffaloes (*Bubalus bubalis*) are native to Thailand (DLD 2010), major meat-producing animals and play an important role in the conventional agricultural practice. The majority of farms in Thailand are smallholder operations that are closely located to other farms often without barriers between water buffaloes and other wildlife, domestic, and companion animals. Furthermore, buffaloes in Thailand are commonly reared on public pastures to minimize management costs, thus increasing the chances that these animals will be exposed to an environment shared by other species. The role of *Cryptosporidium* spp. in the occurrence of diarrhea of water buffaloes in Thailand remains unclear. Therefore, it is important to understand the prevalence of bovine cryptosporidiosis in order to understand interspecies transmission of *Cryptosporidium* and to prevent zoonotic transmission of *Cryptosporidium* spp. The aims of this study were to determine the individual and herd-level prevalence and genotype of *Cryptosporidium* and to identify putative risk factors associated with *Cryptosporidium* spp. infections in water buffaloes in the northeast Thailand.

Materials and methods

Six provinces with the highest water buffaloes density in northeast Thailand (DLD 2010) were chosen for this study: Ubon Ratchathani, Surin, Buri Ram, Si Sa Ket, Sakon Nakhon, and Roi Et. Between March 2010 and May 2011, a total of 600 fresh fecal samples were collected directly from rectum of native water buffalo in 287 small farm holders. The animals

were randomly selected and classified into four age groups: pre-weaned calves (2 samples, <3 months), post-weaned calves (22 samples, 3–12 months), juveniles (22 samples, 12–18 months), and adults (541 samples, >18 months) of age and 89 out of 600 samples (14.8 %) were from males and 85.2 % (511/600) from females. In addition, buffalo herd size sampled were divided into two groups, ≥5 buffalo per farm, and <5 buffalo per farm. All specimens were transported to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand, for further processing. All fecal samples were analyzed for *Cryptosporidium* oocysts using DMSO-modified acid-fast stain microscopy as previously described (Inpankaew et al. 2010).

Total DNA was extracted from buffalo stool samples using QIAamp DNA Stool Mini Kit (Qiagen, GmbH, Germany) according to manufacturer's instructions, with the addition of 5–6 cycles of freeze-thaw after the addition of buffer ATL to the pellet. *Cryptosporidium* spp. were genotyped by nested PCR amplification of an approximately 830-bp fragment of the small subunit (SSU) rRNA gene (Xiao et al. 1999). The secondary PCR products were visualized by 1 % agarose gel electrophoresis.

The SSU rRNA PCR products were purified using the QIAquick PCR purification kit (Qaigen GmbH, Germany). Sequencing of the PCR products were conducted using the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI Genetic Analyzer 3730 (Applied Biosystems). The obtained sequences were compared with reference sequences of known *Cryptosporidium* species and genotypes by BLAST analysis on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The obtained *C. parvum* sequences (10 samples) and *C. ryanae* sequences (four samples) were analyzed using Finch TV 1.4.0 (Geospiza, Inc.) and aligned using BioEdit version 7.2.0 (Hall 1999) with the SSU-rRNA gene of the following *Cryptosporidium* species: *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni, Cryptosporidium ryanae* cattle variant, *C. ryanae* buffalo variant (GenBank accession no. DQ286403, AB513881, AB628204, JQ313931, AB513679, and AB712388, respectively). Neighbor-joining analyses were conducted with Tamura-Nei parameter distance estimates using Mega 4.1 (www.megasoftware.net).

Characteristics of individual water buffaloes and information based on the different farms were analyzed in relation to *Cryptosporidium* positivity to identify putative risk factors associated with water buffalo exposure to *Cryptosporidium*. Potential risk factors were tested including province, geographical area (i.e., basin), herd size, host sex, and host age, which were analyzed for each province by the Chi-square (X^2) with Number Cruncher Statistical System version 2000 (Kaysville, UT) programs. All these factors were also assessed for potential association with exposure to *Cryptosporidium* at the 95 % confidence interval with WinEpiscope software version 2.0 (Thrusfield et al. 2001).

Results

Thirty-four (5.7 %) of the 600 samples were positive by both DMSO-modified acid-fast stain and PCR (Table 1), and results of detection of the two techniques were in complete agreement.

Table 1 Prevalence of *Cryptosporidium* infection in water buffaloes in northeast Thailand

Parameter	*Cryptosporidium* positive/ negative (%)	Statistical parameters	95 % CI
Individual prevalence			
Province		χ^2=19.6, df=5, p=0.001	
Ubon Ratchathani	9/132 (6.8 %)		
Si Sa Ket	0/55 (0.0 %)		
Surin	0/67 (0.0 %)		
Buri Ram	0/61 (0.0 %)		
Roi Et	4/81 (4.9 %)		
Sakon Nakhon	21/204 (10.3 %)		
Total	34/600 (5.7 %)		
Basin		χ^2=9.7, df=1, p=0.001	
Sakon Nakhon	25/286 (8.7 %)		1.5–6.8
Khorat	9/314 (2.9 %)		0.1–0.6
Age group		χ^2=4.1, df=3, p=0.3	
Pre-weaned calves	0/2 (0.0 %)		
Post-weaned calves	1/35 (2.9 %)		
Juveniles	1/22 (4.5 %)		
Adults	32/541 (5.9 %)		
Sex		χ^2=0.3, df=1, p=0.6	
Male	4/89 (4.5 %)		0.3–2.2
Female	30/511 (5.9 %)		0.5–3.8
Herd prevalence			
Province		χ^2=15.1, df=5, p=0.01	
Si Sa Ket	0/22 (0.0 %)		
Ubon Ratchathani	6/59 (10.2 %)		
Buri Ram	0/22 (0.0 %)		
Surin	0/28 (0.0 %)		
Sakon Nakhon	16/98 (16.3 %)		
Roi Et	3/58 (5.2 %)		
Total	25/287 (8.7 %)		
Herd size		χ^2=1.3, df=1, p=0.2	
<5 animals	23/276 (8.3 %)		0.1–1.8
>5 animals	2/11 (18.2 %)		0.6–8.6

The individual prevalence varied from 0 to 10.3 % among six provinces ($p<0.05$), but only three of the six provinces (Ubon Ratchathani, Roi Et, and Sakon Nakhon) were positive for *Cryptosporidium* infections in this study. Sakon Nakhon had the highest individual prevalence (10.3 %, 21/204) of *Cryptosporidium* infection followed by Ubon Ratchathani (6.8 %, 9/132) and Roi Et (4.9 %, 4/81). Similarly, the herd prevalence among six provinces were between 0 and 16.3 % ($p<0.05$), and Sakon Nakhon had the highest herd infection rate (16.3 %, 16/98) followed by Ubon Ratchathani (10.2 %, 6/59) and Roi Et (5.2 %, 3/58). A total of 25 of 287 (8.7 %) herds were infected with *Cryptosporidium* in this study (Table 1).

Thirty-four PCR-positive samples were sequenced, and 30 (88.2 %) of the 34 positive samples were *C. parvum*, and their sequences were 99 % homologous to the *C. parvum* SSU rRNA gene (GenBank accession no. AB513881). On the other hand, 11.8 % (4/34) sequences were *C. ryanae* that was identical to *C. ryanae* from cattle (GenBank accession no. AB513679). However, *C. andersoni* and *C. bovis* were not found in this study.

For the risk assessment of infection with *Cryptosporidium*, several factors were examined. Prevalence was associated with basin region and herd size of northeast Thailand (Table 1). The areas sampled primarily consisted of hilly terrain with small mountainous or highland areas and were geographically divided into the Sakon Nakhon and Khorat basins. The northern Sakon Nakhon basin includes the Sakon Nakhon and Roi Et provinces, and the southern Khorat basin contains Ubon Ratchathani, Surin, Buri Ram, and Si Sa Ket provinces. Likewise, a higher prevalence was measured among water buffaloes from the northern Sakon Nakhon basin (8.7 %, 25/286; 95 % CI=1.5–6.8) compared to the southern Khorat basin (2.9 %, 9/314; $p<0.001$, $\chi^2=9.7$, df=1; 95 % CI=0.1–0.6). Farms with >5 animals had higher prevalence (30 %) than those with ≤5 (16.2 %). However, these values were not statistically significant ($p=0.2$). In addition, a statistically significant association was not detected between prevalence, host age, and host sex in this study.

Discussion

The overall prevalence of *Cryptosporidium* spp. from water buffaloes in Thailand was 5.7 % in the present study. While Abu Samra et al. (2011) reported a 5.5 % *Cryptosporidium* infection in African buffaloes, Amer et al. (2013) recorded *Cryptosporidium* infection rates of 9.5 % in water buffaloes in Egypt, Cacciò et al. (2007) reported 14.5 % infection rate of *Cryptosporidium* in Italian buffalo calves, and Bhat et al. (2012) and Feng et al. (2012) reported *Cryptosporidium* infection rates of 38.3 and 37.5 % in water buffalo calves in India and Nepal, respectively. In other studies on Thailand, the prevalence of *Cryptosporidium* in cattle was reported between 0.6 and 15.5 % (Jittapalapong et al. 2006; Nuchjangreed et al. 2008; Inpankaew et al. 2009, 2010).

The identification of *C. parvum* as the dominant species in water buffalo is in agreement with the previous finding of the dominance of *C. parvum* in cattle in Thailand (Nuchjangreed et al. 2008; Inpankaew et al. 2010). This might be the evidence of the occurrence of interspecies transmission of *C. parvum* in the study area. Thus, water buffaloes are carriers for *C. parvum* in Thailand, and asymptomatic water buffaloes can serve as an important natural reservoir for transmission to humans through food and water contamination or from livestock directly. More attention should be paid to this parasite for the control of livestock and human cryptosporidiosis.

Host age is an important factor that influences the occurrence and distribution of *Cryptosporidium* species (Xiao et al. 2004). Thus, Nguyen et al. (2007) revealed that calves younger than 6 months old had significantly higher infection rate of *C. parvum* than those of cattle older than 6 months. However, *C. parvum* infection was identified in all age groups with no significant difference in infection rates among groups in this study. As suggested by Feng et al. (2007), cattle of all ages are susceptible to infection with *C. ryanae.* Our results suggest that this is also the case in water buffaloes.

Previously, it was shown that higher animal density caused the higher prevalence of *Cryptosporidium* infection (Garber et al. 1994; Mohammed et al. 1999). Likewise, in this study, there was an effect of the herd size on the occurrence of *Cryptosporidium* infections. Farms with >5 animals had higher infection rates two times than farms with less than five animals; however, these were not statistically significant ($p=0.2$).

Rivers are commonly used for agriculture, livestock husbandry, human and animal consumption, and transportation in Thailand. The consistently higher prevalence values measured from the Sakon Nakhon Basin could be associated with the presence of rivers and their branches in the Sakon Nakhon Basin. Therefore, it is possible that *Cryptosporidium* oocysts in these areas are less subjected to desiccation due to moisture in the soil that ungulates are more likely to be exposed to *Cryptosporidium* oocysts when drinking and grazing in areas near a water source shared by other hosts of the parasite especially human (*C. parvum*) and other cattle (*C. ryanae*).

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Conflict of interest The authors wish to declare that they have no conflict of interests.

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