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High prevalence of *Cryptosporidium* infection caused by *C. scrofarum* and *C. suis* among pigs in Thailand

Umaporn Thathaisong[a], Suradej Siripattanapipong[b], Tawin Inpankaew[c], Saovanee Leelayoova[d], Mathirut Mungthin[d,*]

[a] Department of Microbiology, Faculty of Science, Burapha University, Chonburi, Thailand
[b] Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand
[c] Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Thailand
[d] Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand

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ABSTRACT

Cryptosporidium spp. is an important intestinal protozoan causing diarrhea among both healthy and immunocompromised patients especially those with HIV/AIDS. *Cryptosporidium* spp. can be transmitted via foodborne, waterborne and person-to-person routes. In addition, several *Cryptosporidium* species are zoonotic. This study aimed to determine the prevalence of *Cryptosporidium* infection among pigs raised in both smallholder (< 50 heads/farm) and large scale farms (50–500 heads/farm) in Chonburi Province, eastern Thailand using nested PCR amplifying the small subunit of the ribosomal RNA (SSU-rRNA) gene. DNA sequencing was also performed to identify the species of *Cryptosporidium*. A total of 245 fecal samples were collected from 11 pig farms. The overall prevalence of *Cryptosporidium* infection was 20.8% (51/245) which were found in both smallholder and small large scale pig farms. The prevalence of *Cryptosporidium* infection among pigs aged ≤6 months was significantly higher than those aged > 6 months ($p < .001$). Among 51 *Cryptosporidium* positive samples, *Cryptosporidium scrofarum* (42/51, 82.4%) and *Cryptosporidium suis* (9/51, 17.6%) were identified. The prevalence of *C. scrofarum* infection observed among pigs aged ≤6 months was significantly higher when compared with those aged > 6 months (20.7% and 2.1%, respectively, $p < .001$). The high prevalence of *C. scrofarum* and *C. suis* infections among pigs could be a potential source of infection to humans.

1. Introduction

Cryptosporidiosis is one of diarrheal diseases distributed worldwide and caused by an intestinal protozoan, *Cryptosporidium* spp. Cryptosporidiosis appears among both immunocompetent and immunocompromised patients especially among patients with HIV/AIDS. A wide range of animals including pets, farm and wild animals are also infected by *Cryptosporidium* spp., which could serve as a source of human infection [1]. Cryptosporidiosis in humans is commonly caused by a few species including *Cryptosporidium hominis* and zoonotic species, *Cryptosporidium parvum* and *Cryptosporidium meleagridis*. Other animal *Cryptosporidium* species such as *Cryptosporidium canis*, *Cryptosporidium cuniculus* and *Cryptosporidium felis* were also reported in humans especially in immunocompromised patients [2].

In Thailand, most human cryptosporidiosis cases have been reported among patients with HIV/AIDS with prevalences ranging from 8.8 to 94.4% [3–7]. Among these patients, the most prevalent *Cryptosporidium* spp. was *C. hominis*, followed by *C. parvum* and *C. meleagridis*. Other species with less prevalence of infection were *C. muris, C. felis,* and *C. canis* [5,7–10]. A few studies reported the prevalence of *Cryptosporidium* in animals such as dogs (1.5 to 7.6%) [11–12], dairy cows (0.5 to 51.0%) [8,13–16] and cats (2.5%) [17].

Pigs serve as a natural reservoir of host-specific species of *Cryptosporidium*, that is, *Cryptosporidium suis* (previously named *Cryptosporidium* pig genotype I) and *Cryptosporidium scrofarum* (previously named *Cryptosporidium* pig genotype II). *C. suis* infection was previously reported among patients with HIV/AIDS in Peru [18–20] as well as among immunocompetent patients in Madagascar [21] and England [22]. A recent retrospective analysis of *Cryptosporidium* species collected from 155 Thai patients with HIV/AIDS from 1999 to 2004 showed that 6 patients were infected by *C. suis* [23]. However, C. *scrofarum* infection was reported in an immunocompetent individual in the Czech Republic [24]. Pig production has significantly increased in Thailand during the last decade. The intensification of pig production

* Corresponding author at: Department of Parasitology, Phramongkutklao College of Medicine, 315 Ratchawithi Rd., Ratchathewi, Bangkok 10400, Thailand.
E-mail address: mathirut@hotmail.com (M. Mungthin).

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may contribute to the emergence and spread of zoonotic pathogens including *Cryptosporidium* spp. To date, no situation of *Cryptosporidium* infection among pigs in Thailand has been reported. Thus, the present study aimed to investigate the prevalence and species of *Cryptosporidium* infection among pigs raised in pig farms in eastern Thailand.

2. Material and methods

2.1. Specimen collection

This study was conducted in two types of pig farms namely, smallholder (< 50 heads/farm) and small large-scale farms (50 to 500 heads/farm) [25]. All pig farms were under the open farm system with sanitary conditions. Strains of these pigs included three cross breeds of the Large White, Landrace, and Duroc. Two hundred and forty-five fecal specimens were collected from 11 pig farms in Chonburi Province, eastern Thailand from May 2015 to January 2016. In each farm, feces were collected from approximately 20% of the total number of pigs. To collect fecal samples, feces were randomly collected from individual pigs by evacuating directly from the rectum using disposable gloves and put in a plastic container. The strain, sex, and age of each pig were recorded during sampling. The fecal samples were kept in cool condition during transportation to the laboratory and then kept frozen at –20 °C until extracting genomic DNA.

2.2. Genomic DNA extraction, PCR amplification

Genomic DNA was extracted from each stool sample using the QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany) according to manufacturer instructions and then kept at −20 °C until further analysis. To determine *Cryptosporidium* infection, the extracted genomic DNA from stool specimens was subjected to amplify the portion of the SSU-rRNA gene using nested PCR. The PCR reactions were carried out using specific primers and conditions described by Feltus et al. (2006) [26]. The primary primers were 18SFor1 (5′-TTCTAGAGCTAATACATGCG-3′) and 18SRev1 (5′-CCCATTTCCTTCGAAACAGGA-3′). The secondary primers were 18SFor2 (5′-GGAAGGGTTGTATTTATTAGATAAAG-3′) and 18SRev2 (5′-AAGGAGTAAGGAACAACCTCCA-3′). All PCR reactions were carried out using 400 μM of each primer, 3 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate (dNTP), 1× PCR buffer (Promega, Madison, WI, USA), 1 U of *Taq* DNA polymerase, and 1 μl of nonacetylated bovine serum albumin (BSA; 10 mg/ml) (Promega, Madison, WI, USA), except that in the secondary reactions, 1.5 mM $MgCl_2$ was used. Primary PCR cycling conditions consisted of an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 60 s, with a final extension of 72 °C for 7 min. Cycling conditions for the secondary PCR consisted of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 2 min. The PCR product was gel electrophoresed using 1.5% agarose gel and visualized by gel documentation (Uvitech, Cambridge, UK).

2.3. Sequence analysis and phylogenetic tree analysis of the SSU-rRNA gene

To identify the species of *Cryptosporidium*, fragments of the 830-bp PCR product of the SSU rRNA gene was purified using the QIA Quick Gel Purification Kit (QIAgen, Hilden, Germany) according to manufacturer instructions. The purified PCR products were processed for bidirectional nucleotide sequencing using primers 18SFor2 and 18SRev2 by 1st BASE Laboratories, Malaysia. Chromatograms were manually checked and edited using BioEdit, Version 7.0.1 [27]. The nucleotide sequences were compared with the relevant sequences of the *Cryptosporidium* in GenBank using BLAST. Nucleotide sequences in this study have been assigned GenBank accession numbers as MN243567-MN243617. Phylogenetic analysis of 693 bp of the SSU-rDNA sequences was performed using the Neighbor Joining Method in MEGA, Version 7.0 [28].

Table 1
Characteristics of pigs and *Cryptosporidium* infections among pigs.

Characteristics	No. examined	*Cryptosporidium*	*p*-value
		No. positive (%)	
Farms			
1	21	4 (19.0)	0.02
2	17	3 (17.6)	
3	22	1 (4.5)	
4	41	19 (46.3)	
5	7	3 (42.9)	
6	7	1 (14.3)	
7	13	1 (7.7)	
8	3	0 (0.0)	
9	60	7 (11.7)	
10	30	8 (26.7)	
11	24	4 (16.7)	
Size of farm			
Smallholder (< 50 heads)	17	4 (23.5)	0.488
Large-scale farm (50–500 heads)	228	47 (24.6)	
Age (months)	Mean age = 9.1 ± 16.1 mo (min-max = 1–84 mo)		
≤6.0	198	50 (25.3)	< 0.001
> 6.0	47	1 (2.1)	
Sex			
Male	105	19 (18.1)	0.277
Female	140	32 (22.9)	
Total	245	51 (20.8)	

Significant differences among groups were tested by chi-square or Fisher's exact test.

2.4. Statistical analysis

Data were analyzed using STATA/MP, Version 12. (STATA Inc., TX, USA). Descriptive analyses were performed to determine characteristics of pigs and prevalence of *Cryptosporidium* infection. Association factors of *Cryptosporidium* infection were analyzed using Chi square or Fisher's exact test. All statistical parameters were calculated with a p value of 0.05.

3. Results

As shown in Tables 1, 3 of 11 pig farms were smallholder farms (Farms no. 5, 6 and 8) while the 8 remaining farms were small large-scale farms. Specimens were collected from 245 pigs aged from 1 to 84 months (mean age = 9.1 ± 16.1 months). Of 245 fecal samples, an overall prevalence of *Cryptosporidium* infection was 20.8% (51/245) which were detected in both smallholder and small large-scale pig farms. The positive results were obtained from all farms except Farm no. 8. Farms no.4 and 5 revealed a high prevalence of *Cryptosporidium* infection, i.e.46.4 (19/41) and 42.9% (3/7), respectively. No significant difference was observed among pigs regarding sex (p = .364) and farm size (p = .488). However, the prevalence of *Cryptosporidium* infection was significantly less among pigs aged > 6 months (p < .001).

Based on sequence analysis, 42 (82.4%) and 9 (17.6%) of 51 *Cryptosporidium* positive samples were identified as *C. scrofarum* and *C. suis*, respectively. Thus, the prevalence of *C. scrofarum* and *C. suis* infection was 17.1 and 3.7%, respectively. All these pig showed no gastrointestinal symptoms. Among the ten positive pig farms, *C. suis* was detected in pigs raised from Farms no. 1, 4, 9, 10 and 11 while *C. scrofarum* was detected in all ten pig farms (Table 2). In addition, the prevalence of *C. scrofarum* infection was significantly higher among pigs aged ≤6 months old (20.7%, 41/198) compared with those aged > 6 months old (2.1%, 1/47) (p = .001). However, no significant difference was found between the prevalence of *C. suis* infection and age (p = .213).

The phylogenetic tree reconstructed using the SSU-rRNA sequences

Table 2
Cryptosporidium scrofarum and *Cryptosporidium suis* infections among pigs.

Characteristics	No. examined	*Cryptosporidium* species (%)			
		C. scrofarum	*p*-value	*C. suis*	*p*-value
Farms					
1	21	3 (14.3)	0.009	1 (4.8)	0.596
2	17	3 (17.6)		0 (0.0)	
3	22	1 (4.5)		0 (0.0)	
4	41	16 (39.0)		3 (7.3)	
5	7	3 (42.9)		0 (0.0)	
6	7	1 (14.3)		0 (0.0)	
7	13	1 (7.7)		0 (0.0)	
8	3	0 (0.0)		0 (0.0)	
9	60	6 (10.0)		1 (1.7)	
10	30	5 (16.7)		3 (10.0)	
11	24	3 (12.5)		1 (4.2)	
Size of farm					
Smallholder (< 50 heads)	17	4 (23.5)	0.329	0 (0)	0.518
Large-scale farm (50–500 heads)	228	38 (16.7)		9 (3.9)	
Age (months)					
≤6.0	198	41 (20.7)	0.001	9 (4.5)	0.142
> 6.0	47	1 (2.1)		0 (0.0)	
Sex					
Male	105	16 (15.2)	0.305	3 (2.9)	0.410
Female	140	26 (18.6)		6 (4.3)	
Total	245	42 (17.1)		9 (3.7)	

Significant differences among groups were tested by chi-square or Fisher's exact test.

showed that all *C. scrofarum* and *C. suis* sequences were clustered with their corresponding clades of *C. scrofarum* (GenBank accession no. GU254170) and *C. suis* sequences (GenBank accession no. AF108861).

4. Discussion

Cryptosporidium infection in pigs has been reported worldwide with varied prevalences depending on differing geographical areas. Other factors such as management of pig farms, i.e., cleaning and hygiene operations and study age group influenced the prevalence of *Cryptosporidium* infection as well. Studies revealed the prevalences of 3.3 to 55.8% in China [29–31], 6 to 22.1% in Australia [32–33], 22.5% in Spain [34], 1.4% in Germany [35], 31.9 to 40.9% in Denmark [36–37], 26% in Canada [38], 16.5 to 26.4% in the Czech Republic [24,39–41], 14.4% in Switzerland [42] and 14.5% in central Vietnam [43]. In this study, using nested PCR, the prevalence of *Cryptosporidium* infection among pigs in Chonburi Province, eastern Thailand was 20.8%, which was similar to related reports in many countries.

Cryptosporidium infection in pigs occurs mainly through the oral-fecal route. Host-specific species of *Cryptosporidium* infection in pigs are C. *scrofarum and C. suis*. However, studies showed that pigs are also susceptible to other species that is *C. muris*, *C. tyzzeri*, *C. parvum, C. felis, C. hominis, C. andersoni and C. meleagridis* [44]. Both *C. scrofarum* and *C. suis* are thought to be host-adapted parasites, due to the absence of clinical symptoms in most infections [32–34,45]. However, severe diarrhea and weight loss can be found and caused death in neonatal and immunodeficient pigs [46–47].

In this study, sequences and phylogenetic analyses based on the SSU rRNA gene revealed a high prevalence of *Cryptosporidium* infection in pigs (20.8%) caused by two species namely, *C. scrofarum* (17.1%) and *C. suis* (3.7%). A few studies showed a higher prevalence of *C. scrofarum* compared with *C. suis* infections [33,37–39,48]. However, *C. suis* was more commonly found in pigs in some areas of China [29] and Vietnam [49]. Zoonotic transmission of both *C. scrofarum* and *C. suis* was also indicated. *C. suis* has been identified among HIV patients in Peru [18–20], Madagascar [21] and England [22]. *C. scrofarum* has been reported among humans in the Czech Republic [50]. Recently, a few cases of *C. suis* infection among Thai patients with HIV/AIDS were reported from a retrospective analysis of collected samples from 1999 to 2004, Bangkok, Thailand [23].

Young animals were more susceptible to the infection than those in the older age groups [51]. In the present study, age-specific prevalence of *Cryptosporidium* infection was observed. Our study found that *Cryptosporidium* infection in pigs aged ≤6 months was more frequent than those of > 6 months ($p < .001$). Our findings were consistent with related reports that *Cryptosporidium* infections occurred more frequently in 1 to 6 months old domestic pigs with prevalences of 24 to 60% [34,52–54]. However, some studies have reported that the prevalence of *Cryptosporidium* showed no age difference between infected and non-infected groups [39,48,55–56]. In this study, no significant difference was observed between *Cryptosporidium* infection and sex similar to a report in China [48].

From our results, *C. scrofarum* was significantly detected among pigs aged ≤6 months than those aged > 6 months. Thus, due to less immunity, pigs aged ≤6 months were more susceptible to *C. scrofarum* infection. This finding agreed with a related study that the prevalence of *C. scrofarum* infection was found only in pigs aged 2 to 6 months [34]. Johnson et al. (2008) also reported that *C. scrofarum* infection was found only in post-weaned pigs aged 1 to 6 months [33]. In contrast, in the Czech Republic, China, Denmark, and Ireland, *C. scrofarum* was commonly detected in > 6 month-old pigs [29,54,57–58]. Moreover, this study showed that the prevalence of *C. suis* infection in pigs did not significantly differ between ≤6 and > 6 months old pigs. This observation is in agreement with a study of pigs in the Czech Republic showing *C. suis* infection had no age specificity [57].

5. Conclusion

This study reported the high prevalence of *Cryptosporidium* infection in healthy pigs. Two species were detected, that is, *C. scrofarum* and *C. suis*. Age-specific prevalence of *Cryptosporidium* infection was observed among ≤6 month-old pigs. These asymptomatic pigs could serve as a potential source of *Cryptosporidium* infection to humans. Due to the zoonotic nature of *Cryptosporidium* spp., control measures should be taken using proper cleaning and hygienic operations in pig farms to control possible transmission of infective oocysts to humans and other animals.

Declaration of Competing Interest

None.

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