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▣ ORIGINAL ARTICLE

Molecular Identification of *Cryptosporidium* Species from Pet Snakes in Thailand

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Abstract: *Cryptosporidium* is an important pathogen causing gastrointestinal disease in snakes and is distributed worldwide. The main objectives of this study were to detect and identify *Cryptosporidium* species in captive snakes from exotic pet shops and snake farms in Thailand. In total, 165 fecal samples were examined from 8 snake species, boa constrictor (*Boa constrictor constrictor*), corn snake (*Elaphe guttata*), ball python (*Python regius*), milk snake (*Lampropeltis triangulum*), king snake (*Lampropeltis getula*), rock python (*Python sebae*), rainbow boa (*Epicrates cenchria*), and carpet python (*Morelia spilota*). *Cryptosporidium* oocysts were examined using the dimethyl sulfoxide (DMSO)-modified acid-fast staining and a molecular method based on nested-PCR, PCR-RFLP analysis, and sequencing amplification of the SSU rRNA gene. DMSO-modified acid-fast staining revealed the presence of *Cryptosporidium* oocysts in 12 out of 165 (7.3%) samples, whereas PCR produced positive results in 40 (24.2%) samples. Molecular characterization indicated the presence of *Cryptosporidium parvum* (mouse genotype) as the most common species in 24 samples (60%) from 5 species of snake followed by *Cryptosporidium serpentis* in 9 samples (22.5%) from 2 species of snake and *Cryptosporidium muris* in 3 samples (7.5%) from *P. regius*.

Key words: *Cryptosporidium parvum*, *Cryptosporidium serpentis*, *Cryptosporidium muris*, snake, nested PCR, SSU rRNA, Thailand

INTRODUCTION

Cryptosporidium spp. are apicomplexan protozoan parasites that infect a wide variety of vertebrate hosts, including reptiles, birds, fish, amphibians, and mammals, and cause cryptosporidiosis [1]. Cryptosporidiosis is characterized by acute gastrointestinal disturbances, mucoid or hemorrhagic watery diarrhea, fever, lethargy, anorexia, and death in humans and mammals [2]. *Cryptosporidium* infections have been described in at least 57 reptilian species consisting of 40 species of snakes, 15 species of lizards, and 2 species of tortoises [3]. Unlike in other animals in which infection with *Cryptosporidium* spp. is usually self-limiting in immunocompetent individuals, cryptosporidiosis in reptiles is frequently chronic and sometimes lethal in snakes [4]. Two species, *Cryptosporidium serpentis* and *Cryptosporidium varanii* (syn. *C. saurophilum*) have been described in snakes and lizards to date [4,5]. *C. serpentis* is an important parasite in snakes and is usually found in the gastric epithelium [6]. Clinical signs of cryptosporidiosis in snakes have been described as anorexia, lethargy, postprandial regurgitation, midbody swelling, and weight loss [1]. The infection occurs more frequently in adults rather than in young reptiles, unlike in mammals and birds [7]. While *C. varanii* was originally described in lizards as causing weight loss, abdominal swelling and mortality, it can be found in snakes but has no significant signs [5].

Stressed animals, which have been raised in a limited living space together with various types of species, are more likely to contribute to the spread of the parasite [8]. The above factors have been reported to suppress the immune responses and increase the opportunity for pathogens to cause infections and consequently spread to other animals including humans [8,9]. *Cryptosporidium* infection in snakes is difficult to identify, especially in those with a subclinical infection [10]. Conventional methods for detection of *Cryptosporidium* oocysts (including microscopic examination of fecal smears with acid-fast stains) are not capable of identification to the species level. Therefore, molecular techniques have been developed to detect and dif-

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ferentiate *Cryptosporidium* at the species/genotype and subtype levels [11]. Previously, molecular analysis of *Cryptosporidium* infection in snakes has identified *C. parvum*, *C. muris*, and *Cryptosporidium* mouse genotype, which probably originated from the ingestion of infected rodents or other prey [4,6,12-14]. Likewise, molecular techniques will help ensure accurate species identification of *Cryptosporidium* oocysts in snakes.

The pet snake business has become popular in Thailand. However, there is a lack of information regarding *Cryptosporidium* infection in snakes in Thailand. The present study aimed to identify *Cryptosporidium* species in captive snake fecal samples using microscopic and molecular examinations. This study will be relevant to disease surveillance and to the improvement of the management of aliments in captive snakes in Thailand.

MATERIALS AND METHODS

Sample collection

In total, 165 fecal samples were collected from asymptomatic snakes of 8 species in 6 genera (Table 1). Of these, 34 snakes were housed in 5 exotic pet shops, and 131 were from 2 snake farms. Fecal samples were stored at 4˚C before analysis.

Microscopy

Cryptosporidium oocysts were concentrated using Sheather's sugar flotation technique [15]. One drop from the top of the supernatant was smeared on a slide followed by staining using DMSO-modified acid-fast stain as previously described [16].

DNA extraction

DNA was extracted from the supernatant produced using Sheather's sugar flotation technique with a commercial kit (E.Z.N.A.® Stool DNA Kit, Omega Biotek Inc., Norcross, GA, USA) following the manufacturer's protocol. DNA was stored at -20˚C before molecular analysis.

Nested PCR amplification and PCR- RFLP analysis

Amplification of the 819-825 bp polymorphic fragment of the SSU rRNA using nested PCR was performed as previously described [17]. Briefly, the PCR conditions were composed of pre-denaturation at 94˚C for 5 min, then 35 cycles of denaturation at 94˚C for 45 sec, annealing at 55˚C for 45 sec, and extension at 72˚C for 1 min, followed by final extension at 72˚C for 10 min. RFLP of the secondary PCR products of *C. parvum* positive samples was performed using *Vsp* I (Thermo Fisher Scientific Inc., Rochester, New York, USA) for the genotyping of *C. parvum* [17]. The reaction mixture contained 0.5 µl of *Vsp*

Table 1. Detection of *Cryptosporidium* in snakes using microscopic and molecular methods

Scientific name	Common name	No. of samples		No. of positive samples				Species and/or genotype (no. of samples)	
				Acid fast stain		PCR		Sequence analysis	RFLP
		Farm	Pet shop	Farm	Pet shop	Farm	Pet shop		
Boa constrictor constrictor	Boa constrictor	14	3	0	0	4	1	*C. parvum (4)*	*C. parvum* mouse genotype
								Cryptosporidium mouse genotype (1)	-
Elaphe guttata	Corn snake	71	5	7	2	17	2	*C. parvum (10)*	*C. parvum* mouse genotype
								C. serpentis (8)	-
								C. saurophilum (1)	-
Epicrates cenchria	Rainbow boa	0	1	0	0	0	0	-	-
Lampropeltis getula	King snake	19	2	0	0	1	0	*C. serpentis (1)*	-
Lampropeltis triangulum	Milk snake	4	3	0	0	1	1	*C. parvum (2)*	*C. parvum* mouse genotype
Morelia spilota	Carpet python	0	4	0	1	0	4	*C. parvum (3)*	*C. parvum* mouse genotype
								C. andersoni (1)	-
Python regius	Ball python	23	15	2	0	0	9	*C. parvum (5)*	*C. parvum* mouse genotype
								C. muris (3)	-
								Cryptosporidium mouse genotype (1)	-
Python sebae	Rock python	0	1	0	0	0	0	-	-
Total		131	34	9	3	23	17		

I (Thermo Fisher Scientific Inc.), 2.2 µl of restriction buffer, and 5 µl of PCR product at 37˚C for 30 min, under conditions recommended by the manufacturer. The digested products were analyzed using 2% agarose gel electrophoresis.

DNA sequencing and phylogenetic analysis

The positive *Cryptosporidium* samples were submitted for sequencing (1st Base Laboratory, Selangor, Malaysia). The DNA sequences were compared with those in the GenBank database using the basic local alignment search tool (BLAST) algorithm, and the species of *Cryptosporidium* present in the sample was determined. The nucleotide sequences of the partial SSU rRNA gene of the *Cryptosporidium* parasites were deposited in the GenBank™ database under the accession nos. KM870564 - KM870603. Multiple alignments were done using the ClustalW program [18,19]. A neighbor-joining tree was constructed from the aligned sequences using the MEGA version 5 software [20].

Statistical analysis

Statistical analysis was performed using a chi-square (χ^2) test in the Number Cruncher Statistical System (NCSS) version 2000 to determine the association between the prevalence of *Cryptosporidium* infection vs host genders and host locations. Values were tested for significance at $P \leq 0.05$.

RESULTS

Of the 165 fecal samples from captive snakes, 12 (7.3%) were detected as positive for *Cryptosporidium* oocysts using the DMSO-modified acid-fast stain, and 40 (24.2%) were positive using nested PCR (Table 1). Seventeen out of 34 samples (50.0%) from pet shops and 23 out of 131 samples (17.6%) from private farms were positive for *Cryptosporidium*. Among the positive results from nested PCR testing, sequencing analysis identified 24 (60.0%); 9 (22.5%); 3 (7.5%), 2 (5.0%), 1 (2.5%), and 1 (2.5%) as *C. parvum*, *C. serpentis*, *C. muris*, *Cryptosporidium* mouse genotype, *C. andersoni*, and *C. saurophilum*, respectively (Table 1). *C. parvum* was detected from *Boa constrictor constrictor* (4), *Elaphe guttata* (10), *Lampropeltis triangulum* (2), *Python regius* (5), and *Morelia spilota* (3). *C. serpentis* was detected from *Lampropeltis getula* (1) and *Elaphe guttata* (8). *C. muris* was detected from *Python regius*. *Cryptosporidium* mouse genotype was detected from *B. constrictor constrictor* (1) and *P. regius*. *C. andersoni* was detected from *M. spilota*. *C. saurophilum* was detected from *E. guttata* (Table 1).

Sequencing analysis indicated that *C. parvum*, *C. serpentis*, *C. muris*, *Cryptosporidium* mouse genotype, *C. andersoni*, and *C. saurophilum* were 99% identical to GenBank accession nos. DQ898158, AF093499, EU553588, EU553589, JX515549, and EU553551, respectively. Differentiation of the *C. parvum* genotype by PCR-RFLP showed that all 24 *C. parvum* positive samples were *C. parvum* mouse genotype (Fig. 1). This genotype is

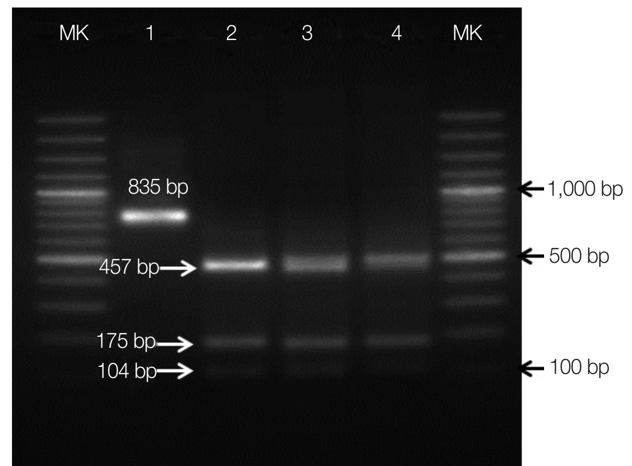

Fig. 1. PCR-RFLP analysis. Lane 1 is *Cryptosporidium parvum* without digestion showing a single 835 bp band and lanes 2-4 are *C. parvum* "mouse genotype" showing digested bands of 104, 175, and 457 bp. The molecular weight marker size, 100 bp ladder. Black arrow, the DNA marker size at 100 bp, 500 bp, and 1,000 bp.

Table 2. Risk factors associated with *Cryptosporidium* spp. infection in snakes

Variable	No. (%) of infected snakes	No. (%) of non-infected snakes	Total sample	χ^2	*P*-value[a]
Sex[b]	25 (17.4)	119 (82.6)	144	1.027 df = 1	0.310
Male	6 (12.8)	41 (87.2)	47		
Female	19 (19.6)	78 (80.4)	97		
Location	40 (24.2)	125 (75.8)	165	15.470 df = 1	0.00008
Pet shops	17 (50.0)	17 (50.0)	34		
Farms	23 (17.6)	108 (82.4)	131		

[a]$P \leq 0.05$ was considered statistically significant.
[b]Gender data were missing in 21 samples.

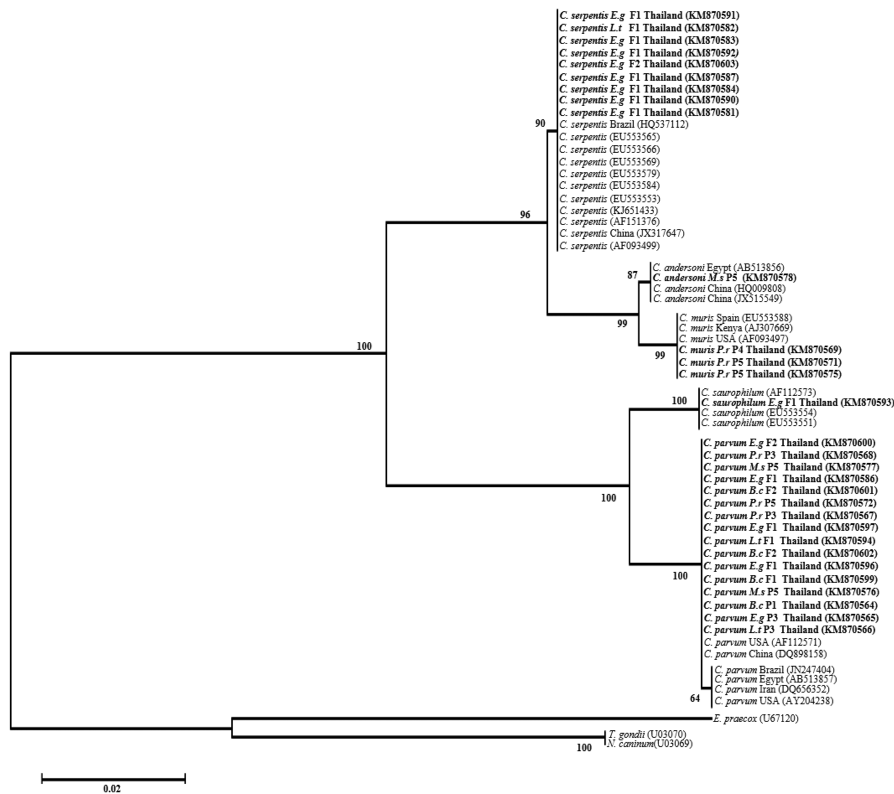

Fig. 2. Phylogenetic relationships among *Cryptosporidium* species found in this study and other *Cryptosporidium* spp. performed using neighbor-joining analysis of the SSU rDNA fragment. The samples with local origin "Thailand" were in current study. Captive snake: *B. c* = *Boa constrictor constrictor*, *E.g* = *Elaphe guttata*, *L.t* = *Lampropeltis triangulum*, *M.s* = *Morelia spilota*, *P.r* = *Python regius*. Location: P1 = Pet shop, P2 = Pet shop, P4 = Pet shop 4, P5 = Pet shop 5, F1 = Farm 1, F2 = Farm 2.

considered as nonpathogenic in snakes [21].

Risk factor analysis showed that there were infection rates of 12.8% and 19.6% of *Cryptosporidium* spp. in male and female snakes ($\chi^2 = 1.027$, $P = 0.310$), respectively, and the infection rates were 50.0% and 17.6% on pet shops and farms ($\chi^2 = 17.470$, $P = 0.00008$), respectively. These results indicated that location was a statistically significant factor associated with *Cryptosporidium* spp. infection in snakes (Table 2). The neighbor-joining analysis results showed that 2 distinctive clades of intestinal and stomach *Cryptosporidium* species from this study were clearly separated (Fig. 2). Intestinal *Cryptosporidium* species consisted of *C. parvum*, *Cryptosporidium* mouse genotype, and *C. saurophilum*, while stomach *Cryptosporidium* species consisted of *C. serpentis*, *C. muris*, and *C. andersoni*.

DISCUSSION

Cryptosporidiosis is a well-known cause of chronic hypertrophic gastritis, and is possibly lethal in captive snakes [5,22]. The present study was the first identification of *Cryptosporidium* infection in pet snakes in Thailand. We found 7.3% of the *Cryptosporidium* oocysts using the acid-fast stain technique and 24.2% of the *Cryptosporidium* DNA using a molecular method. The acid-fast staining technique is less sensitive for the detection of *Cryptosporidium* infection. However, it is the easiest and most effective method for veterinary clinical diagnosis [23]. Moreover, PCR is a sensitive and specific detection technique, which resulted in a much higher proportion of *Cryptosporidium* positive samples in comparison to acid-fast staining [24]. Additionally, PCR is an important method for the identification of *Cryptosporidium* species or genotypes in samples with low numbers of oocysts [25,26]. Thus, PCR is still hindered by its high cost and time-consuming DNA extraction, PCR amplification, and gel electrophoresis [27,28]. A combination of several diagnostic techniques for the detection of *Cryptosporidium* oocysts is still needed [29].

In the present study, sequencing analysis of the 18S rRNA gene revealed the presence of *C. serpentis* in the corn snake (*E.*

guttata) and king snake (*L. getula*). These findings were similar to previous studies, which have found that *C. serpentis* is most common in snakes [4,6,9,12,14,30]. Additionally, we found *C. saurophilum* in the corn snake (*E. guttata*), which is similar to previous reports [4-6,13]. *C. saurophilum* was originally described as an intestinal parasite mainly in lizards [31]. The presence of *C. saurophilum* in reptiles other than lizards might have resulted from the fact that they were housed together [4-5]. Moreover, none of the infected animals showed any clinical signs of the disease in the present study, which was in agreement with previous reports [6,9]. However, the subclinical stage can last for years in these animals [32].

We have confirmed the presence of *C. parvum*, *C. muris*, and *C. andersoni* in captive snakes. *C. parvum* and *C. muris* were considered as *Cryptosporidium* from mammals. Therefore, they were not pathogenic in snakes [33,34]. On the other hand, we have also confirmed *C. andersoni* in captive snakes. Interestingly, *C. andersoni* was commonly found in infected cattle abomasum. Possibly, the finding of other *Cryptospodium* genotypes in snakes might be due to infection from infected prey animals [35]. This is the first report of *C. andersoni* in captive snakes.

The study of farm management found that the sanitary conditions on the farm and inadequate management influenced the rate of infection of *Cryptosporidium* in dairy cows [36]. Farms with poor management and bad sanitary conditions present a high risk of *Cryptosporidium* and other gastrointestinal protozoan infections. In this study, risk factor analysis indicated that location was significantly associated with *Cryptosporidium* spp. infection. The risk of infection was higher in pet shops than in farms even though the former did not use for breeding, but only for selling snakes. However, our observation during sample collection found that some pet shops have many species of animals, and keep them at high densities in small cages. They also used contaminated equipment. The result is that pet shops tend to have a higher percentage of *Cryptosporidium* spp. infection than private farms.

Phylogenetic analysis of SSU rRNA gene fragments within the genus *Cryptosporidium* has proven to be a useful tool for both the systematic analysis of the presently recognized species and the possibility of definitive identification of new species or genotypes within this genus [4]. In this study, we identified 6 different *Cryptosporidium* species or genotypes in reptiles. These results confirmed that *C. serpentis* and *C. parvum* are the main species found in snakes.

A high proportion of *Cryptosporidium* species from mammals (75.0%) was detected in this study consisting of *C. parvum*, *C. muris*, *Cryptosporidium* mouse genotype, and *C. andersoni*. However, these *Cryptosporidium* are non-pathogenic in snakes but potentially zoonotic. *Cryptosporidiosis* causes mucoid or hemorrhagic diarrhea, fever, lethargy, anorexia, and death especially in immunocompromised patients [11,37-39]. Additionally, *C. muris* is probably the zoonotic *Cryptosporidium*, which was reported in HIV patients in Perú, Thailand, Indonesia, France, and Kenya [40]. Recently, *C. andersoni* has been found in 21 diarrhea patients out of 232 outpatients in China, whereas *Cryptosporidium hominis* (the human genotype) was found only in 2 patients [41]. In 2015, *C. andersoni* was first reported in a captured lesser panda in China [42]. Therefore, the present identification of *C. andersoni* might have a public health impact. Additionally, *Cryptosporidium* has an environmentally resistant oocyst, which is a public health risk factor for handlers and owners, especially when they are children, elderly people, or immunocompromised patients. Consequently, feeding snakes with *Cryptosporidium* infected mice or any other prey could possibly transmit the pathogen to humans via feces, water, and contaminated equipment. Although a substantial study of *Cryptosporidium* transmission from reptiles to humans has not been reported yet, disinfection processes have been recommended as the best option for reducing the transmission risk by the application of 4-cholr-M-cresol, 5% ammonium solution, or hydrogen peroxide-based disinfectants [14,21,32]. The presence of *Cryptosporidium* infection in snakes should not be ignored in snake collections because infection can be transmitted from animal to animal by the fecal-oral-route when animals are housed together. The lack of effective treatment for cryptosporidiosis almost always results in euthanasia of the infected snakes, which often leads to the loss of valuable animals in a collection [43]. Hyperimmune bovine colostrum (HBC) is recommended to combat clinical and subclinical *C. serpentis* infections in captive snakes [44].

In conclusion, applying a molecular method for the detection of the pathogen will probably prove the presence of the *Cryptosporidium* species or genotype, because it is difficult to identify oocysts of pathogenic *C. serpentis* or *C. saurophilum* from those of non-pathogenic *Cryptosporidium* species [45]. The study has shown that molecular techniques can separate different species and genotypes of *Cryptosporidium*. The sequencing of the PCR products revealed that *C. parvum*, *C. serpentis*, *C. muris, C.* mouse genotype, *C. andersoni*, and *C. sauro-*

philum are different from each other. In addition, the PCR-RFLP technique can differentiate various *C. parvum* genotypes (e.g., *C. parvum* human, *C. parvum* mouse, and *C. parvum* bovine genotypes) [45]. This study found a high percentage of non-pathogenic *Cryptosporidium* in snakes in Thailand. However, some species were zoonotic *Cryptosporidium*, which might have been ingested from prey and passed through intestinal tract of the snake.

Cryptosporidium oocysts from food items can cause the misidentification of cryptosporidiosis in snakes. In addition, paying more attention to cryptosporidiosis in snakes is required due to public health concerns. Moreover, the sanitary conditions associated with snake feeding should be improved along with avoiding the purchase of infected feeds. Thus, practicing good sanitation and hygiene, including at snake prey suppliers, should be considered, and these places tested for specific pathogens and to prevent the killing of snakes infected with non-pathogenic *Cryptosporidium*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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