Molecular detection of Cryptosporidium spp. in captive snakes in Thailand

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ABSTRACT

Cryptosporidiosis is an important cause of gastro-intestinal disease in snakes and distributed worldwide. The objective of this study was to detect and identify Cryptosporidium spp. in captive snakes based on molecular technique. A total of 32 fecal samples of eight snake species were examined, including 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 analyzed by DMSO-Modified acid fast stain and molecular method based on nested PCR and sequencing amplification of the 18s rRNA gene. DMSO-Modified acid fast stain detected the presence of Cryptosporidium oocysts in 5 out of 32 (15.2%) samples, whereas PCR detected 17 (50%) samples. BLASTn and phylogenetic analyses of DNA sequences showed Cryptosporidium parvum as the most common species with 11 samples (34.4%) from Boa constrictor constrictor, Elaphe guttata, Lampropeltis triangulum, Python regius and Morelia spilota followed by C. muris with 3 samples (9.4%) from Python regius. These results suggested that the mammalian Cryptosporidium was not the pathogenic species in snake.

Key word: Cryptosporidium spp., snake, reptile, endoparasite, 18s rRNA

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INTRODUCTION

Cryptosporidium is a protozoan parasite of vertebrate host including reptiles, birds, fish, rodents, cattle, sheep, goats, pigs and humans. (Fayer, 1997). Cryptosporidiosis is a zoonotic disease caused by Cryptosporidium parvum. In humans, cryptosporidiosis is characterized by mucoid or haemorrhagic diarrhea, fever, lethargy, anorexia and death (Navin and Juranek, 1984). The Cryptosporidium infections are common in reptiles and have been described at least 57 reptile species (O’Donoghue, 1995). The cryptosporidiosis infection of other animals is self-limiting in immunocompetent individuals but cryptosporidiosis in reptiles is generally chronic and can sometime lead to death (Xiao et al., 2004).

Cryptosporidium serpentis is an important parasite in snakes and infects usually the gastric epithelium (Pedraza-Díaz et al., 2009). Anorexia, depression, regurgitation of food, abdominal enlargement, and weight lost are major in snakes (Fayer, 1997). Infections in reptiles are often symptomatic in adult animals. It is difficult to diagnose infected animals, especially those in the subclinical infection (Graczyk, 1996). Animals under stressful life, concentration of animals and the presence of different species in a small living space can promote the spreading of parasites (Rataj et al., 2011). Conventional methods for identification of Cryptosporidium include microscopic examination with fecal stain but this method is not enough for species identification. In addition, the molecular technique is use to identify the causative pathogen of the disease (Sevá et al., 2011). Previous studies of molecular analysis of Cryptosporidium infection in snakes reported that C. parvum, Cryptosporidium “mouse genotype” and C. muris were detected. (Morgan et al., 1999; Xiao et al., 2004 Pedraza-Diaz et al., 2009; Richter et al., 2011; Sevá et al., 2011; Pedraza-Diaz et al., 2013). The presence of those Cryptosporidium from reptiles reported that they are not infectious to reptiles, including snakes and oocysts could be from ingested prey or feeder mice (Xiao et al., 2004; Pedraza-Diaz et al., 2009). Molecular techniques will aid the accurate identification of the species of Cryptosporidium oocysts in snake.

The present study aimed to identify of Cryptosporidium species from captive snakes by sequencing of a polymorphic region of the 18s rRNA gene.

MATERIALS AND METHODS

1. Sample collection

A total of 32 snakes from eight species, were as following, 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 pythons (Morelia spilota) from exotic petshops and private farms. Fecal samples were stored at 4 °C until used or preserved in 2.0% potassium dichromate (Sevá et al., 2011).

2. Analysis of Cryptosporidium oocysts

The fecal samples were prepared in thin smear technique. The samples-slide were fixed with absolute methanol for 5-10 min, stained with Carbol-fuchsin-DMSO for 5 min and washed with tap water. The slide were dried immersed in decolorized solution and counterstained in malachite green for 1-2 min. The slide was rinsed in running tap water to stop the decolorizing and let them dried. The slides were mounted with permount solution and then examined the stained oocysts under the microscope (Bronsdon, 1984).

3. DNA extraction

Fecal samples were extracted using a commercial kit (E.Z.N.A.® Stool DNA Kit, Omega Bio-Tek) with the protocol following the manufacturer’s advice. DNA was stored at -20 °C before it was used in molecular analysis.

4. Polymerase chain reaction

For detection of Cryptosporidium spp., nested PCR assay was modified from the protocol reported by Xiao et al (1999). Nested PCR was performed for amplification of an 835 bp polymorphic fragment of the 18S rRNA. Twenty five microliters μl reactions contained 1× buffer (10mM Tris–HCl pH 8.8, 50mM KCl and0.1% Triton X-100), 9mM MgCl2 for the first round and the second round used 3mM, 0.4 pmol of each primer, 0.08 mM of each dNTP, 1.5 Units of Taq DNA polymerase (DyNAzyme, FINNZYMES) and 1 μl of DNA template. The first PCR primer; F1 (AL 1687) 5’-TTCTAGAGCTAATACATGCG-3’ and R1 (AL3417) 5’-CCCATTTCCTTCGAAACAGGA-3’ amplified approximately 1,325 bp of the Cryptosporidium spp. 18s rRNA gene. The PCR condition was compose 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 and followed by final extension at 72°C for 10 min. The nested primers were F2 (AL3032) 5’-GGAAGGTTGTTATTTATTAGAAGG-3’ and R2 (AL1598 5’-AAGGAGTAAAGGAACCTCCA-3’ and the nested PCR protocol was similar to the first protocol. The nested PCR product was sized at 835 bp.
Both of PCR protocols were processed in MyCyclerTM Thermal Cycler (BioRad Laboratories, USA). The amplified PCR products were checked in 1% agarose (SeaKem ME:FMC, USA) gel electrophoresis and stained with ethidium bromide. Amplicons were processed with UltraCleanTM GelSpin DNA purification Kit (MO BIO LABORATORIES Inc, CA, USA) and submitted for sequencing (1st Base Laboratory, Malaysia).

5. DNA Sequencing and phylogenetic analysis

DNA sequences were subject to similarity identification by comparing with Genbank database using BLAST algorithm. The phylogenetic tree was constructed by analysis sequences from the MAFFT program (Katoh et al., 2002). A bootstrap value calculation was used to estimate the confidence levels for relation of separating branch of the tree.

RESULTS AND DISCUSSIONS

The Cryptosporidium positive were found 17 out of 33 samples (50%) by PCR. The positive DNA target was approximately size at 835 bp for nested PCR products as shown in figure 1 and the results of Cryptosporidium spp. from partial sequencing of the 18s rRNA gene were shown in table 1. The positive samples were found several species including 34.4% C. parvum (11/33), 9.4% C. muris (3/33), 3.1% C. andersoni (1/33), 3.1% Cryptosporidium “mouse genotype” (1/33) and 3.0% not identifiable from sequencing (1/33). These Cryptosporidium speciations were found in mammalian hosts but not found the species caused of disease in snake. Analysis of Cryptosporidium oocyst by DMSO-Modified acid fast stain method found positive only 5 samples (15.2%) that shown in Table1.

Sequence analysis of the secondary PCR products indicated that C. parvum, C. muris, Cryptosporidium “mouse genotype” and C. andersoni were 99% identical to GenBank accession numbers DQ898158, EU553588, EU553589 and JX515549, respectively. The PCR products from 16 positive samples were used in phylogenetic analysis by MAFFT program using neighbor – joining algorithm for Cryptosporidium spp. 18s rRNA gene. The phylogenetic tree demonstrated the protozoan speciation in Thailand were grouped together and separated from the other isolations as shown in figure 2.

The previous studies have been reported that the reptile could be infected with nine species of Cryptosporidium but only two species cause disease in reptiles as C. serpentis which was mostly found in snakes and C. varanii was mostly found in lizards (Xiao et al., 2004).
Figure 1 The positive DNA bands of Cryptosporidium spp. from 18s rRNA gene presented at 835 bp and lane 1 was a positive control, lane 9 was a negative control, lane 3-7 were positive sample. MK: DNA marker 100 bp, Black arrow: the DNA marker size at 500 pb.

Table 1. Detection of Cryptosporidium spp. in snakes by microscopic and molecular methods.

<table>
<thead>
<tr>
<th>No.</th>
<th>Snakebreed</th>
<th>Acid fast</th>
<th>PCR</th>
<th>sequencing</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Boa constrictor constrictor</em></td>
<td>(0/3)</td>
<td>(1/3)</td>
<td><em>C. parvum</em> (1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Epicrates cenchria</em></td>
<td>(0/1)</td>
<td>(0/1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Python sebae</em></td>
<td>(0/1)</td>
<td>(0/1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>Lampropeltis getula</em></td>
<td>(0/2)</td>
<td>(0/2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Elaphe guttata</em></td>
<td>(2/4)</td>
<td>(2/4)</td>
<td><em>C. parvum</em> (2)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>Lampropeltis triangulum</em></td>
<td>(0/3)</td>
<td>(1/3)</td>
<td><em>C. parvum</em> (1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>Python regius</em></td>
<td>(2/15)</td>
<td>(9/15)</td>
<td><em>C. parvum</em> (4)</td>
<td><em>C. muris</em> (3) <em>C. &quot;mouse genotype&quot;</em> (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NI (1)</td>
</tr>
<tr>
<td>8</td>
<td><em>Morelia spilota</em></td>
<td>(1/4)</td>
<td>(4/4)</td>
<td><em>C. parvum</em> (3)</td>
<td><em>C. andersoni</em> (1)</td>
</tr>
</tbody>
</table>

*Ni = not identifiable.
Figure 2: The phylogenetic relationship among *Cryptosporidium* spp. 18s rRNA gene compared with the sequences in Genebank database. The phylogenetic tree was constructed by using neighbor-joining algorithm with 1000 bootstrap value calculation and *Eimeria meleagritits* as outgroup.

The present result showed the DMSO-Modified acid fast stain technique had low sensitivity than molecular technique. Additionally, by amplification the 18s rRNA gene of the parasite, the PCR technique presented high sensitivity which detected 1 oocyst in 1 gram of animal's feces (Paul et al., 2009).

The result of sequencing demonstrated no infection from *Cryptosporidium* spp. that infected in snake host. The most infection was mammalian typically *Cryptosporidium* spp. included C. parvum, C. muris and C. "mouse genotype". These informations were related to the previous studies which found the same organisms infected in snake host (Pedraza-Díaz et al., 2009; Pedraza-Díaz et al., 2013; Richter et al., 2011; Xiao et al., 2004). These results suggested that the mammalian *Cryptosporidium* was not the
pathogenic species in snake. The infection probably occurred from the ingestion of prey or feeder mice (Morgan et al., 1999). From the author’s knowledge, this study present the first infection of *C. andersoni*, the *Cryptosporidium* specific to cattle host, in snake might be the snake infected from substrate such as hay. The phylogenetic analysis demonstrated the cluster of Thailand-snake *Cryptosporidium* that completely separated from the other species.

ACKNOWLEDGMENTS

This study was supported by a research grant from Faculty of Veterinary Medicine, Kasetsart University.

REFERENCES


