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Detection of *Cryptosporidium* Species in Feces from Snakes

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Abstract: Cryptosporidiosis is an important gastrointestinal disease in snakes. In the current study, 143 feces samples of cobra snakes and Oriental rat-snake were examined for the presence of cryptosporidia by morphology and Polymerase Chain Reaction (PCR) Method targeting a part of the 18S ribosomal *RNA* gene. A consecutive sequencing reaction was used to identify the cryptosporidian species present in PCR-positive samples. The oocyst of *Cryptosporidium serpentis* was found in 19 out of 143 (13.29%). The results stress the importance for diagnostic methods to be specific for *Cryptosporidium* species especially in snakes.

Key words: *Cryptosporidium serpentis*, snakes, morphology, polymerase chain reaction, prevalence, sequences

INTRODUCTION

Cryptosporidiosis is a hot topic in terms of hygiene and health worldwide which can cause diarrhoea not only in mammal but also in reptiles (Fayer and Ungar, 1986; Richter *et al*., 2011). Currently, *Cryptosporidium serpentis* is acknowledged as valid species in reptiles and has been associated with disease of the gastrointestinal tract especially in snakes (Brownstein *et al*., 1977; Deming *et al*., 2008; Fayer *et al*., 1995).

C. serpentis in snakes which is different from other *Cryptosporidium* species in reptiles on morphology. The oocysts of *C. serpentis* are bigger than those of *C. saurophilum* and former one is a gastric parasite whereas the latter is an intestinal parasite (Upton *et al*., 1989; Xiao *et al*., 2004). The identification of *Cryptosporidium* pathogenic species from which are ingested with the prey (pseudoparasites) and excreted as intact oocysts is important for correct diagnosis (Morgan *et al*., 1998; Pedraza-Diaz *et al*., 2009). Although, the genetic diversity within *C. serpentis* organisms is not clear but *C. serpentis* infection in snakes frequently causes clinical diseases (Fayer *et al*., 1997).

There are several methods in the detection of cryptosporidia. Many of these methods diagnose cryptosporidia are at the genus level and do not allow a distinction of different species (Pedraza-Diaz *et al*., 2009). Other methods used for the differentiation of certain Cryptosporidium species usually comprise important human pathogenic or possibly zoonotic, pseudoparasitic species and genotypes but not all reptilian pathogenic types (Kimbell *et al*., 1999; Limor *et al*., 2002). In snakes, the absence of an effective diagnosis and treatment for cryptosporidiosis necessitates euthanasia to eliminate patient suffering and prevent further spread of the parasite. The aim of the study was to diagnose Cryptosporidium infection of snakes from Lingshan and Nanning, China using morphology and PCR Methods to identify the species and evaluate the importance of Cryptosporidium in snakes.

MATERIALS AND METHODS

Stool samples: In this study, 143 stool samples were collected from cobra snakes (46) and Oriental rat-snake (97) (the code of positive samples can be seen in Table 1): a part was stored at 4°C for PCR and the other fresh stool were identified by acid fast stain and Auramine stain for routine screening of oocyst.

Morphology identification: Firstly, fecal smears were fixed by formaldehyde. After dried in air, they were stained by

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Kinyoun acid fast stain (Chan *et al*., 2000). Slides were observed by optical microscopy using a magnification of x1,000 (immersion). Moreover, oocysts were identified according to morphological characteristics and size.

DNA extraction, PCR protocols and sequencing: Samples of DNA were extracted using a commercial DNA extration card (FTA card). Next, a two-step nested PCR protocol for the 18s small subunit ribosomal *RNA* gene was used as the aim gene and amplification as earlier described (Xiao *et al*., 1999). Positive amplicons were purified and sequenced in both directions using an ABI 377 automated DNA sequencer (using BigDye Terminator Chemistry).

Phylogeny: Clustal X 1.83 was used to align the part sequences of 18s small subunit ribosomal *RNA* genes (Thompson *et al*., 1997). Pairwise comparisons were conducted of the level of sequence Differences (D) among and within *Cryptosporidium* sp. taxa using the equation:

$$D = 1 - \frac{M}{L}$$

Table 1: Posituve samples of Cryptosporidium from Lingshan and Nanning, China used in the present study as well as their GenBank™ accession numbers for sequences of the 18S small subunit ribosomal *RNA* gene

Sample codes	Genotype	GenBank™ accession number
CsLS7	*Cryptosporidium serpentis*	JX317647
CsLS35	*Cryptosporidium serpentis*	No submitted
CsLS46	*Cryptosporidium serpentis*	No submitted
CsLS84	*Cryptosporidium serpentis*	No submitted
CsLS107	*Cryptosporidium serpentis*	No submitted
CsLS108	*Cryptosporidium serpentis*	No submitted
CsLS109	*Cryptosporidium serpentis*	No submitted
CsLS110	*Cryptosporidium serpentis*	No submitted
CsLS113	*Cryptosporidium serpentis*	No submitted
CsLS115	*Cryptosporidium serpentis*	No submitted
CsLS116	*Cryptosporidium serpentis*	No submitted
CsLS117	*Cryptosporidium serpentis*	No submitted
CsNN123	*Cryptosporidium serpentis*	No submitted
CsNN125	*Cryptosporidium serpentis*	No submitted
CsNN126	*Cryptosporidium serpentis*	No submitted
CsNN127	*Cryptosporidium serpentis*	No submitted
CsNN130	*Cryptosporidium serpentis*	No submitted
CsNN158	*Cryptosporidium serpentis*	No submitted
CsNN183	*Cryptosporidium serpentis*	No submitted
C. suis	*Cryptosporidium suis*	GU254177
C. parvum	*Cryptosporidium parvum* (strain Human)	AF093492
C. serpentis	*Cryptosporidium serpentis*	AF151376
C. baileyi	*Cryptosporidium baileyi*	AF093495
C. andersoni	*Cryptosporidium andersoni*	HQ009808
C. ryanae	*Cryptosporidium ryanae*	HQ179574
C. galli	*Cryptosporidium galli*	GU816051
G. intestinalis	*Giardia intestinalis*	AY826207

There was no variation among the 19 samples. Only the sequence of CsLS7 has been submitted

Where:

M = The number of alignment positions at which the two sequences have a base in common

L = The total number of alignment positions over which the two sequences are compared (Chilton *et al*., 1995)

In order to study the phylogenetic relationships, the 18s small subunit ribosomal *RNA* gene sequences in Cryptosporidium were obtained in the research and from GenBank (Table 1) with Giardia (Gd) as the outgroup (GenBank™ accession number can be seen in Table 1).

The methods of Maximum Parsimony (MP) were used for phylogenetic re-constructions which were carried out using PAUP 4.0 Beta 10 programme (Swofford, 2002). The consensus tree was obtained after bootstrap analysis with 1000 replications with values above 50% reported. Phylograms were drawn using the Tree View Program Version 1.65 (Page, 1996).

RESULTS AND DISCUSSION

The clinical signs reported in some cryptosporidium-positive reptiles are consistent with chronic gastrointestinal disease (Kimbell *et al*., 1999; Plutzer and Karanis, 2007). However, not all the pathogen with gastrointestinal diseases were cryptosporidia. The symptom of abdomen swelling, malodorous feces and diarrhea can also be caused by other microorganisms, for example enteropathogenic bacteria (Taylor *et al*., 1999). Thus, reptilian cryptosporidiosis should not be diagnosed merely based on gastrointestinal clinical signs.

In this research, the morphological detection was performed by Kinyoun acid-fast stain to observe the oocysts of cryptosporidium (Fig. 1). In acid-fast stain, the oocyst has been stained in red colour.

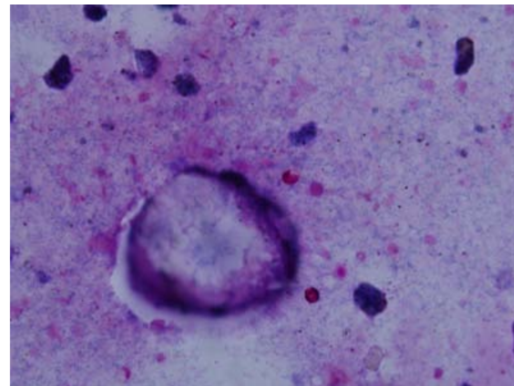

Fig. 1: Kinyoun acid-fast stain of the oocysts of *Cryptosporidium serpentis*

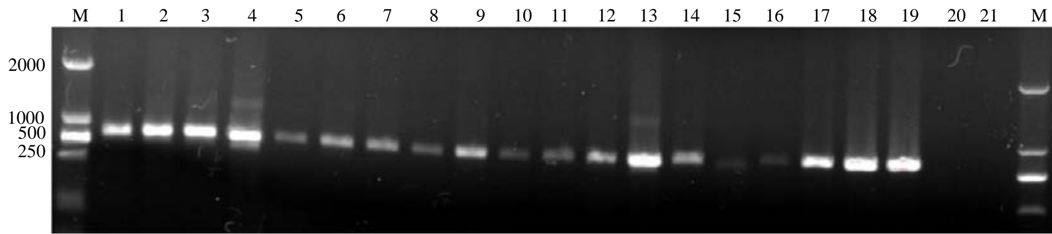

Fig. 2: Representative PCR products of samples from snakes for 18s small subunit ribosomal *RNA* gene. Lanes 1-21 represent samples CsLS7, CsLS35, CsLS46, CsLS84, CsLS107, CsLS108, CsLS109, CsLS110, CsLS113, CsLS115, CsLS116, CsLS117, CsNN123, CsNN125, CsNN126, CsNN127, CsNN130, CsNN158, CsNN183 and negative control (20, 21), respectively. M represents a DNA size marker (ordinate values in bp)

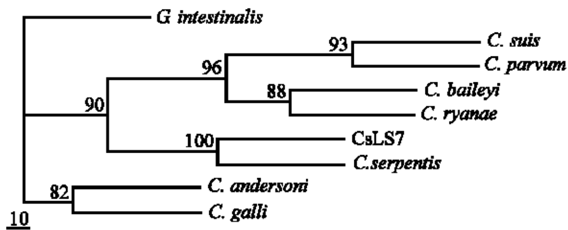

Fig. 3: Phylogenetic relationship of *Cryptosporidium serpentis* with other species of *Cryptosporidium* sp. inferred by Maximum Parsimony (MP) using the 18s small subunit ribosomal *RNA* gene with *Giardia intestinalis* as outgroup. Bootstrap values (in percentage) above 50% from 1,000 pseudo-replicates are shown for MP. Scale bar indicates an evolutionary distance of 10 substitutions per site in the sequence.

Of the 143 fecal samples from snakes, 19 (13.29%) were positive by PCR including 6 from cobra snakes (6/46, 13.04%) and 13 from Oriental rat-snake (13/97, 13.40%). Meanwhile, there was no size variation was detected on agarose gel among any of the amplicons examined (Fig. 2). After trimming some base pairs at the beginning and end of the sequences, sequences size for part *18s* gene of the two samples were 807 bp and with the similar base composition. When blasted in NCBI, the highest similarity was 100% with *C. serpentis*. The accession numbers has been shown in Table 1.

The two methods in the diagnosis of cryptosporidium infection in snakes show the same results. The morphology detection was the gold-standard method in parasitic infection with the character of direct-viewing. However, it needs professional training and rich clinical experiences. Moreover, PCR Method can reveal the genotype and convient to operation. On the contrary, it is expensive for a small number of samples which is the situation in many parasitology laboratories.

The phylogenetic tree (Fig. 3) was consisted of two large clades: the first one contained *C. suis*, *C. parvum*, *C. baileyi*, *C. ryanae* and *C. serpentis* strains including the sample CsLS7 within the second clade, *C. andersoni* and *C. galli* were clustered together then the two clade were clustered together, respectively. In the first branch, CsLS7 was clustered with *C. serpentis* strains together. This clustering is in agreement with the results of traditional classifications.

CONCLUSION

Morphology method in parasitic disease diagnosis is the gold standard approach to find the pathogen. However, this assay is not convenient for needing trained microscopists.

The described PCR assay and sequencing reaction proved to be useful in the diagnostics of cryptosporidiosis in live reptiles. Common laboratories are also capable of carring out the conventional PCR reaction and the sequencing reaction can be outsourced to specialized facilities to identify the cryptosporidian species. Sometimes a single negative sample could not rule out an active infection, since oocysts might temporarily be below the detection limit of the assays. Thus, a combination of different methods can lead to a more reliable result than a single approach.

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