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A Diagnosis of *Cryptosporidium suis* Infection of Baird's Tapir

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Abstract: Cryptosporidiosis in animals is always with the symptom diarrhea which is not easily diagnosed and even be overlooked. Currently, researchers present here a case of this disease in Baird's tapir with watery diarrhea in Shanghai wild zoo. The morphology identification, fecal antigen and PCR Method have been used in the detection. Moreover, all the testing assaies have shown coincidental result and species of *Cryptosporidium* has been ascertained as *C. suis* by sequencing. Therefore, the three examination methods may be useful tools for the diagnosis of cryptosporidiosis.

Key words: Cryptosporidiosis, *Cryptosporidium suis*, morphology identification, fecal antigen, PCR, China

INTRODUCTION

Cryptosporidium sp. are important zoonotic intestinal protozoon which can infect a wide range of vertebrate hosts and can cause diarrhoea (Xiao and Ryan, 2004; Xiao, 2010). Currently, the genus of *Cryptosporidium* sp. has been identified with extensive genetic variation such as *C. parvum*, *C. felis*, *C. canis*, *C. muis* and *C. suis* (Xiao *et al*., 2004).

Hitherto, there have been 21 species of *Cryptosporidium* sp. reported however, also >60 *Cryptosporidium* sp. genotypes without no designated species names are continually being discovered (Xiao *et al*., 2004; Plutzer and Karanis, 2009; Fayer, 2010).

The commonly diagnosing assay in cryptosporidiosis are including stool smears with acid-fast stains (Mambo *et al*., 1992; Morgan *et al*., 1998), immunological detection of antigen or antibody (Chan *et al*., 2000; Sharp *et al*., 2001), PCR Methods (Chen *et al*., 2002; El-Shazly *et al*., 2002) and electron microscopy or with various stains (Janoff and Reller, 1987) in intestinal biopsy specimens.

The aim of this study was to diagnose *Cryptosporidium* sp. infection of the Baird's tapir (*T. bairdii*) in Shanghai wild zoo using morphology, immunology and PCR Methods to evaluate the importance of *Cryptosporidium* sp. as a cause of diarrhoea in two tapirs.

MATERIALS AND METHODS

Stool samples: In the study period, 2 tapirs with diarrhoea were admitted. Stool samples were collected from each animal; a part was stored at -4°C for antigen detection immunoassay and PCR and the other fresh stool were identified by acid fast stain for routine screening of oocyst.

Morphology identification: Fecal smears were prepared from fresh samples and stained by Kinyoun acid fast stain then dried in air, fixed with 95% methanol for 5 min (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.

The detection using colloidal gold test paper of fecal antigen: For *in vitro* diagnostic use of *Cryptosporidium* sp., the fecal samples collected above has been tested by RIDA®QUICK Parasite Combi Control kit (R-Biopharm company, Germany) which specially used for detection of *Cryptosporidium* sp. infection. Firstly, the stool samples must be mixed in proportion of 1+1 with the respective extraction buffer and then the strip has been ensured that is not immersed beyond the line marked with an arrow. After an incubation of 10 min the result can be read out.

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Table 1: Sequences significant alignments

Accession	Description	Max score	Total score	Query coverage (%)	ΔE-value	Max ident (%)
GU254177.1	*Cryptosporidium suis* isolate XY-04 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GU254176.1	*Cryptosporidium suis* isolate TH-01 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GU254175.1	*Cryptosporidium suis* isolate LH-B06 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GU254174.1	*Cryptosporidium suis* isolate LH-C35 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GU254173.1	*Cryptosporidium suis* isolate LH-C08 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GU254172.1	*Cryptosporidium suis* isolate ZM-38 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GU254171.1	*Cryptosporidium suis* isolate QX-104 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99
GQ227705.1	*Cryptosporidium suis* isolate LY11 18S ribosomal *RNA* gene, partial sequence	1229	1229	100	0.0	99

ELISA testing of fecal antigen: This detection was practiced by RIDASCREEN *Cryptosporidium* sp. kit. First, the wash buffer was diluted with distilled water 1:10. Next, 50-100 mg stool samples were diluted by sample dilution buffer (diluent) 1:11. Then, place the required microwell strips in the frame and add 2 drops samples and 2 drops conjugate.

After the steps, the mixed testing samples were incubated at room temperature (20-25°C) for 60 min and then wash 5 times with 300 μL diluted wash buffer. Moreover, add 2 drops substrate and incubate at room temperature in the dark for 15 min. The last step was adding 1 drop stop reagent and carrying out a photometric measurement at 450 nm. Additionally, positive control (provided in the kit) and negative control (dilute) also were setting in the detection.

DNA extraction, PCR protocols and sequencing: Total genomic DNA was extracted from individual flukes by using SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega) and eluted into 20 μL H_2O according to the manufacturer's recommendations (Ai *et al*., 2010a, b).

A two-step nested PCR protocol for the 18s small subunit ribosomal *RNA* gene was performed as previously 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) employing the same primers used in the PCR.

Phylogeny: Sequences of the part of 18s small subunit ribosomal *RNA* genes were aligned using the computer program Clustal X 1.83 (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-(M/L) where M is the number of alignment positions at which the two sequences have a base in common and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al*., 1995). To study the phylogenetic relationships between the samples in this research and other species of Cryptosporidium which the 18s small subunit ribosomal *RNA* gene sequences were obtained from GenBank were used for phylogenetic analyses with Giardia (Gd) as the outgroup (GenBank™ accession number can be shown in Table 1 and 2).

Table 2: Samples of Cryptosporidium from Shanghai wild zoo, 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 no.
Csui1	*Cryptosporidium suis*	Not submitted
Csui2	*Cryptosporidium suis*	Not submitted
Cstb1	*Cryptosporidium suis*	GU254176
Cstb3	*Cryptosporidium suis*	GU254177
Cph	*Cryptosporidium parvum* (strain Human)	AF093492
Cpb	*Cryptosporidium parvum* (strain Bovine)	AF093490
Cser	*Cryptosporidium serpentis*	AF151376
Cbc	*Cryptosporidium baileyi*	AF093495
Ca	*Cryptosporidium andersoni*	HQ009808
Cr	*Cryptosporidium ryanae*	HQ179574
Cg	*Cryptosporidium galli*	GU816051
Gd	*Giardia intestinalis*	AY826207

Three methods, namely Neighbor Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) were used for phylogenetic re-constructions. NJ and MP analysis were carried out using PAUP 4.0 Beta 10 programme (Swofford, 2002) and ML analyses were performed using PUZZLE 4.1 (Strimmer and von Haeseler, 1996) under the default setting. 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 oocysts of *Cryptosporidium* sp. was found by Kinyoun acid-fast stain in both samples (Fig. 1). Furthermore, it was also evident that the positive results has been shown in the testing of colloidal gold test paper of fecal antigen (Fig. 2) and ELISA. PCR analysis also confirmed the detection results. No size variation was detected on agarose gel among any of the amplicons examined.

After trimming some base pairs at the beginning and end of the sequences, sequences size for part *18s* gene of the two samples were 674 bp and with the similar base composition. When blasted in NCBI, the highest similarity was 99% with *C. suis* (Fig. 3).

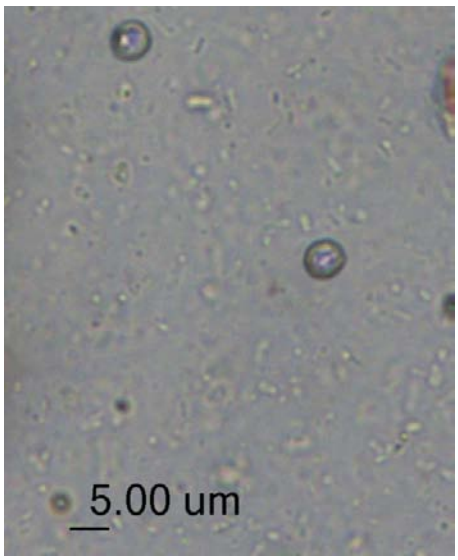

Fig. 1: Kinyoun acid-fast stain of the oocysts of *Cryptosporidium* sp.

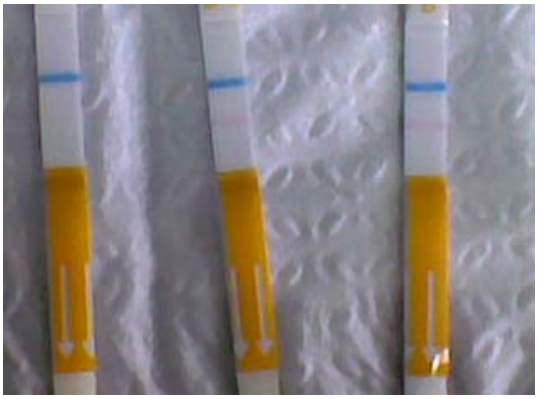

Fig. 2: Colloidal gold test paper of fecal antigen of both the samples (the middle and the right paper strips were for the testing of the samples, the left one was the negative using healthy person)

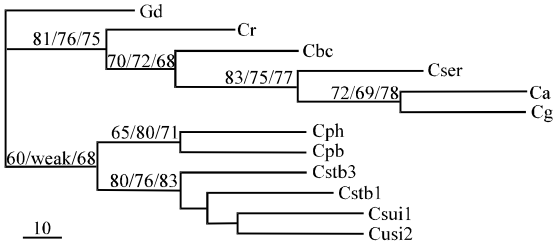

Fig. 3: The blast results in NCBI

The four methods for the diagnosis of Cryptosporidium infection indicated the same results. The morphology detection was the standard method in Cryptosporidium infection with the character of direct-viewing. However, it needs professional training and rich clinical experiences. Moreover, the rapid immunological test detects stool antigens which may persist after the ceases shedding intact organisms. Samples positive only on this test may therefore not be false-positives but recent recoveries. There were reports that some samples were positive on microscopy but negative on the ImmunoCard Cryptosporidium/Giardia Rapid Assay may be because the latter often fails to detect samples with a small number of parasites (Garcia *et al*., 2003) and/or because there is antigenic variability among species of *Cryptosporidium*. PCR also indicated positive in both samples and can reveal the genotype. On the contrary it is expensive for a small number of samples which is the situation in many parasitology laboratories.

Topologies of the 18s sequences inferred by different methods (NJ, MP and ML) with different building strategies and/or different distance models were similar (Fig. 3). The phylogenetic tree was consisted of two large clades; the first one contained *C. serpentis*, *C. andersoni*, *C. baileyi*, *C. ryanae* and *C. galli* strains within the second clade, *C. suis* including the samples in the research and the ones that have been found in NCBI were clustered together then they were clustered together with *C. parvum*, respectively. This clustering is in agreement with the results of traditional classifications.

CONCLUSION

For diagnostic analysis of samples from immunocompetent cases, microscopy test with Kinyoun stain currently seems to be the best approach in the hands of trained microscopists examining a large number of microscopic fields. Besides this method is cheap even when used for a small number of samples.

The study indicates that Cryptosporidium is a common enteric pathogen in the area and suggests that routine examination for oocyst in stools from patients and animals with diarrhoea is worthwhile.

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