Research Article

OPEN ACCESS

Detection and molecular characterisation of intestinal parasites in the South China tiger *Panthera tigris amoyensis* (Hilzheimer)

Hung-chuan Chiu^{1,2#}, Kewei Fan^{1,2#}, Xiaoshuang Sun^{1,3}, Kaixiong Lin⁴, Tengting Chen⁴, Fei Yang⁵, Yunfei Qiu⁶, Dongxia Wei^{7,*} and Cuiqin Huang^{1,2,*}

¹ Fujian Provincial Key Laboratory for the Prevention & Control of Animal Infectious Diseases & Biotechnology, College of Life Sciences, Longyan University, Longyan, Fujian Province, China;

² Engineering Research Center for the Prevention and Control of Animal Original Zoonosis, Fujian Province, College of Life Science, Longyan University, Longyan, Fujian Province, China;

³ College of Animal Sciences (College of Bee Science), Fujian Agriculture and Forestry University, Fuzhou, Fujian Province, China;

⁴ South China Tigers Breeding Institute of Fujian Meihuashan, Shanghang County, Fujian Province, China;

⁵ College of Animal Science, Tibet Agriculture & Animal Husbandry University, Nyingchi, Tibet, China;

⁶ Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture, Lanzhou, Gansu Province, China;

⁷ Jiangsu Agri-animal Husbandry Vocational College, Jiangsu Taizhou, China

#These authors contributed equally to this work.

Abstract: Parasitic infections of the South China tigers in the Meihua Mountains have not been explored previously. Faeces of 22 South China tigers from the China Tiger Park in the Meihua Mountains were examined. Eggs of ascaridoid nematodes and oocysts of coccidia were detected by Mini-FLOTAC assay. Morphological observation and molecular characterisation of the oocysts were carried out. The prevalence of *Toxascaris leonina* (von Linstow, 1902) was 18% (4/22), and the highest egg per gram (EPG) count in the faeces was 27,150. The prevalence of *Cystoisospora* sp. was 45% (10/22) and the highest oocysts per gram (OPG) in the faeces was 6,000. In addition, we found one ascaridoid nematode in the South China tiger's faeces and was molecularly and morphologically identified as *T. leonina*. The oocysts in the faeces were sporulated *in vitro* and identified as *Cystoisospora* sp. Amplification of full-length internal transcribed spacers (ITS) resulted in sequences 1,622 bp long. Using the sequences, *Cystoisospora* sp. of the South China tiger was closest to *Isospora belli* (Wenyon, 1923) and *Cystoisospora suis* (Biester, 1934).

Keywords: Mini-FLOTAC, *Toxascaris leonina*, *Cystoisospora* sp., Felidae, Meihua, Mountain, China

The South China tiger *Panthera tigris amoyensis* (Hilzheimer) is a large nocturnal mammal of the family Felidae, and it is a unique subspecies of the tiger that occurs in China. Studies on captive tigers have shown that infectious diseases threaten the breeding and restoration projects vital for survival of this critically endangered species (Liu et al. 2013).

The China Tiger Park (Longyan, Fujian, China) is the largest field domestication base in China and currently contains 35 adult and juvenile South China tigers. This place is in a suitable region for the captive breeding and release of South China tigers into the wild. However, the species composition and distribution of parasites of this mammal remain largely unknown. There are a variety of landforms in the Meihua Mountains, and it is necessary to

study whether the distribution of parasite infections and the dominant parasitic species involved differ between tigers in different regions.

In this study, parasites found in faeces of the South China tiger by Mini-FLOTAC assay and Polymerase Chain Reaction (PCR) are reported. New data presented may help in the prevention and control of the parasites of this endangered big cat in the Meihua Mountains.

MATERIALS AND METHODS

Faecal samples from 22 South China tigers (*Panthera tigris amoyensis*) were collected in July 2018, marked with the host number, age and gender (Table 1), and transported to the laboratory for analysing immediately. Nematodes were collected from the faeces and fixed in 70% ethanol and then preserved in 80% ethanol.

Address for correspondence: Cuiqin Huang, E-mail: cuiqinh@126.com

Detection of eggs of ascaridoid nematodes and oocysts of coccidia in the South China tiger by Mini-FLOTAC assay

The Mini-FLOTAC (University of Naples Federico II, Naples, Italy) procedure (Cringoli et al. 2017) was modified with the following detail: 2 g of fresh faecal samples were poured in one of the chambers for flotation in 36 ml of saturated sodium chloride solution. Then, faeces were crushed and stirred thoroughly with the collector rod. The faecal suspension in Fill-FLOTAC was used to fill two flotation chambers, a and b, through the sample filtration channel. After 10 minutes, the translation disc was turned clockwise, into the position for microscopic examination. The number of oocysts or eggs in the two flotation chambers of the Mini-FLOTAC was counted and oocysts per gram (OPG), which was the sum of the oocysts eggs in the two flotation chambers, was multiplied by a factor of 10 to obtain an estimate of the oocysts eggs per gram of faeces.

Separation, purification and sporulation of oocysts of coccidia from faecal samples

The faecal samples were mixed with appropriate proportion of ddH₂O and passed through a size 80 mesh followed by size 200 mesh sieves. The mixtures were then centrifuged at 370 g for 1 min and supernatants discarded. Pellets were washed twice with ddH₂O followed by resuspension in the saturated sodium chloride solution and centrifuged at 570 g for 2 min. Supernatants were added to ddH₂O (supernatants: ddH₂O = 1 : 9) and centrifuged at 830 g for 3 min. After discarding the resulting supernatants, the pellets contained purified oocysts. The oocysts were sporulated in an aqueous solution of potassium dichromate 2.5 % (W/V) at 28 °C for 48 h, and sporulation was observed.

Morphology evaluation. Morphology of eggs of ascaridoid nematodes and sporulated oocysts of coccidia in faeces

Ascarid eggs or sporulated oocysts were examined using light microscope under magnifications × 400 light microscopy. Shapes and internal structures of ascarid eggs were observed and dimensions measured. Nematodes were identified using morphological characteristics provided by Alexander et al. (2018) and Hadi (2019).

The length and width of the sporulated oocysts were also measured. Oocysts were identified on the basis of the two sporocysts and four sporozoites as well as a large round residual body (Lindsay et al. 1997, Madani et al. 2018, Genovez-Oliveria et al. 2019).

DNA isolation

A small portion (1–2 cm) of adult nematode tail was cut using sterile microscissors and placed in a 1.5-ml sterilised microcentrifuge tube after rinsing three times with ddH₂O. The samples were thoroughly mixed with 30 µl of proteinase K (50 ng/ml) and 270 µl of tissue lysis (TL) buffer, placed in a 55 °C incubator for 15–18 h, and shaken once every 2 h. After complete digestion, DNA was extracted using E.Z.N.A.® tissue DNA extraction kit (Omega Bio-tek, Norcross, GA, USA), and the extracted DNA samples were stored at -20 °C.

Lysis buffer was added to the purified sporulated oocysts, mixed thoroughly, and 100 µl of the mixture added to separate microcentrifuge tubes. The tubes containing the sporulated oocysts were placed in a 100 °C water bath for 5 minutes and then placed at -80 °C for 5 minutes. After repeated freezing and thaw-

Table 1. Detection of eggs of ascaridoid nematodes and oocysts of coccidia in the South China tigers.

Case	Sex	Age	Egg (<i>Toxascaris leonina</i>)	EPG value	Oocyst (<i>Cystoisospora</i> sp.)	OPG value
1	M ^a	1Y9M	- ^c		+	300
2	M	1Y9M	-		+	150
3	M	7Y8M	-		-	
4	M	1Y9M	-		-	
5	M	1Y9M	-		-	
6	M	8Y6M	-		+	150
7	F ^b	13Y6M	-		+	150
8	F	9Y6M	-		-	
9	F	7Y5M	-		-	
10	M	1Y9M	-		+	6,000
11	M	2Y11M	+ ^d	27,150	-	
12	F	4M	-		-	
13	F	14Y10M	-		-	
14	M	7Y8M	-		+	150
15	M	1Y5M	+	7,550	-	
16	M	1Y5M	+	150	-	
17	F	1Y5M	-		+	150
18	M	1Y5M	-		-	
19	M	1Y5M	-		+	450
20	M	1Y9M	-		+	750
21	F	2Y6M	+	8,264	-	
22	M	2Y11M	-		+	750

^a M – male; ^b F – female; ^c – negative; ^d – positive

ing five times, the digestion continued with magnetic beads, and the DNA of oocysts was extracted by EZNA tissue DNA extraction kit (Omega Bio-tek.). The samples were stored at -20 °C until further use.

Identification of the ascaridoid nematode and oocysts of coccidia by PCR

Primers for internal transcribed spacers (ITS) conserved sequences of *Toxascaris leonina* (Linstow, 1902) were designed according to a previous report (Zhu et al. 2002). PCR was performed with 50 ng of template DNA, 2 µl of 2.5 mM dNTP (Takara Bio, Mountain View, CA, USA), 10 µM forward primer: NC5: 5'-GTAGGTGAACCTGCGGAAGGATCATT-3', 10 µM reverse primer: NC2: 5'-TTAGTTTCTTTTCTCCGCT-3', 0.5 µl of rTaq DNA polymerase (Takara Bio), 5 µl of 10 × Taq buffer and adjusted with ddH₂O to a final volume of 50 µl. PCR conditions were as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min 30 s, followed by a final 10 min extension at 72 °C.

Based on the ITS conserved sequences of species of *Cystoisospora* Frenkel, 1977, we designed forward and reverse primers using Primer Premier software (PREMIER Biosoft International, San Francisco, CA, USA). PCR was performed as described above except using 10 µM forward primer: CITS-F: 5'-GAGGGACTTTGCGTGCTA-3' and 10 µM reverse primer: CITS-R: 5'-TCCTCCGCTTAATAATATGCT-3'. PCR conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min 30 s, followed by a final 10 min extension at 72 °C. PCR products were examined using 1% agarose gel electrophoresis, gel extracted and purified, and blunt-end ligated into the pEASY-Blunt vector (Thermo Fisher Scientific Inc., Waltham, MA, USA). Thereafter, the recombinant plasmids were transformed into *Escherichia coli* Top10 competent cells. The transformed *E. coli* cells were

Fig. 1. Egg of *Toxascaris leonina* (Linstow, 1902) (A) and oocyst of *Cystoisospora* sp. (B). The oval egg measured 78 µm (L1) × 73 µm (L2) (A). The sporulated oocyst of *Cystoisospora* sp. contained two sporocysts and each sporocyst contained four sporozoites. The size of sporulated oocyst was about 39 µm (L3) × 40 µm (L4).

selected with Luria-Bertani (LB) plate with 100 µg/ml of ampicillin at 37 °C. The candidates were checked by PCR and DNA sequencing.

Sequence and genetic evolution analysis

PCR products were analysed using DNASTAR software (DNASTAR Inc., Madison, WI, USA) and compared to sequences published in GenBank. Additionally, DNA sequences were aligned using ClustalW in Mega 7.0 (MEGA Inc., University Park, PA, USA). A phylogenetic tree was constructed using the maximum parsimony method.

RESULTS

The prevalence of intestinal parasites

The intestinal tracts of the South China tigers were mainly infected with ascaridoid nematodes and coccidia. The prevalence of *Toxascaris leonina* was 18% and the highest EPG count of 27,150. The prevalence of *Cystoisospora* sp. was 45% and the highest OPG value 6,000. No tigers were co-infected with both *T. leonina* and *Cystoisospora* sp. (Table 1).

Morphology of eggs of ascaridoid nematodes and oocysts of coccidia

The eggs detected in the faeces were microscopically identified as *T. leonina*. They were elliptical with a thick

shell wall; the outer wall surface was smooth and the interior contained an embryo. The inner surface of the egg wall was rough or wavy because of the presence of yolk membrane (Fig. 1A).

Morphology of oocysts of coccidia

Oocysts contained two sporocysts and each sporocyst contained four sporozoites and a large round residual body. The oocysts were preliminarily identified as *Cystoisospora* sp. (Fig. 1B).

Morphology of the ascaridoid nematode

The nematode expelled from the host was 37 mm-long and has a thick body (Fig. 2A). The morphological features included three developed triradiate lips (Fig. 2B) surrounding the mouth. The caudal end of this male parasite possessed spicules (Fig. 2C) and the tail tip of this male parasite lacked the finger-like appendage typical of *Toxocara canis* (Werner, 1782). Based on the observation of these morphological characteristics, we concluded it was *T. leonina*.

PCR-based identification

DNA from ascaridoid nematode was extracted using E.Z.N.A.® tissue DNA extraction kit as described in materials and methods. The PCR products of were examined with gel electrophoresis and sequenced, and showed that ITS fragments of ascaridoid nematode were 953 bp. Comparison of this ascaridoid nematode ITS sequence with *T. leonina* showed 100% similarity.

Sporulated oocysts of coccidia was lysed with lysis buffer following by repeated freezing and thawing five times, and oocysts DNA was extracted using E.Z.N.A.® tissue DNA extraction kit as described in materials and methods. PCR products from oocyst DNA were sequenced and their size was 1,622 bp. Using NCBI blast, *Isospora* sp. of the South China tiger showed similarity of 91.66% to *Isospora belli* (Wenyon, 1923) (DQ060659.2), 88.55% to *Isospora suis* (Biester et Murray, 1934) (KR139985.1), and 86.78% to *Cystoisospora ohioensis* (Dubey, 1975) (GU292307.1).

Fig. 2. Male of *Toxascaris leonina* (Linstow, 1902) from *Panthera tigris amoyensis* (Hilzheimer). A – total view; B – the anterior end with triradiate lips (arrow) (10×); C – posterior end with spicules (arrow) (10×).

Fig. 3. Phylogenetic tree of coccidia. ITS sequences were aligned using Clustal X (version 2.1). MEGA (7.0) was used to construct phylogenetic tree by maximum parsimony method. Bootstrap percentages from 1000 replicates were shown for the clades that were supported in analysis.

Construction of the phylogenetic tree based on ITS sequences of coccidia

Comparative analysis of ITS sequences has revealed the genera *Eimeria* Schneider, 1875 and *Cystoisospora* Frenkel, 1977 formed a separate branch. Two coccidia found in the South China tiger named *Cystoisospora* sp. clone 1 and clone 2, belonged to the genus *Cystoisospora* and were most closely matched to *C. suis* and *I. belli* (Fig. 3).

DISCUSSION

This study identified two intestinal parasites of the South China tiger in the Meihua Mountains, namely the nematode *Toxascaris leonina* and coccidium *Cystoisospora* sp. The ITS sequences of the nematode found in the tiger faeces were 100% similar to reported sequences from *T. leonina*. Toxascariasis can cause malnutrition, anemia and stunted growth in animals (Dong et al. 2019). This parasite may pose a threat to South China tiger breeding and restoration efforts. Previous reports on parasitic infections of felines from Heilong Jiang Amur Tiger Park reported *T. leonina* and *Toxocara cati* (Schrunk, 1788) in the faeces (Liu et al. 2007).

In 1988, a faecal sample from a 4-month-old female Siberian tiger, held in captivity at a private zoo in Limburg, was found to be positive for oocysts of $12.4 \mu\text{m} \times 10.5 \mu\text{m}$, which were identified as *Toxoplasma gondii* (Nicolle et Manceaux, 1908) after sporulation and inoculation into mice. This was the only report thus far to describe a tiger infected by *T. gondii* (Dorny and Franssen 1989).

Our study in the Meihua Mountains is the first to identify *Cystoisospora* sp. in the South China tigers. This unidentified coccidium appeared closely related to *Isospora belli* and *Isospora suis* (Fig. 3).

Faecal egg count (FEC) techniques such as FLOTAC (Cringoli 2006, Cringoli et al. 2010, 2017, Catalano et al. 2019) and McMaster (Paras et al. 2018) have been used to diagnose human soil-transmitted helminth and parasites (Levecke et al. 2012). Recently, Barda et al. (2013) compared Mini-FLOTAC method with other techniques that were previously used for diagnosis of intestinal parasitic infections and found it was a sensitive and potentially low-cost alternative technique that could be used in resource-limited settings. In the present study, the Mini-FLOTAC method was used for the first time for detecting eggs and oocysts of intestinal parasites from the South China tigers.

In view of intestinal parasites infecting the South China tigers, we can take some prevention and control measures. For *T. leonina*, the main sources of infection are parasitic eggs in the soils. Therefore, it is necessary to clean faeces and disinfect the environment frequently. In addition, infected tigers should be treated with albendazole, fenbendazole and ivermectin regularly.

In the case of *Cystoisospora* sp., the living environments of tigers should be kept dry, and anti-coccidial drugs, such as, amprolium should be added to drinking water for South China tigers.

Acknowledgments. This work was supported by a project of Natural Science Foundation of Fujian Science and Technology Department (grant No. 2020J01371), Educational Research Project for Young and Middle-Aged Teachers in Fujian Province (grant No. JAT190737), Special Fund for Local Science and Technology Development Guided by the Chinese Government (grant No. 2018L3011), and the major project of Longyan city (grant No. 2018LYF1003).

REFERENCES

- ALEXANDER U., LIM C.W., KIM B., HONG E.J., KIM H.C., PARK B.K. 2018: Morphological and molecular characterization of *Toxocara tanuki* (Nematoda: Ascaridae) from Korean raccoon dog, *Nyctereutes procyonoides koreensis*. Kor. J. Parasitol. 56: 567–575.
- BARDA B.D., RINALDI L., IANNIELLO D., ZEPHERINE H., SALVO F., SADUTSHANG T., CRINGOLI G., CLEMENTI M., ALBONICO M. 2013: Mini-FLOTAC, an innovative direct diagnostic technique for intestinal parasitic infections: experience from the field. PLoS Negl. Trop. Dis. 7: e2344.
- CATALANO S., SYMEOU A., MARSH K.J., BORLASE A., LÉGER E., FALL C.B., SÈNE M., DIOUF N.D., IANNIELLO D., CRINGOLI G., RINALDI L., BÂ K., WEBSTER J.P. 2019: Mini-FLOTAC as an alternative, non-invasive diagnostic tool for *Schistosoma mansoni* and other trematode infections in wildlife reservoirs. Parasit. Vectors 12: 439.
- CRINGOLI G. 2006: FLOTAC, a novel apparatus for a multivalent faecal egg count technique. Parassitologia 48: 381–384.
- CRINGOLI G., MAURELLI M.P., LEVECKE B., BOSCO A., VERCURYSSE J., UTZINGER J., RINALDI L. 2017: The Mini-FLOTAC technique for the diagnosis of helminth and protozoan infections in human and animals. Nat. Protocols 12: 1723–1732.
- CRINGOLI G., RINALDI L., MAURELLI M.P., UTZINGER J. 2010: FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat. Protocols 5: 503–515.
- DONG Y.H., ZOU Z.Y., HU P.J., DONG B., WANG Z.H., YANG Z.G., WEN BO., MA Y.H., SONG Y., MA J., LAU P. 2019: Secular trends of ascariasis infestation and nutritional status in Chinese children from 2000 to 2014: evidence from 4 successive national surveys. Open Forum Infect. Dis. 6: ofz193.
- DORNY P., FRANSEN J. 1989: Toxoplasmosis in a Siberian tiger (*Panthera tigris altaica*). Vet. Rec. 125: 647.
- GENOVEZ-OLIVEIRA J.L., CARDOZO S. V., OLIVEIRA A.A.D., LIMA V.M.D., FERREIRA I., BERTO B. P. 2019: Morphological and molecular Identification of *Isospora sepetibensis* (Chromista: Miozoa: Eimeriidae) from a New Host, *Trichothraupis melanops* (Passeriformes: Thraupidae: Tachyphoninae) in South America. Acta Protozool. 58: 17–23.
- HADI A. 2019: Morphology study of *Toxascaris canis* (Werner, 1788) worms by scanning microscope. Int. J. Biosci. 14: 293–298.
- LEVECKE B., RINALDI L., CHARLIER J., MAURELLI M.P., BOSCO A., VERCURYSSE J., CRINGOLI G. 2012: The bias, accuracy and precision of faecal egg count reduction test results in the cattle using McMaster, Cornell-Winsconsin and FLOTAC egg counting methods. Vet. Parasitol. 188: 194–199.
- LINDSAY D.S., DUBEY J.P., BLAGBURN B.L. 1997: Biology of *Isospora* spp. from humans, nonhuman primates, and domestic animals. Clin. Microbiol. Rev. 10: 19–34.
- LIU H., YAN Q., ZHAO B., LUO J., WANG C., DU Y., YAN J., HE H. 2013: Isolation, molecular characterization, and phylogenetic analysis of encephalomyocarditis virus from South China tigers in China. Infect. Genet. Evol. 19: 240–243.
- LIU J.L., HOU Z.J., HUA Y.P., LIU D., WANG W. 2007: [Survey of endoparasites infection in feline animals in the Heilong Jiang Amur Tiger Park, China.] Chin. J. Wildlife 28: 26–28. (In Chinese.)
- MADANI S.A., ARABKHAZAEI F., SHAKERI E., NABIAN S. 2018: Molecular and morphological description of *Isospora* sp. from the common mynah (*Acridotheres tristis*) and a preliminary survey of two anticoccidial drugs in natural infection. Avian Pathol. 47:206-212.
- PARAS K.L., GEORGE M.M., VIDYASHANKAR A.N., KAPLAN R.M. 2018: Comparison of fecal egg counting methods in four livestock species. Vet. Parasitol. 257: 21–27.
- ZHU X.Q., D'AMELIO S., PALM H.W., PAGGI L., GEORGE-NASCIMENTO M., GASSER R.B. 2002: SSCP-based identification of members within the *Pseudoterranova decipiens* complex (Nematoda: Ascaridoidea: Anisakidae) using genetic markers in the internal transcribed spacers of ribosomal DNA. Parasitology 124: 615–623.

Received 18 December 2020

Accepted 24 September 2021

Published online 31 December 2021

Cite this article as: Chiu H-C., Fan K., Sun X., Lin K., Chen T., Yang E., Qiu Y., Wei D., Huang C. 2021: Detection and molecular characterisation of intestinal parasites in the South China tiger (*Panthera tigris amoyensis*). Folia Parasitol. 68: 029.