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Evidence of red panda as an intermediate host of *Toxoplasma gondii* and *Sarcocystis* species

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ABSTRACT

Toxoplasma gondii has been found to infect almost all warm-blooded animals; however, some hosts lack direct evidence of *T. gondii* infection. The red panda (*Ailurus fulgens*) is an endangered species that mainly lives in temperate forests of South Asia. Here, *T. gondii* infection in red pandas from zoos in China were reported. Antibodies to *T. gondii* were found in 14.3% (2/14) of red pandas via the modified agglutination test (MAT) with a cut-off titer of 1:25. One viable *T. gondii* strain was isolated from tissues of red panda and designated as TgRedpandaCHn1. DNA from tachyzoites obtained from cell culture was characterized by PCR-RFLP with 10 markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29–2, L358, PK1, and Apico) and virulence genes of ROP5 and ROP18. The results indicate that this isolate belonged to ToxoDB genotype #20. The ROP18/ROP5 genotype combination predicated that this strain is non-lethal to mice, which is supported by the infection in mice. *T. gondii* tissue cysts were readily formed and mice survived. Tissue cysts observed in the histopathological sections of the tongue and diaphragm of one red panda were speculated as sarcocysts, but not *T. gondii* base on morphological characteristics. To our knowledge, this study is the first to report on the isolation of *T. gondii* from red panda. Additionally, this report provides direct evidence of red panda as an intermediate host of *T. gondii* and *Sarcocystis* species.

1. Introduction

Toxoplasma gondii has been found to infect almost all warm-blooded animals and humans (Dubey, 2010). Felids are the only known definitive hosts in which *T. gondii* can complete their full sexual life cycle, while birds and small mammals are usually intermediate hosts. Given the wide range species of warm-blooded animals, some hosts lack direct evidence of *T. gondii* infection. The red panda (*Ailurus fulgens*) is an endangered species that primarily lives in temperate forests of China, Bhutan, India, Myanmar, and Nepal (Hunter, 1991). To date, only one study reported the presence of antibodies to *T. gondii in* red pandas (Qin et al., 2007), whereas other reports found no evidence of *T. gondii* infection in red panda by serology, molecular or histology methods (Langan et al., 2000; Zoll et al., 2015; Loeffler et al., 2007). There is no direct evidence to confirm that they can serve as an intermediate host of *T. gondii*.

Sarcocystis spp. is obligate two-host life cycle parasite, with herbivores or omnivores serving as the intermediate host and carnivores as the definitive host. These species undergo multiple development stages within different host cells and can found incidentally in the tissues of mammals, birds and reptiles (Dubey et al., 2016). However, there is only one report documented schizonts of a *Sarcocystis*-like organism infection in red panda (Zoll et al., 2015).

In this study, eight red pandas developed pneumonia and subsequently died, after which their carcasses were submitted for post-mortem investigation. *T. gondii* were identified based on serological examination, bioassay in mice and molecular genotyping. The tissue cysts of *Sarcocystis* species were identified based on morphological characteristics. To our knowledge, this is the first demonstration of *T. gondii* infection in red panda.

2. Materials and methods

2.1. Naturally infected red pandas and sampling

From May to July of 2017, eight dead red pandas (3–6 years old)

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and six fecal samples from healthy red pandas (2–6 years old) were collected from zoos in Zhengzhou city, Shangqiu city, Henan province. Henan province is located in central China (33°N, 113.30°E) and has a humid and subtropical climate. One week prior to their demise, the red pandas had dyspnea, pyrexia, or both. Treatments included florfenicol and trimethoprim sulfa, which alleviated their clinical symptoms. However, they ultimately died because it was difficult to give treatment. The bodies were then submitted to the Laboratory of Veterinary Pathology of Henan Agricultural University (Zhengzhou, Henan Province, China) for pathological diagnosis, which also allowed us to survey for parasitic infection.

2.2. Histopathology

Red panda tissues samples (myocardium, liver, spleen, lung, kidney, leg muscle, tongue, and diaphragm) were fixed in 10% (v/v) neutral buffered formalin. They were processed using routine histological processing techniques, and then embedded in paraffin. Paraffin sections (5 μm thick) of the samples were then prepared and stained with hematoxylin and eosin (H&E). Based on observation of cysts in the H&E sections, the serial paraffin sections were stained with immunohistochemistry (IHC). The primary antibodies were rabbit anti-*T. gondii* polyclonal antibody and rabbit anti-*Neospora caninum* polyclonal antibody. Brain sections of a VEG *T. gondii*-infected mouse (kindly provided by Dr. Dubey, ARS, USDA) and a NC-1 *N. caninum*-infected mouse (kindly provided by Dr. Jing Liu, China Agriculture University, China) were used as positive controls. Anti-rabbit IgG conjugated with HRP/DAB as the second antibodies (IHC detection kit, Abcam, ab64264).

2.3. Serological examination by MAT

Eight serum samples and six fecal fluid samples from captive red pandas were serologically assessed for antibodies against *T. gondii* using modified agglutination test (MAT) (Dubey and Desmonts, 1987). Whole formalin fixed *T. gondii* antigens were obtained from the University of Tennessee Research Foundation (Knoxville, TN, USA). A titer of 1:25 was considered indicative of exposure to *T. gondii*. Additionally, a titer of 1:200 was used to test the antibodies against *T. gondii*. Control, negative and positive was performed on the same plate.

2.4. Isolation of viable T. gondii from red panda tissues by bioassay in mice

Fifty gram tissue samples (heart, tongue, diaphragm, and leg muscle) of eight red pandas were bioassayed in mice respectively. Tissues from each red panda were pooled, homogenized and digested in pepsin. The homogenates were then inoculated into BALB/c mice (n = 5) and/or gamma interferon (γ-IFN) knockout mice (n = 2) subcutaneously as previously described (Dubey, 2010). Tissues (lung, brain or mesenteric lymph nodes) smears of dead mice were examined for *T. gondii* tachyzoites or cysts individually. Survivors were bled on day 60 post-inoculation (DPI) and a 1:25 and 1:200 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. If tissue cysts or tachyzoites were not found in mouse tissues, homogenized lung, brain and heart tissues were subpassaged into new groups of mice subcutaneously.

2.5. DNA isolation and polymerase chain reaction (PCR) identification of T. gondii, N. caninum and Sarcocystis neurona

The DNA was extracted from digestion striated muscle using a commercial DNA extraction kit (Tiangen Biotec Company, DP304). The DNA isolated from *T. gondii* (CT1 strain) or *N. caninum* (NC1 strain) was used as a reference for PCR, they were kindly provided by Dr. JP Dubey (ARS, USDA) and Dr. Jing Liu (China Agricultural University, China). PCR assays for *T. gondii*, *N. caninum* and *S. neurona* were performed using the specific primer pairs TOX5/TOX8, NP6/NP21 and JNB33//JNB54, the products were expected to be 450 bp, 328 bp, and 1100 bp, respectively (Schares et al., 2008; Yamage et al., 1996; Dubey et al., 2001).

2.6. In vitro cultivation and genotyping

Brain homogenates of *T. gondii* positive mice were seeded on to CV-1 cell culture flasks as previously described (Dubey, 2010). DNA was extracted from cell culture derived tachyzoites. Multiplex PCR of the *T. gondii* isolates was performed using 10 PCR-RFLP genetic markers, SAG1, SAG2 (5′-3′ SAG2, alt.SAG2), SAG3, BTUB, GRA6, c22-8, c29–2, L358, PK1, and Apico as previously described by Su et al. (2010). The virulence proteins of ROP5 and ROP18 were also measured as previously reported (Rego et al., 2017; Cheng et al., 2017). References *T. gondii* DNA were included in all batches (Su et al., 2010).

2.7. Evaluation the virulence of T. gondii tachyzoites isolated from red panda by mice

Virulence of *T. gondii* isolated from the red panda was evaluated in BALB/C mice. *T. gondii* tachyzoites were counted in a disposable hemocytometer, then diluted 10-fold from 10^{-1} to10^{-6} to reach an endpoint of < 1 tachyzoite. Next, < 1, 10^0, 10^1, 10^2, 10^3, and 10^4 tachyzoites were intraperitoneally inoculated into five BALB/C mice for each dilution. The clinical symptoms were recorded daily. At 60 DPI, all surviving mice were bled and tested for IgG antibodies to *T. gondii* by MAT using titers between 1:25 and 1:200. The mice were then euthanasia at 61 DPI, after which their brains were examined and enumerated for tissue cysts based on a squash preparation (Dubey et al., 2012). The mice were considered infected when antibodies of *T. gondii* or parasites were detected in their sera or tissues.

2.8. Ethics

This study was approved by the Institutional Animal Use Protocol Committee of the Henan Agricultural University, China. The Beijing Association for Science and Technology (Approval SYXK [Beijing] 2007–0023) approved the protocol used in this study. All mice were handled in strict accordance with the good animal practices of the Animal Ethics Procedures and Guidelines of the People's Republic of China.

2.9. Statistical analysis

Statistical analysis was conducted using the Graph Pad Prism 4.0 software (Graph Pad Software Inc., San Diego, CA, USA). Data were analyzed using the chi-squared test or Fisher's exact test. A $p < 0.05$ was considered significant.

3. Results

3.1. Pathological findings

The degree of fat storage and muscle development were poor for all eight red pandas. Suppurative bronchopneumonia (8/8), bacteremia (2/8), liver fatty change (4/8), and spleen necrosis and hyperemia (5/8) were observed. Pathological diagnosis showed that pneumonia was the major cause of death in all of the red pandas.

No cysts were found in tissue sections except for red panda case 8. Paraffin blocks were deposited in the Animal Pathology Museum of Henan Agricultural University (accession numbers: Path#2513). Light microscopy revealed the presence of fusiform or oval cysts in the leg muscles and diaphragm (Fig. 1 A, B and C). The cysts loads were 5.2 ± 2.1 per square centimeter in leg muscles, and 1.4 ± 1.3 per square centimeter in the diaphragm. The size of the cysts was within the

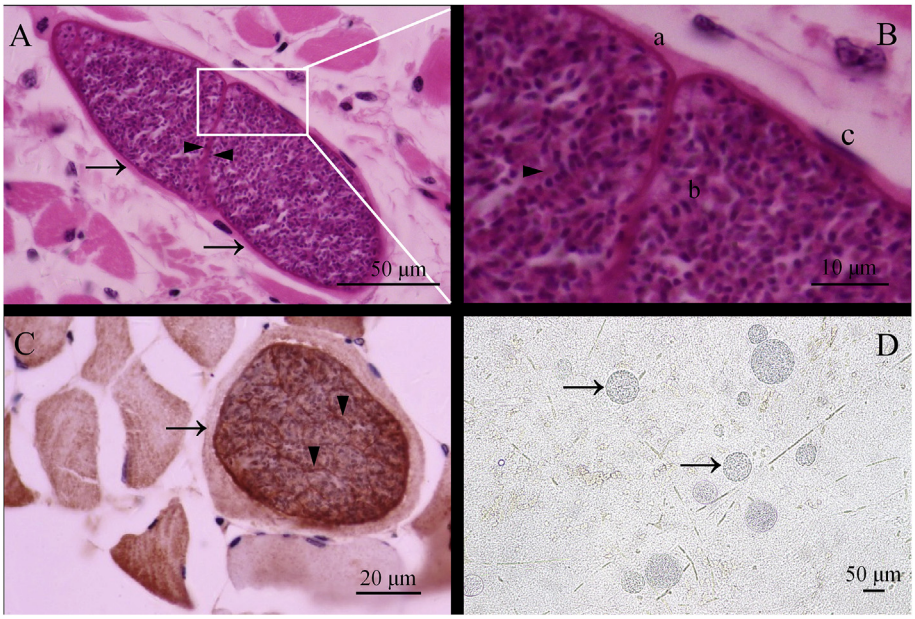

Fig. 1. Sarcocysts and *Toxoplasma gondii* cysts in red panda or mice. A: Tissue cysts, leg muscle, red panda. Two oval cysts (arrow) were observed in the skeletal muscle cell. The walls of the two cysts (arrowhead) were deeply stained by eosin. Red panda, H&E. B: Partial magnification of figure A. a, The wall of the cyst was clearly and deeply stained by eosin. b, The bradyzoites were like cresent or banana, they were arranged in packets (arrowhead). c, Necleus of host cell. C: Tissue cyst (arrow) was cross reacted with *T. gondii*, the cysts were separated by septa and formed many compartments (arrowhead), leg muscle. *T. gondii* antibody, red panda, IHC. D: Tissue cysts of *T. gondii* in brain of mouse (arrows), brain squash, unstained, 75DPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

range of 20.96 ± 6.47 μm × 17.43 ± 4.67 μm. The cyst walls were clearly and deeply stained by eosin, and had a thickness of less than 1 μm (Fig. 1 B). Tissue cysts were cross reactive to *T. gondii* antibody (Fig. 1 C). The cysts were separated by septa and formed many compartments (Fig. 1 C), while bradyzoites were arranged in packets (Fig. 1 B).

3.2. Serological examination by MAT and PCR

Antibody to *T. gondii* was found in 14.3% (2/14, 1 of 8 sera and 1 of 6 fecal fluids) of red pandas with titers of 1:200. Tissue digestive lysates were analyzed for the DNA of *T. gondii*, *N. caninum* and *S. neurona* by PCR; however, the results were all negative.

3.3. Isolation of viable T. gondii from red panda muscles by bioassay in mice

The striated muscle homogenates from the eight red pandas were bioassayed in mice individually. One group of mice inoculated with red panda case 3 tissues sample had antibodies to *T. gondii*. After continuous passage, mice showed 100% infection with *T. gondii* successfully. Furthermore, cysts were found in their brain (Fig. 1 D) and identified as *T. gondii* based on IHC and molecular characteristics. The average survival time of *T. gondii* infected γ-IFN knockout mice was 17.0 ± 1.0 DPI. This isolate from mouse brain was successfully propagated in cell culture (20 DPI) and designated as TgRedpandaCHn1. Its genetic typing revealed that it was ToxoDB genotype #20 based on ten genetic makers. In addition, virulence gene analysis showed that it was 3/4 for ROP18/ROP5.

3.4. Virulence evaluation of TgRedpandaCHn1

After inoculation of BALB/C mice with gradient concentration of TgRedpandaCHn1 tachyzoites, the mice were asymptomatic, and no deaths were observed within 60 DPI. Inoculation with $> 10^2$ tachyzoites induced *T. gondii* infection in all of the mice (Table 1). *T. gondii* tissue cysts from the brain were detected in these mice when euthanasia at 61 DPI. As shown in Table 1, when the brain cyst number in mice inoculated with 10^2 tachyzoites (20.0 ± 63.3) and 10^3 tachyzoites (4.0 ± 12.7) compared with those inoculated 10^4 tachyzoites (198.0 ± 244.1), the mice inoculated with the highest level showed a significant increase in cyst load ($P < 0.05$).

Table 1
Virulence of *T. gondii* strain TgRedpandaCHn1 in BALB/C mice.

No. of counted tachyzoites	No. infected of 5 mice inoculated	*T. gondii* IgG titer by MAT[b]	Tissue cysts in brain
10^4	5	≥1:200/5	198.0 ± 244.1
10^3	5	≥1:200/5	4.0 ± 12.7[a]
10^2	5	≥1:200/5	20.0 ± 63.3[a]
10^1	3	≥1:200/3, < 1:25/2	30.0 ± 67.5
1	2	≥1:200/2, < 1:25/3	Not found
< 1	0	< 1:25/5	Not found
Blank control	0	< 1:25/5	Not found

[a] $p < 0.05$, 100 vs 10,000; 1000 vs 10,000.
[b] *T. gondii* IgG titer/No. of mice.

4. Discussion

In the current study, we found 14.3% (2/14, 1 of 8 sera and 1 of 6 fecal fluids) of red pandas were IgG positive to *T. gondii*, indicating prior exposure to this parasites. Detection of IgG from fecal sample is of interest given the easier access of the material. However, the sensitivity and specificity of immune globulin assays from fecal fluid need further verify.

Viable *T. gondii* parasites were successfully isolated from tissues of one red panda (case 3) in this study. Genotyping of the *T. gondii* isolate revealed it was ToxoDB#20. This genotype has been found in stray or feral animals from North Africa, East Africa, the Middle East and South Asia (Dubey et al., 2007; Dubey et al., 2010; Dubey et al., 2013; Tian et al., 2014; Al-Kappany et al., 2010; EI Behairy et al., 2013; Chaichan et al., 2017). According the virulence evaluation for ToxoDB#20 strains, most isolates (n = 9) were not pathogenic to mice (Dubey et al., 2007). The ROP18/ROP5 genotype combination suggests that this strain is non-lethal to mice (Shwab et al., 2016). Here, our test in mice supported the prediction. Further comparison of genotypes indicated that ToxoDB#20 differs from *Chinese 1* (ToxoDB#9) at locus PK1 of the ten PCR-RFLP markers, suggesting they probably belong to the same ancestral clade with Type II lineage.

The origin of *T. gondii* in red pandas from zoos was predicted. The diet of red pandas from zoos is primarily herbivorous, including bamboo, formulated biscuits or other dietary supplements; they also will eat rodents, birds and insects when available. Accordingly, red

pandas may have ingested oocysts shed by stray cats or captive felids, or eaten infected birds or rodents. Stray cats are ubiquitous in Chinese cities and generally have easy access to zoos. The seroprevalence of *T. gondii* in stray cats is about 50%, and can be as high as 100% in adult felids in zoos (Yang et al., 2015, 2017). In addition, animal breeders in zoos also showed at high risk of *T. gondii* infection (Xie et al., 2010). High prevalence of *T. gondii* in felines indicated that high number of oocyst shedding and widespread of oocyst in the zoo.

In this study, *T. gondii* DNA was not detected in the tissue digests from all of red pandas which indicated a low level of infection of *T. gondii* in case 3. Further investigation is needed to determine if the efficiency of DNA extraction procedure is low or parasite tissue burden is low in red pandas.

There was no *N. caninum* DNA detected in any tissue digests from red pandas, nor was *S. neurona*. However, tissue cysts were found in skeletal muscles of red panda case 8. Although the cysts were partially cross reacted with *T. gondii* antibody, they were separated by septa and formed many compartments, which may indicated that they were sarcocysts, but not *T. gondii*. The closely related cystforming members of the subphylum apicomplexa, such as *T. gondii*, *N. caninum* and *Sarcocystis* spp., may cross-react to antibodies generated to one of these species (Gondim et al., 2017; Baszler et al., 1996; Sundermann et al., 1997). *S. hirsuta* could be recognized by anti-*T. gondii* antibody (Baszler et al., 1996). However, there is no report about which specie of *Sarcocystis* spp. from red panda cross-reactive *T. gondii* antibody. No inflammation was found around the sarcocyst-like cysts, indicating that the infection was subclinical. To date, only one study of sarcocystosis outbreak in red panda cubs has been document (Zoll et al., 2015), it maybe attribute to *S. neurona* or *S. dasypi*.

In this study, red panda was found to be an intermediate host of *T. gondii* and *Sarcocystis* species. The clinical significance of these parasites and its biological characteristics in wild red pandas need further investigation.

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Authors'contributions

YRY designed the study protocol, performed the laboratory tests, analyzed the results and wrote the manuscript. HD performed PCR-RFLP analysis and performed the laboratory tests. RJS, TYL and NJ participated in laboratory testing and collecting the samples. CLS helped in analyze genotypes and writing this manuscript. LXZ helped in the writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.02.006.

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