Additional evidence of tigers (Panthera tigris altaica) as intermediate hosts for Toxoplasma gondii through the isolation of viable strains

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A B S T R A C T

Toxoplasmosis is one of the most common zoonotic diseases in the world. Felines excrete Toxoplasma gondii oocysts, which play a key role in the transmission of this protozoon. Pathological diagnoses were performed on four carcasses of captive tigers collected from 2019 to 2021 in China, and T. gondii was surveyed using serology, molecular analysis, and aetiology. Striated muscle samples of the tigers (n = 4) were bioassayed in mice. DNA derived from T. gondii tachyzoites was isolated and characterized using PCR-RFLP. The pathological diagnoses revealed that ageing, declined immune function, liver, and kidney failures caused the deaths in the tigers examined. A modified agglutination test (cut-off: 1:25) revealed that IgG antibodies to T. gondii were 100% (4/4) in the captive tigers. Two viable T. gondii strains (TgTigerCHn3 and TgTigerCHn4) were isolated from tiger striated muscles and seeded on the Vero cell culture for further propagation. The genotypes of TgTigerCHn3 and TgTigerCHn4 were ToxoDB#20 and ToxoDB#2, respectively. The two strains were avirulent for Swiss mice, which matched the ROP18 and ROP5 gene alleles of TgtigerCHn3 (3/4) and TgtigerCHn4 (3/3). Few brain tissue cysts (0–213) were observed in the mice after inoculation with TgTigerCHn3 and TgTigerCHn4. This is the first documented isolation of T. gondii ToxoDB#20 and ToxoDB#2 from tigers. The results provide additional direct evidence of tiger as intermediate hosts for T. gondii. Tigers in the zoos may potentially transmit T. gondii to other animals and humans.

1. Introduction

Toxoplasma gondii is one of the intracellular protozoan parasites that can infect mammals and birds, including humans and felines (Dubey, 2010; Pappas et al., 2009; Waldman et al., 2020). Generally, felines serve as the definitive host of T. gondii; they specifically excrete millions of environmentally resistant oocysts. Furthermore, cats could re-shed oocysts after secondary or tertiary T. gondii infection (Dubey, 1995; Zulpo et al., 2018). Under the mild environmental condition, the infectious sporulated oocysts could survive more than 1 year (Dubey, 2010). Hence, captive felids (e.g., in zoos) infected with T. gondii can be a possible contamination source for other animals and humans.

Currently, the pooled seroprevalence of T. gondii in wild felids worldwide is approximately 64%, and the highest and lowest seroprevalence species were Panthera leo and Leopardus colocolo, respectively (Dubey et al., 2020; Hatam-Nahavandi et al., 2021). In China, the seroprevalence of T. gondii is approximately 24% in cats (Ding et al., 2017; Dubey et al., 2020), there is no report on wild felids, and only T. gondii infection has been reported in captive felids (Dubey et al., 2020).

Tigers are listed by the international Union for Conservation of Nature as threatened species, and 2154–3159 mature tigers exist, inhabiting under 7% of their historic range (IUCN, 2021). Different subspecies of wild tigers are in a precarious state worldwide. It is necessary to protect tigers from zoos. However, to date, little is known about T. gondii infection in tigers, and only two viable T. gondii strains have been

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successfully isolated from tigers (Yang et al., 2019a). In this study, we identified *T. gondii* infection in tigers, isolated the parasites using mouse bioassay, and characterized them using multilocus PCR–RFLP genotyping. The results could serve as a reference for a better understanding of the genetic diversity, pathogenicity, and transmission dynamics of *T. gondii*.

2. Materials and methods

2.1. Sample collection and sites

Between 2019 and 2021, four captive tigers died in one zoo in Henan (34°46′N, 113°39′E), China and their tissues (heart, liver, spleen, lung, kidney, brain, intestines, lymph, tongue, diaphragm, and skeletal muscles) were submitted to the laboratory of Veterinary Pathology of the Henan Agricultural University (Zhengzhou, Henan, China) for pathological diagnosis (Table 1). The tigers were fed raw beef, raw chicken, and raw pork before they died.

2.2. Isolation of viable *T. gondii* from tiger tissues using bioassay in mice

Tissue samples (50 g: namely heart, tongue, skeletal muscle, diaphragm, or brain) from the four tigers were homogenized and digested in pepsin solution, respectively (Dubey, 2010). The homogenates were injected subcutaneously into Swiss mice (n = 3–5) and IFN–γ⁻/⁻ mice (n = 1). Specific pathogen–free Swiss mice were supplied by the Laboratory Animal Center at the Zhengzhou University (China, Grant No. 41003100000236). IFN–γ⁻/⁻ mice were supplied by Jackson Laboratory (USA, product code: 002287). The remaining homogenate was saved at −20 °C for molecular analysis. After inoculation, the clinical signs of the mice were recorded every day. Impression smears of the lungs, mesenteric lymph nodes, and brain of dead mice were examined for *T. gondii*. The survivors were bled on day 30 post-inoculation (DPI), and 1:25 and 1:200 dilutions of mice serum samples were tested for *T. gondii* antibody or parasites were detected in their serum samples or tissues.

2.3. Detection of antibodies against *T. gondii*

The heart fluids of the tigers were collected, double diluted from 1:25 to 1:12800, and tested for IgG antibodies against *T. gondii* using the MAT (Dubey and Desmonts, 1987). Whole formalin fixed *T. gondii* antigens were obtained from the University of Tennessee Research Foundation (Knoxville, TN, USA). The tigers were exposed to *T. gondii* when the titre of antibodies against *T. gondii* is greater than 1:25. The reference serum was kindly provided by Dr. J. P. Dubey (ARS, USDA). The serum samples of the positive and negative controls were included in the same 96-well U plate.

2.4. Histopathological analysis

Fresh tiger tissues, including brain, myocardium, liver, spleen, lung, kidney, tongue, leg muscle, intestines, and diaphragm were fixed in 10% formalin and used for histopathological examination. Tissues were stained using haematoxylin and eosin (HE) and immunohistochemistry (IHC). Rabbit anti-*T. gondii* serum was used as the primary antibody and mouse anti-rabbit IgG conjugated with HRP/DAB as the secondary antibody (IHC detection kit, ab64264, Abcam, Waltham, Massachusetts, USA). The distribution of *T. gondii* antigens in the tissues was observed under a light microscope.

### Table 1

| Batch No. Samples (T. gondii ID) | Sample received date (month, year) | Age (month), Sex | Clinical symptoms and pathology finding | Antibody titer by MAT | T. gondii nucleic acid test positive tissues by PCR | Mice bioassay Experimental date and group | Swiss mice | IFN–γ⁻/⁻ mice (survival days) |
|-------------------------------|---------------------------------|-----------------|-----------------------------|----------------------|-----------------------------------------------|-----------------------------------------|-----------|-----------------------------|
| Tiger 1 Path 2986 (TgTigerChn3) | Jul 14, 2019 18, M | It died suddenly without obvious clinical signs. Necrotic splenitis, acute proliferative glomerulonephritis, hepatic hemorrhage and diffuse hepatitis necrosis, acute pulmonary edema. | 1:400 Kidney. | | | | | 3/4\(^a\) |
| Tiger 2 Path 2980 (TgTigerChn4) | Sep 13, 2019 7, F | Anorexia, paralysis in hind legs, mucus sticky. Multi focal necrotizing hepatitis, necrotic splenitis, interstitial pneumonia, necrotic enteritis, neuron necrosis. | 1:200 Striated muscle digestive juice, spleen, liver, myocardium, brain. | | | | 4/5 | 2/2\(^b\) (9, 11) |
| Tiger 3 Path 2983 | Sep 22, 2019 240, F | Periarial fistula, tooth atrophy. Ascites, chronic sclerosing glomerulonephritis, liver cirrhosis and abundant lipofuscin deposition in hepatocytes, interstitial pneumonia, acute splenitis, hemorrhagic necrotizing lymphadenitis. | 1:1600 Striated muscle digestive juice, spleen, liver, kidney, lung, lymph gland, myocardium, tongue, diaphragm, leg muscle. | | | | 0/3 | 0/2 |
| Tiger 4 Path 3124 | Mar 02, 2021 60, F | Depressed, anorexia, hematuria. Hydrothorax, hydropericardium, multi focal necrotizing hepatitis, interstitial pneumonia, kidney atrophy, acute splenitis, neuron necrosis, necrotizing atrophie enteritis. | 1:25600 Striated muscle digestive juice, spleen, liver, kidney, lung, lymph gland, tongue. | | | | 0/4 | 0/1 |

\(^a\) Modified agglutination test.

\(^b\) Polymerase Chain Reaction. The myocardium of Tiger 1 (Path 2295) was found *T. gondii* nucleic acid by PCR, with MAT titer 1:50 (Yang et al., 2019a).

\(^c\) Number of infected *T. gondii* mice/number of inoculated mice.
DNA extraction and polymerase chain reaction (PCR) amplification of *T. gondii*

DNA was extracted from the tiger myocardium, liver, spleen, lung, kidney, tongue, leg muscle, brain, diaphragm, and pepsin digestion liquids using a commercial DNA extraction kit (Tiangen Biotech Company, DP304, China). PCR assays were performed to detect *T. gondii* using the specific primer pairs TOX5/TOX8. The products for *T. gondii* were expected to be 450 bp in length (Reischl et al., 2003).

2.6. Cell cultivation and genotyping

Tissue (brain, lung, or mesenteric lymph nodes) homogenates of *T. gondii* positive mice were seeded into Vero cells (RPMI 1640, 10% foetal bovine serum, 37 °C, and 5% CO₂). DNA was extracted from the *T. gondii* tachyzoites, which were collected using cell culture. The *T. gondii* genotypes were differentiated using PCR–RFLP with 10 genetic markers, namely SAG1, SAG2 (5'- and 3'-SAG2, alt. SAG2), SAG3, GRA6, BTUB, L358, PK1, c22-8, c29-2, and Apico (Su et al., 2010). The *T. gondii* virulence factor was identified by genotyping the polymorphisms in the following genes: ROP5 and ROP18 (Shwab et al., 2016a; b). *T. gondii* reference DNA was included in all the batches.

2.7. Evaluation of the virulence of *T. gondii* isolated from tigers using swiss mice

The *T. gondii* tachyzoites were collected from cell culture and diluted 10-fold after counting with a blood count plate from 10⁻¹ – 10⁻⁴ to reach an endpoint of <1 tachyzoite. Next, < 1, 10⁰, 10¹, 10² and 10³ tachyzoites were inoculated intraperitoneally into five Swiss mice for each dilution. Clinical signs, including illness and death, and the gross signs of the mice were observed and documented every day. Lung, mesenteric lymph node, and brain impression smears of the dead mice were examined for *T. gondii*, and the tissues of the dead mice were fixed in 10% (v/v) neutral buffered formalin. On 30 DPI, serum samples from the surviving mice were analyzed for *T. gondii* antibodies using MAT with titres 1:25 and 1:200, and euthanized on 60 DPI. The cysts in the brain of the mice were observed and counted under a microscope (Dubey et al., 2012). The virulence was evaluated based on the percentage of dead mice among *T. gondii*-positive mice.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed using the Chi-squared test or Fisher’s exact test. The numerical values are expressed as mean ± SE.

3. Results

3.1. Clinical, pathological findings and prevalence of *T. gondii* infection in captive tigers

The background, clinical signs, and main pathological findings of the four captive tigers are summarized in Table 1. They include multifocal necrotizing hepatitis (2/4), liver cirrhosis (1/4), hepatic hemorrhage (1/4), necrotic splenitis (2/4), acute splenitis (2/4), acute pulmonary edema (1/4), interstitial pneumonia (3/4), acute proliferative gliomerulonephritis (1/4), chronic sclerosing glomerulonephritis (1/4), kidney atrophy (1/4), necrotic enteritis (1/4), neuron necrosis (2/4). The pathological diagnosis showed that hepatic failure, renal insufficiency, declined immune function, and age-related multiple organ atrophy were responsible for the death of the four tigers. *T. gondii* parasites were not found in IHC and H&E tissue sections of the four tigers.

*T. gondii* IgG antibodies were found in the heart fluid of all the captive tigers using the MAT (100%, 4/4), with titres of 1:400, 1:200, 1:1600 and 1:25600 (Table 1). An interesting phenomenon discovered was that the *T. gondii* antibody titre in elder tigers was higher than that in younger tigers. *T. gondii* DNA was amplified in the tissues of all the tigers with the primer pair TOX5/TOX8. Striated muscle digestive fluid, spleen, kidney, liver, and lung had high-density *T. gondii* (Table 1).

3.2. Isolation of viable *T. gondii* from the muscle of the tigers using bioassay in mice

Striated tissue homogenates from the four tigers were bioassayed in the mice individually.

For Tiger#11, Tox#18–46 group (mice group number, the mice from Tox18 were inoculated tigers tissues), three (M#1182–1184) of the four Swiss mice showed *T. gondii* antibody seroconversion on 35 DPI (MAT ≥1:200). *T. gondii* cysts were found in the mice brain (M#1183–1184) on 50–54 DPI. *T. gondii* tachyzoites were found in IFN-γ/-/- mice lymph nodes (M#1124, and M#142) on 9–11 DPI. The *T. gondii* strain from the lymph nodes of IFN-γ/-/- mouse M#142 was successfully propagated in cell cultures (on 15 DPI) and designated TgtigerCHn3.

For Tiger#12, Tox#18–52 group, *T. gondii* tachyzoites were found in the lungs of three Swiss mice (M#1123, M#1125, and M#1237) on 9–19 DPI. *T. gondii* tachyzoites were found in the lung of the IFN-γ/-/- mouse (M#1227) on 8 DPI. The *T. gondii* strain from the lungs of the IFN-γ/-/- mouse (M#1227) was successfully propagated in cell cultures (on 9 DPI) and was designated TgtigerCHn4.

For Tiger#13, Tox#18–54 group, all the mice (three Swiss mice, and two IFN-γ/-/- mice) died on 8–13 DPI. Parasites were not found in the mice tissues. After sub-passagging, none of the mice (n = 5) had antibodies for *T. gondii*, and no bradyzoites were observed on 49 DPI.

For Tiger#14, Tox#18–74 group, none of the parasites was found in the four Swiss mice on 23–96 DPI. After sub-passagging, none of the mice (n = 2) had antibodies for *T. gondii*, and no bradyzoites were observed on 69 DPI.

3.3. Genotyping and virulence of TgTigerCHn3 and TgTigerCHn4

DNA from the tachyzoites of TgTigerCHn3 and TgTigerCHn4 in cell culture medium was analyzed using PCR–RFLP with 10 genetic markers, as well as the polymorphic ROP5 and ROP18 genes. The genotype of TgTigerCHn3 was ToxoDB#20; ROP18 and ROP5 showed a 3/4 allele combination, it was predicted that TgTigerCHn3 was avirulence in mice. The genotype of TgTigerCHn4 was ToxoDB#2 (type III); ROP18 and ROP5 had allele 3/3 combination, it was also predicted that TgTigerCHn4 was avirulence in mice.

After inoculating Swiss mice with different doses of TgTigerCHn3 tachyzoites, the survival time of most *T. gondii* infected mice was more than 40 days. Ten tachyzoites of *T. gondii* could infect 100% (4/4) of the mice. In the 10³ and 10⁴ tachyzoites groups, only a few *T. gondii* cysts (2–5) were detected in the mice brains when euthanized on 61 DPI (Table 2). Compared with the group of one tachyzoite, the number of cysts did not increase significantly in the higher tachyzoites groups (P > 0.05).

After inoculating Swiss mice with different doses of TgTigerCHn4 tachyzoites, the survival time of most *T. gondii* infected mice was more than 39 days. Ten tachyzoites of *T. gondii* infected 100% (4/4) of mice. *T. gondii* cysts (15–213) were detected in the brain of the mice when euthanized on 61 DPI (Table 2). Compared with the group of one tachyzoite, the number of cysts did not increase significantly in the higher tachyzoites groups (P > 0.05).

4. Discussions

The existence of wild tigers is being threatened in many ways, including hunting, habitat loss, human-wildlife conflict, and various anthropogenic activities (Kang et al., 2010; Mahmood et al., 2021). The tiger (Panthera tigris altaica) population has rebounded as its habitat in
Table 2 Evaluation of the virulence of Toxoplasma gondii TgTigerCHn3 and TgTigerCHn4 strains in Swiss mice.

| No. of parasites  | No. of infection/No. of inoculation (%) | Days of survival | No. of brain cysts |
|-------------------|----------------------------------------|------------------|-------------------|
| TgTigerCHn3       |                                        |                  |                   |
| 10⁴                | 5/5 (100%)                             | 40 ± 10          | 2 ± 2             |
| 10³                | 4/4 (100%)                             | 61 ± 11          | 5 ± 5             |
| 10²                | 5/5 (100%)                             | 53 ± 11          | Not found         |
| 10¹                | 4/4 (100%)                             | ≥80DPI/4         | Not found         |
| 1                  | 2/5 (40%)                              | 63 ± 17          | Not found         |
| <1                 | 0/5 (–)                                | ≥80DPI/5         | Not found         |
| Blank control      | 0/5 (–)                                | ≥80DPI/5         | Not found         |
| TgTigerCHn4        |                                        |                  |                   |
| 10⁴                | 5/5 (100%)                             | 64 ± 1           | 74 ± 34           |
| 10³                | 4/4 (100%)                             | 50 ± 13          | 137 ± 87          |
| 10²                | 4/4 (100%)                             | 48 ± 14          | 213 ± 37          |
| 10¹                | 4/4 (100%)                             | 39 ± 8           | Not found         |
| 1                  | 3/4 (75%)                              | 39 ± 3           | 15 ± 15           |
| <1                 | 0/5 (–)                                | ≥60DPI/5         | Not found         |
| Blank control      | 0/5 (–)                                | ≥60DPI/5         | Not found         |

Avirulence: The dose of 10⁴ T. gondii tachyzoites or oocysts was non-lethal to mice (within 30 DPI).

Intermediate virulence: Mice were dead (mortality between 1%-99%) after inoculation with 10⁴ T. gondii tachyzoites or oocysts (within 30 DPI).

Virulence: Mice were 100% mortality after inoculation with 10⁴ T. gondii tachyzoites or oocysts (within 30 DPI) (Yang et al., 2021).

China has gradually improved (Zhang and Ma, 2010). Zoo tigers are often used as a resource for public science education, scientific research, and tourism. Between 2019 and 2021, a limited number of tissues from four dead tigers were sent to the laboratory, which allowed us to conduct T. gondii research.

In this study, the frequency of positive animals in tigers from the zoo was 100% (4/4), which was at a high level, indicating that they may have been infected with T. gondii and shed oocysts. The tigers might have been infected with T. gondii by ingesting viable cysts from raw meat or oocysts from contaminated food, water, and the environment. Furthermore, the antibody titre of T. gondii in senior tigers was higher than that in young tigers, consider the few cases, we are not sure that they were postnatal infection. The lactation period of a tiger usually lasts five to six months; the main diet of the cub is breast milk from the dam, and then, the milk could be selected to isolate T. gondii. This finding is consistent with other reports on cats or macropods (Dubey et al., 2020, 2021). Parasite load may be related to animal species, infection status, strain, or genotypes of T. gondii and the environment.

Viable T. gondii strains were successfully isolated from the tissues of Tiger#11 and Tiger#12 in this study. Both were novel genotypes discovered in tigers. The genotype of TgTigerCHn3 was ToxoDB#20, the ROP18/ROP5 genotype combination (3/4) suggests that this strain was avirulence for mice (Shwab et al., 2016a, b), which matched with the mouse virulence evaluation in this study. The genotype of TgTigerCHn4 was ToxoDB#2, and 3/3 for the ROP18/ROP5 combination, indicating avirulence for mice. The genotype of ToxoDB#20 has been reported in cats, dogs, sand cats, red pandas, and serval from Africa and Asia (Dong et al., 2019; Dubey et al., 2007, 2010, 2013; El Behairy et al., 2013; Al-Kappany et al., 2010; Tian et al., 2014; Yang et al., 2019b). ToxoDB#20 T. gondii strains have been found in red pandas and serval from central China, both were 3/4 for the ROP18/ROP5 combination, avirulence for mice and low rate of brain cyst formation (Dong et al., 2019; Yang et al., 2019b). This indicates that TgTigerCHn3, TgRedpandaCHn1 and TgServalCHn1 may share common origins. Furthermore, ToxoDB#20 only differs from the local endemic genotype of Chinese1 (ToxoDB#9) at locus PK1, they probably belong to the same ancestral clade with Type II lineage (Chaichan et al., 2017).

ToxoDB#2 (type III) is widely distributed worldwide, including Asia, Africa, South Europe, North America, South, and Central America (Chaichan et al., 2017; Dubey, 2010; Dubey et al., 2013, 2014; Shwab et al., 2018). ToxoDB#2 T. gondii strains have been found in cats (Yang et al., 2015), lambs (Jiang et al., 2020a), caracal (Jiang et al., 2020b), white Spoonbill (Yang et al., 2020), and kangaroo (Yang et al., 2023) from central China, all of them were 3/3 for ROP18/ROP5 and most of them were avirulence for mice (except TgRooCHn3). They share the same genotype, and ROP18/ROP5 allele, this indicates that TgTigerCHn4 may be of local origin. It is speculated that in addition to ToxoDB#9, ToxoDB#2 is another one of the major endemic genotypes in China.

The rate of isolation of T. gondii increased with antibody titre against T. gondii in lambs and sows (Jiang et al., 2020a; Dubey et al., 1995), and a positive correlation was observed between the T. gondii antibody titre and brain parasite burden in mice (Bezerra et al., 2019; Yang et al., 2021). However, no viable T. gondii was isolated from the tissues of Tiger#13 and Tiger#14 with high MAT titre (1:1600 and 1:25600). This probably may be species specific for tigers, or a limited number of tissue samples, or Tiger#13 and Tiger#14 maybe at acute infection phase of toxoplasmosis, parasites maybe kill by pepsin digested solution.

Tiger (Panthera tigris altaica) has been proven as the definitive host of T. gondii through bioassay of faecal samples in mice (Yang et al., 2019a). Here, two viable strains were successfully isolated from two tigers, they may have eliminated oocysts in the feces, the results provide additional direct evidence of tiger (Panthera tigris altaica) as an intermediate host for T. gondii. Therefore, cleaning the feces and inactivating the oocysts from captive felids by burning or incubating them at high temperatures are very important strategies for controlling T. gondii infection. The other effective way to reduce the risk of T. gondii exposure would be to freeze meat to −12 °C for more than seven days before feeding it to the felids (Kotula et al., 1991; Ramos Silva et al., 2007).

5. Ethics approval and consent to participate

Verbal consent was obtained to collect samples from veterinary hospitals. This method is widely used in China and was approved by the ethics committee of Henan Agricultural University (China). The mouse experiments, isolation and virulence assay were approved by the Beijing Association for Science and Technology (SYXK [Beijing] 2007–0023). All animals were handled in strict accordance with good animal practices according to the Animal Ethics Procedures and Guidelines of the People’s Republic of China. All experiments reported here were approved by the Institutional Animal Use Protocol Committee of the Henan Agricultural University, China.

Data availability statement

The datasets used and/or analyzed in this study are available from...
the corresponding author upon request.

Disclosure statement

The authors report there are no competing interests to declare.

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Authors’ contributions

HJR performed the laboratory tests, analyzed the data, and wrote the manuscript. LLY, NPZ, and JBL participated in sample collection and laboratory testing. CLS and YBJ helped revise the manuscript. YRY designed the study protocol, analyzed the results, and wrote the manuscript. All authors have read and approved the final version of this manuscript.

Declaration of competing interest

None.

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