

Preliminary Studies on the Relevance of Antibodies and Bioassay to Experimental Infection with *Toxoplasma gondii* In Ducks

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ABSTRACT

Little is known about the relationship between antibody dynamics and positive bioassay with *Toxoplasma gondii* (*T. gondii*) infection in ducks. In this study, 42 ducks were allocated randomly into groups A, B and C with 14 birds of 14 days of age in each group. Three groups were infected intravenously with 10^5 , 10^6 and 10^8 of *T. gondii* tachyzoites, respectively. Blood samples were collected on day 7, 14, 21, 28, 42, 56, 70 post-infection (p.i.). Sera was prepared from each group to evaluate the anti-*T. gondii* antibodies by the modified agglutination test (MAT); from each group 2 duck samples were used to bioassay *T. gondii* in mice. The *T. gondii* were identified by PCR and nucleic acids sequencing. In group A, the parasite was successfully bioassayed from day 7, 14 and 21 in post-infected ducks, with the MAT titers of 1:2, 1:5 and 1:80, respectively. However, in ducks with MAT titers 1:80, 1:40, 1:20 and 1:4 on the following days 28, 42, 56 and 70 p.i., the bioassay was negative. In group B, *T. gondii* was successfully bioassayed at all the time points, regardless of the seropositivity or seronegativity for *T. gondii* antibodies. All birds of group C died before day 4 p.i., and therefore they were not bioassayed. PCR and sequencing results confirmed that all the isolates belong to *T. gondii*. This study demonstrated that successful bioassay does not completely correlate with positive MAT diagnosis. The existence of *T. gondii* and the concentration of the parasite may be the key parameters during the bioassay studies.

Keywords: Duck; *Toxoplasma gondii*; Antibody; Bioassay; Artificial Infection

INTRODUCTION

Toxoplasma gondii is an important intracellular protozoan parasite, widely prevalent in humans and animals and also birds throughout the world (1-3). Humans can be infected by ingestion of raw, or undercooked meat from the infected animals, and by consuming food or water contaminated with oocysts excreted by cats (4). Ducks are an important intermediate host for *T. gondii*. The consumption of raw duck meat or improperly cooked duck meat is a risk factor for *T. gondii* infection in humans and other animals (3, 5).

In recent years, seroprevalence studies of *T. gondii* in ducks have had been conducted extensively in various parts of the world (3, 6-8), and *T. gondii* strains have been constantly isolated from this host (7-9). It has been already shown that successful bioassay of *T. gondii* in ducks is crucial to the further research about this parasite. So far, to the best of our knowledge, most of the isolated strains of *T. gondii* from ducks were based on the serologic diagnosis with a modified agglutination test MAT (7, 8). However, to date, little is known about the relationship between the dynamics of antibodies and successful

T. gondii isolation. Therefore, the present study has been designed to evaluate the relevance and relationship of serological response to *T. gondii* and its bioassay in ducks.

MATERIALS AND METHODS

Ethics statement

In this research, all the studies using ducks were submitted as protocols approved by the Animal Care and Ethics Committee of Southwest University (Approval No. 201209025).

Mice and parasites

ICR mice of 4–6 weeks of age were obtained from Center of Laboratory Animals, Chongqing Medical University (Chongqing, PR China). All animals were reared under specific-pathogen-free conditions at Southwest University, and commercial basal diet and tap water was provided *ad libitum*. Mice were infected or challenged intraperitoneally (i.p.) with the highly virulent RH strain of *T. gondii* maintained in the laboratory. The tachyzoites for infection were obtained from peritoneal washings and diluted in phosphate buffer saline (PBS) to obtain the doses of 1×10^5 , 1×10^6 and 1×10^8 in 0.5 ml of inoculum.

Ducks, experimental design, nutrition and management

A total of 42 ducks were obtained from a commercial farm in Rongchang county of Chongqing, China. Blood samples were collected for sera from all ducks before infection. MAT and PCR were performed to confirm the negativity of the ducks for *T. gondii*.

At 14 days of age, the ducks were allocated randomly into three groups (A, B and C) with 14 birds in each group. Ducks were infected intravenously as shown in Table 1. On days 7, 14, 21, 28, 42, 56, 70 post infection (p.i.), two ducks in each group were bled, and the sera were prepared by centrifugation and stored in -20°C until further use.

During the experimental phase, tap water and duck formula feed (Chia Tai Co., Ltd, Chongqing, China) sterilized with high pressure steam before use, were provided to all ducks.

Clinical examinations

Infected ducks were observed throughout the experimental period for any clinical manifestations.

Serological examination

Antibodies to *T. gondii* were determined in duck sera by the modified agglutination test (MAT) as described previously (7, 10, 11). In brief, sera were added to the “U” bottom of 96 well microtiter plates, and diluted two-fold starting from 1:5 to 1:160. Briefly, killed *T. gondii* tachyzoites were used as antigen and serum samples were treated with 0.2M 2-mercapthethanol to destroy the IgM class antibodies. Positive and negative controls were included in each plate. Those sera with suspicious results were retested, and antibodies to *T. gondii* were considered seropositive with $\text{MAT} \geq 1:5$.

Bioassay in mice for *T. gondii* isolation

On days 7, 14, 21, 28, 42, 56, 70 post-infection (p.i.), brains, hearts, spleens, lungs, livers and kidneys of two ducks in each group were bioassayed for *T. gondii* as previously described (12). Briefly, samples were pooled, homogenized in five volumes of 0.85% NaCl solution, mixed with five volumes of acid-pepsin solution and the mixture was incubated in a shaker water bath at 37°C for 60 min. The digest was filtered through gauze, centrifuged at $1200 \times g$ for 10 min, neutralized with 1.2% sodium bicarbonate, mixed with 1000 U/ml ampicillin (Amresco, Solon, USA) and 100 $\mu\text{g}/\text{ml}$ gentamycin sulfate (Amresco, Solon, USA), and immediately the homogenate of tissues was inoculated intraperitoneally into five mice. Tissue imprints of lungs, ascites and brains of dead mice were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were blind passaged 14 days after the inoculation and again at 7 days intervals. After 4 times of blind passage, the mice were killed and all brains were examined for tissue cysts as described (13). Mice were considered infected by *T. gondii* if peritoneal exudates or tissue imprints were positive for tachyzoites or tissue cysts.

Identification of the isolate by PCR

Molecular identification of the isolate was performed with a 341bp fragment of the internal transcribed spacer 1 (ITS-1) gene by a pair of primers 5'-AGTTTAGGAAGCAATCTGAAAGCACATC-3' and 5'-GATTTGCATTCAAG AAGCGTGATAGTAT-3' as described previously (10). In brief, PCR reactions (25 μL) were performed in 2.5 mM of MgCl_2 , 0.4 μM of each primer, 2.5 μL 1 \times rTaq buffer, 0.25 mM of each desoxyribonucleotide,

0.625 U of rTaq DNA polymerase (TaKaRa), and 3 μ L of DNA sample in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 7 min. The positive control of *T. gondii* DNA and negative control (non-DNA) were included in all amplification runs. Each amplicon (13 μ L) was examined by agarose gel electrophoresis to validate amplification efficiency.

RESULTS

Clinical examinations

Ducks in groups A and B appeared clinically normal during the experimental period. In group C, all the 14 ducks displayed typical clinical signs of toxoplasmosis, such as diarrhea, anorexia and apathy, and they died before day 4 p.i.

Serological examination

The specific anti-*T. gondii* antibodies at titers between 1:5 and 1:320 were detected in all infected ducks of groups A and B from day 14 to day 56 p.i. using the MAT (Table 1). On the days 7 and 70 p.i., the titers of these two groups were all lower than 1:5 and were therefore regarded as seronegative. In group C, no samples could be collected as all the ducks had died before day 4 p.i.

Table 1. Antibody titers by MAT in ducks experimentally infected with *T. gondii*.

Group	Dose	Titers of antibody to <i>T. gondii</i> using MAT						
		7d p.i.	14d p.i.	21d p.i.	28d p.i.	42d p.i.	56d p.i.	70d p.i.
A	10 ⁵	1:2	1:5	1:80	1:80	1:40	1:20	1:4
B	10 ⁶	1:4	1:20	1:160	1:320	1:160	1:40	1:4
C	10 ⁸	-	-	-	-	-	-	-

- ducks dying on day 4 post infection (p.i).

Titers were considered seronegative when MAT < 1:5

Table 2. *T. gondii* isolation in bioassayed mice from tissues of experimentally infected ducks

Group	Dose	Isolation results						
		7d p.i.	14d p.i.	21d p.i.	28d p.i.	42d p.i.	56d p.i.	70d p.i.
A	10 ⁵	+	+	+	-	-	-	-
B	10 ⁶	+	+	+	+	+	+	+

p.i.: post infection

Isolation of *T. gondii* from the ducks

All the living ducks (Table 1) were used for *T. gondii* bioassay and the results were shown in Table 2. In group A, *T. gondii* were successfully bioassayed from day 7 p.i., 14 p.i. and 21 p.i. ducks, with the MAT titers negative, 1:5 and 1:80, respectively. However in ducks with MAT titers of 1:80, 1:40, 1:20 and negative titers on the following days 28, 42, 56 and 70 the bioassay was negative. Whereas regarding group B, *T. gondii* were successfully bioassayed at all the time points, regardless of the seropositivity or seronegativity for *T. gondii*.

Identification of the isolate by PCR

All the isolates were identified by PCR using a 341bp fragment of the ITS-1 gene as a target. Alignment of nucleic acid sequences of PCR products shared 100% similarity to RH strain of *T. gondii* (accession No. U16161) homologues, confirmed that they belonged to *T. gondii*.

DISCUSSION

MAT is regarded as a sensitive method for measuring antibodies to *T. gondii* in ducks and is considered as an effective serologic method as a guide to isolating *T. gondii* from tissues of ducks. Utilizing this method, many *T. gondii* strains have been successfully isolated from ducks (7, 8) and chickens (14-20) in previous reports. Thus the MAT method was used in this research to study the relevance and correlation of serological response and bioassay.

In the present study, both groups A and B were successfully bioassayed for *T. gondii* on day 7 p.i, even though the serological response was negative. The reason for this might be that during the early stages infection, antibodies were not yet produced in spite of the parasite already existing in the ducks. These findings were in accordance with that of Wang (21).

From day 14 to 56 p.i., all the ducks in the group A and B were seropositive for *T. gondii* infection. But group A ducks on day 28, 42 and 56 p.i. failed to bioassay. This might be explained on the grounds of a low infective dosage of tachyzoites had been removed by the immune system of the duck, or that the amount of tachyzoites or cysts was too low to be bioassayed. However, the exact reason for this failure of the bioassay still requires further research. At the last time point of the study, *T. gondii* was successfully bioas-

sayed from group B ducks, regardless of the negative MAT antibody titer. This indicated that even if the serodiagnosis of *T. gondii* was negative, the parasite could still be possibly isolated. On the basis of above findings, we may cautiously draw the conclusion that successful bioassay was not entirely depend on the positive MAT diagnosis. Furthermore, the existence of *T. gondii* and the quantity of the parasite may be the major key parameters during the bioassay studies. Our findings were in accordance with those of Dubey *et al.* (22) who reported that successful isolation depended on the amount of tissue-bioassayed and the concentration of the parasite in sampled tissues.

To date, the susceptibility of ducks to toxoplasmosis has been seldom reported. In the present study, 108 tachyzoites administrated intravenously ducks caused death within 4 days, with typical clinical signs of diarrhea, anorexia and apathy. Although the usage of a lower dosage in ducks with 10^6 and 10^5 tachyzoites showed no clinical symptoms, they all bioassayed positively for *T.gondii*. These findings indicated that ducks were relatively susceptible to the infection with *T. gondii*. Our findings are in agreement with the results obtained by Bartova *et al.* (23) using *T. gondii* infected domestic ducks.

In this study, ducks of group A and B presented a relative short period of seropositivity after infection. The exact reasons for this are not clear. However, it might in part be due to the fact that the ducks were infected with tachyzoites using the intravenous route which neither the natural route of infection nor the natural stage of infection. This experiment carried out using oocysts via oral infection may have produced different results.

CONCLUSIONS

The results of the present study demonstrate that successful bioassay of *T. gondii* from experimentally infected ducks with tachyzoites was not entirely depended on the positive MAT diagnosis. The amount of *T.gondii* in the infected host may be the key factor for the bioassay studies.

ACKNOWLEDGEMENTS

This work was supported by Fundamental Research Funds for the Central Universities (XDJK2012C100) and The Doctoral Special Funding of SWU (2013Bsr09).

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