

Real-time PCR-based quantification of *Toxoplasma gondii* in tissue samples of serologically positive outdoor chickens

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This study aimed to quantify Toxoplasma gondii in tissue samples of serologically positive chickens using real-time polymerase chain reaction (PCR). Of 65 chickens evaluated, 28 were positive for T. gondii antibodies. Brain and heart samples were collected from 26 seropositive chickens and DNA was extracted using Trizol® and amplified using real-time PCR with SYBR® Green. Parasite DNA was detected in 24 of the 26 samples analyzed; the number of positive tissue samples and the parasite quantity did not differ between tissue types. The results confirmed the analytical sensitivity of parasite detection in chicken tissue samples and demonstrated the possibility of using other molecular systems for genotypic analysis.

Key words: *Toxoplasma gondii* - chicken - real-time PCR

Toxoplasmosis is a zoonosis that affects millions of people and is caused by the protozoan *Toxoplasma gondii* (Reischl et al. 2003), an intracellular parasite capable of invading several tissues in mammals and birds (Jaurégui et al. 2001).

Domestic chickens (*Gallus gallus*), mainly those bred in free-range conditions, have been studied all over the world because their feeding habits make them indicators of environmental contamination (Dubey et al. 2006). These birds frequently show high infection rates in all countries studied and common rates of infection range from 39% (Dubey et al. 2002) to 66% (Dubey et al. 2006) in Brazil. Most studies of *T. gondii* in chickens use mouse bioassays, but this procedure may take up to two months to complete and the use of animals is an expensive procedure that involves ethical issues. In addition, parasite passage in mice may select for genotypes that are more virulent to this species (Villena et al. 2004), affecting the interpretation and the usefulness of the results. This study aimed to evaluate the use of a real-time polymerase chain reaction (PCR) assay based on a repeat element for the detection of *T. gondii* in brain and heart samples from free-range chickens seropositive for the parasite.

Blood samples from 65 free-range chickens from five farms located in Toledo, state of Paraná (PR), Brazil, were collected by wing vein puncture. Serum obtained by cen-

trifugation was analyzed using the modified agglutination test (MAT) using formalin-fixed RH strain tachyzoites as antigen, with an initial dilution equal to 1:25. Reactive serum samples were titered by successive two-fold dilutions to 1:3.200 (Desmonts & Remington 1980).

MAT reactor birds were killed by cervical dislocation and their hearts and brains were collected. These samples were placed in 30 mL Falcon tubes containing an antibiotic solution (penicillin G + streptomycin) and were refrigerated until they were digested in a pepsin solution, as per Dubey et al. (2002), in the Preventive Veterinary Medicine and Public Health Laboratory at the Paranaense University, Umuarama, PR, Brazil. Following centrifugation, the pellet was weighed and resuspended in five times its volume of 0.9% saline solution to obtain a 200 µg/mL suspension. This suspension was immediately frozen at -20°C for DNA extraction and molecular detection of *T. gondii* in the Molecular Biology Unit at the Álvaro Clinical Analysis and Research Center, in Cascavel, PR.

DNA was extracted from 400 µL of the tissue suspension using Trizol® LS (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. SYBR® Green was used to detect fluorescence in real-time PCR using the StepOne™ Real-Time PCR System (Applied Biosystems). The primer used (Invitrogen) amplified a 529 bp region that is repeated 200-300 times in the genome of the parasite. The forward and reverse sequences were 5'CACAGAAGGGACAGAAGT3' and 5'TCGCCTTCATCTACAGTC3', respectively (Edvinsson et al. 2006). For the assay, each reaction mix had a final volume of 20 µL and contained 10 µL of SYBR Green PCR Master Mix (Applied Biosystems), 1.5 µL of each primer at a concentration of 10 pmol/µL, 5 µL of DNA and 2 µL of sterile distilled water. The reaction was carried out as follows: 5-min hot start at 95°C, 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1.5 min, followed by a final extension for 7 min at 72°C.

Financial support: Universidade Paranaense, Laboratório Álvaro/ Centro de Análises e Pesquisas Clínicas, Fundação Araucária de Apoio Científico e Tecnológico do Paraná

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Received 3 May 2010

Accepted 20 July 2010

The number of parasites per gram of tissue was obtained by the formula $(NF \times 1000)/80$, where the normalization factor (NF) was as follows: (DNA concentration in the standard curve/DNA concentration in the sample) x number of amplicons in the sample. Samples that showed amplification signals lower than the minimum detection threshold of the standard curve underwent a second assay with 40 cycles, followed by melting curve analysis to check for the presence of primer dimers or other non-specific PCR products.

The DNA amplification frequency by tissue type was compared using McNemar's test, whereas DNA quantification in the different tissues was compared by the Wilcoxon test. Values were considered to be significant when $p < 0.05$.

The research protocol was submitted and approved by the Paranaense University's Committee on Ethics in Research Involving Animal Experimentation.

From the 65 serum samples tested, 28 (43.1%) were positive in the MAT and the titres are shown in Table. The brains and hearts of 26 MAT-positive birds were collected for DNA extraction and amplification using real-time PCR. Two seropositive samples were not available for purchase and subsequent collection of tissue samples.

T. gondii DNA was amplified in 22 (84.6%) of the 26 brain tissue samples tested in the quantification assay. Amplifications in five of these samples were lower than the minimum detection threshold of the standard curve and these results were confirmed in a second assay with 40 cycles followed by melting curve analysis. From the 26 heart samples evaluated, 21 (80.8%) were positive for parasite DNA; six of these samples were confirmed positive in the second assay. No significant differences were observed between tissue types in the rate of positive samples ($p = 0.6547$) or in the quantity of DNA per gram of sample ($p = 0.4258$). Considering the results obtained in the brain and heart tissue samples, *T. gondii* DNA was amplified in 24 (92.3%) of the birds analyzed. No statistical difference was observed ($p = 0.6547$) in the detection of *T. gondii* in the tissues analyzed, confirming the evidence that cysts can frequently be found concomitantly in the heart and the brain, organs that are preferred locations for the cysts of this species (Dubey et al. 1993).

In the brain tissue samples, parasite detection ranged from 81-22,742 parasites per gram of tissue, with a median equal to 437 parasites per gram. In the heart tissue samples, detection ranged from 23-982 parasites per gram of tissue, with a median equal to 155 parasites per gram.

T. gondii DNA was not amplified in two samples with MAT titres equal to 800. However, the cysts may not necessarily be located only in the tissues analyzed and it is possible that the DNA degraded during digestion.

The high sensitivity observed using this method and the region of the genome used corresponded with the sensitivity described by Jauregui et al. (2001) and Edvinsson et al. (2006), as demonstrated by the detection of one parasite per mL in both brain and heart tissue samples.

Jauregui et al. (2001) demonstrated that the difficulty of detecting *T. gondii* in the tissues of large animals was due to sampling bias, preferred parasite sites and the

TABLE
Farms, animals, respective antibody titer in modified agglutination test (MAT) and quantification of *Toxoplasma gondii* in samples of brain and heart of outbred chicken in Toledo, Paraná, Brazil, 2008

Farm	Animal	MAT titer	<i>T. gondii</i> quantity (parasites/g)	
			Brain	Heart
1	7	≥ 3200	672.06	ND
1	10	50	114.55	118.58
1	11	≥ 3200	ND	ND
1	15	800	ND	49.26
1	17	800	486.70	ND
1	18	200	109.67	155.21
1	19	800	ND	58.07
2	3	400	ND	571.76
2	4	200	ND	33.49
2	8	1600	234.02	ND
2	9	400	228.23	982.80
2	10	1600	ND	332.98
2	13	800	263.31	23.68
2	16	800	93.02	323.96
2	17	400	435.20	831.65
2	18	400	437.46	578.82
2	19	1600	1470.09	ND
3	1	800	ND	ND
3	2	1600	22742.38	62.81
4	5	200	518.93	ND
4	8	1600	ND	60.06
4	9	800	500.08	ND
5	1	400	1350.80	ND
5	2	800	ND	ND
5	3	≥ 3200	6545.50	ND
5	9	400	81.10	163.75

MAT titer: anti-*T. gondii* antibody titer in modified agglutination test; ND: not detectable.

possibility that, when performing any test for tissue cyst detection, false-negative results may occur due to insufficient sample size. These difficulties and the possibility of false-negatives are reduced with chickens because whole organs can be analyzed, including brains and hearts.

The results obtained in this study confirmed the analytical sensitivity of parasite detection in tissue samples from birds when amplifying a repeat element in real-time PCR using SYBR® Green fluorescent detection. Quantification of the parasite in these tissues demonstrated the possibility of applying other molecular detection systems for genotypic analysis directly to tissue samples. The results obtained using mouse inoculation could be compared with molecular detection systems, which may replace bioassays in the future.

ACKNOWLEDGEMENTS

To Cintia Larissa Schmitt and Marciano Antunes, from the Álvaro Clinical Analysis and Research Center, for their technical help, to Ronaldo César da Rosa and Rodrigo Mattei (PIBIC/UNIPAR), Jacqueline Batista de Araújo and Franciele Rossandra Piassa (Master's degree course in Animal Science), for helping in the collection and preparation of the samples, and to Rodrigo Costa da Silva and Vanessa Yuri de Lima, from Zootechnical and Veterinary Medicine College at UNESP, for helping with the references.

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