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MOLECULAR AND SEROLOGICAL STUDY OF *Toxoplasma gondii* OCCURRENCE IN DOMESTICAL DOVES (*Columba livia*) FROM THE METROPOLITAN REGION OF GOIÂNIA, GOIÁS

(ESTUDO MOLECULAR E SOROLÓGICO DA OCORRÊNCIA DE Toxoplasma gondii EM POMBOS DOMÉSTICOS (Columba livia) DA REGIÃO METROPOLITANA DE GOIÂNIA, GOIÁS)

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RESUMO

O objetivo da realização deste trabalho foi investigar a ocorrência de *T. gondii* em pombos domésticos (*Columba livia*) da região metropolitana de Goiânia, Goiás. A positividade para *T. gondii* foi avaliada em amostras de soro, coração e cérebro de 150 pombos. Das 150 amostras de soros, 34 (22,6%) foram reagentes para o teste de aglutinação direta modificada. Os títulos obtidos foram 1:16 (29/34); 1:64 (3/34) e 1:256 (2/34). Das 150 amostras de cérebro analisadas, apenas três (2%) foram positivas para a detecção de DNA de *T. gondii*. Já em relação as amostras de coração, todas as 150 amostras foram negativas, não tendo gerado nenhuma banda detectável pelo teste de PCR. A análise comparativa ao teste de Kappa entre os dois métodos de diagnóstico empregados, sorologia e PCR, apontou que não houve concordância entre os dois testes, não havendo semelhança entre os resultados dos métodos de diagnóstico utilizados. Isto pode ter ocorrido devido ao baixo número de aves positivas ao teste de PCR, seja pela ausência de cistos do *T. gondii* nestas aves ou pela extração de DNA de apenas um fragmento de cada víscera, o que pode ter resultado em falsos negativos. A ocorrência de resultados positivos para *T. gondii* em pombos capturados na região de Goiânia, Goiás foi baixa, sendo mais elevada na sorologia e menor de acordo com os resultados da PCR. Não houve concordância entre os resultados da MAD e da PCR.

Palavras-chave: contaminação ambiental; parasitologia; pragas urbanas, sinantrópicos; zoonoses.

ABSTRACT

This study aimed to investigate the occurrence of *Toxoplasma gondii* in common pigeons (*Columba livia*) in the metropolitan region of Goiânia, Goiás. The positivity for *T. gondii* was evaluated in serum, heart, and brain samples from 150 pigeons. Of the 150 serum samples, 34 (22.6%) were used as reagents for MAT. The titers obtained were 1:16 (29/34), 1:64 (3/34), and 1:256 (2/34). Of the 150 brain samples analyzed, only three (2%) were positive for *T. gondii* DNA. However, for heart samples, all 150 samples were negative and no detectable bands were detected by PCR. Comparative analysis using the Kappa test between the two diagnostic methods used (serology and PCR) showed that there was no agreement between the two tests, as there was no similarity between the results of the two methods. This may have occurred due to the low number of birds that tested positive by PCR, the absence of *T. gondii* cysts in these birds, or the extraction of DNA from only one fragment of each item of the viscera, which could have resulted in false negatives. The occurrence of positive results for *T. gondii* in pigeons captured in the region of Goiânia, Goiás, was low but higher in serology than in the PCR results. MAT and PCR results were not consistent with those of the pigeons tested.

Key words: environmental contamination; parasitology; urban pests, synanthropy; zoonosis.

INTRODUCTION

Toxoplasmosis is a disease of great importance in public health and is one of the most widespread parasitic zoonoses in the world (SÁFADI, 2000; TENTER, 2009). *Toxoplasma gondii* is an eurixene protozoan capable of infecting domestic animals, wild animals, and humans (HILL et al., 2005). It has a heterogeneous life cycle, with mammals and birds as intermediate hosts and felids as definitive hosts (DUBEY & LAPPIN, 2006). The main mode of transmission of this parasite is by ingestion of food and water contaminated with viable forms of *T. gondii* (DUBEY, 2010).

Bonna et al. (2006) reported that monitoring the seroprevalence of *T. gondii* infection in animals serves as a good parameter for signaling problems in relation to toxoplasmosis, since they are in direct and permanent contact with the environment and are used as sentinels to indicate environmental contamination. Free-living birds with the ability to fly and, therefore, the ability to explore a larger area present a greater risk of exposure to infection and are a good indicator of environmental contamination. Despite its importance, descriptions of the occurrence of infection and isolation of *T. gondii* in synanthropic birds are scarce (LINDSAY & BLAGBURN, 1999; DUBEY, 2002).

Pigeons are excellent fliers and can fly at speeds of 40–50 km/h, they usually remain close to their home territory. The lifespan of these birds in urban centers is 3–5 years; in wild conditions, it can reach up to 15 years (SICK, 1997).

Pigeons represent a reservoir for the spread of disease and can be a source of infection for predators as well as humans (DUBEY, 2010). Pigeons adapt well to urban centers, proliferate without control, have low selectivity in relation to food, and often consume food contaminated with *T. gondii* (GODOI et al., 2010).

Carnivorism, in which cats ingest *T. gondii* tissue cysts, is the most efficient form of infection in definitive hosts (DUBEY, 2006). Therefore, in addition to serving as possible indicators of environmental contamination, pigeons can also prey on cats and contribute to transmission of infection (YAN et al., 2011).

This study aimed to investigate the occurrence of *T. gondii* in domestic pigeons caught in the region of Goiânia, Goiás, and the presence of the parasite in their brains and hearts.

MATERIALS AND METHODS

The study was carried out at the Parasitic Disease Diagnosis and Molecular Diagnosis Laboratories and the Experimental Poultry Disease Center of the Veterinary Medicine Department of the Veterinary and Zootechnics School (EVZ) of the Federal University of Goiás (UFG).

The samples used in this study were obtained from 150 pigeons (Columba livia) caught in the metropolitan region of Goiânia, Goiás. The birds were captured in specific traps and taken to the Experimental Center for Poultry Diseases of the Department of Veterinary Medicine of the Federal University of Goiás, where they were sacrificed by cervical dislocation. All procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Goiás (Protocol 080/11).

After the birds were sacrificed, samples of their blood, brains, and hearts were collected. Blood samples were collected in sterile tubes and then centrifuged at 2500 rpm for 15 min to obtain sera and then stored at -20°C until the serological test was performed. Brain and heart samples were frozen until processing.

The modified direct agglutination test was performed using formalin-fixed antigens, according to DESMONTS and REMINGTON (1980). Sera in which antigen agglutination was observed, characterized by the formation of a mesh or film, which accounts for at least 50% of the area of the microplate cavity, were considered positive. The formation of a compact button or a button that occupies less than 50% of the microplate cavity was considered a negative reaction.

Positive and negative control sera were tested on all plates to guide the interpretation of each reaction. Samples that reacted at a dilution of 1:16 or higher were considered positive.

Before extraction, each sample was macerated in sterile tubes to facilitate the procedure. DNA was extracted from the samples using the Illustra Tissue & Cells Genomic Prep Mini Spin Kit (GE Healthcare), according to the manufacturer's instructions. Heart and brain samples from 150 pigeons were used for the PCR analysis.

The preparation of the PCR reaction mix was based on adaptations of the protocols described by SILVEIRA (2009). Thus, a volume of 50 µL was established for the reaction mix, consisting of 35.75 µL of ultra-pure water (Dnase/Rnase-Free Distilled water—Invitrogen), 5 µL of 10X PCR buffer (PCR buffer 10X Invitrogen), 2.0 µL of 50 mM magnesium chloride ($MgCl_2$) (Invitrogen); 1 µL of 10 mM dNTP (Amersham Biosciences); 0.5 µL (10 pM) of the forward primer, 0.5 µL (10 pM) of the reverse primer, 0.25 µL of Taq 5 U/µL (Invitrogen) and 5 µL of the sample DNA.

To detect *T. gondii* DNA in heart and brain samples, a pair of primers corresponding to a 193 base pair (bp) fragment of the B1 gene of *T. gondii* was used: 5'-GGAACTGCATCCGTTCATGAG-3' and 5'TCTTTAAAGCGTTCGTGGTC-3,' as described by SILVEIRA (2009). The mixture for the PCR reaction was then immediately subjected to the PCR amplification process in a previously programmed thermal cycler (Mastercycler Personal, Eppendorf) at a number of cycles and temperatures as adapted from SILVEIRA (2009): an initial denaturation cycle at 94°C for 30 s, followed by 40 repeated cycles at 94°C for 20 s, 60°C for 25 seconds and 72°C for 25 s, ending the reaction with an additional extension phase at 72°C for 5 min.

Positive and negative controls were included in all reactions performed in this study. Genomic DNA from *T. gondii* (RH strain) from the Institute of Tropical Pathology and Public Health (IPTSP) at UFG was used as a positive control. Sterilized ultra-pure water free of DNase and RNase (DNase/RNase-Free Distilled water, Invitrogen) was used as a negative control.

The PCR products (amplicons) were separated on a 2% agarose gel (Agarose NA, Amersham Biosciences) in TBE1x buffer. A volume of 10 µL of PCR products, homogenized in 2.5 µL of running dye (25% Ficoll 400, 025% bromophenol blue, 0.25% xylene cyanol in nine parts of glycerol), was applied to the gel. DNA ladder 100 pb (Invitrogen) was used as a molecular mass marker. Electrophoresis was performed in a vat (Horizon 11.14, Life Technologies) at 90 V for 60 min.

After each run, the gels were stained by immersion in ethidium bromide solution (0.4 μg/mL). Visualization was performed using a UV transilluminator (UltraLum Electronic UV Transilluminator).

Descriptive statistics were used to present and analyze the cases of *T. gondii* in birds. The correlation between the results obtained by the two methods, serology and PCR, was analyzed using the Kappa concordance test.

RESULTS AND DISCUSSION

Of the 150 serum samples, 34 (22.6%) were reactive in the MAD test. This prevalence is higher than that reported in other studies (MARCIANO, 2004; GODOI et al., 2010; LIMA et al., 2011), indicating that the region analyzed has higher oocyst contamination than other areas already studied. The serological results are presented in TABLE 1.

Table 1 - Results of the MAD test performed on sera of pigeons (*Columba livia*) caught in the region of Goiânia, Goiás between January 10 and November 30, 2012

Results MAD	N birds	Percentage
Positive	34	22.6%
Negative	116	77.4%
Total	150	100.0%

No other studies have investigated the detection of anti-*T. gondii* antibodies in pigeons in the Goiânia region, Goiás, only in other animal species and also in relation to congenital toxoplasmosis in humans (LINHARES et al. 1990; MATOS et al., 1999; AVELINO et al, 2003; RODRIGUES, 2006).

The MAD titers obtained from the 34 seropositive birds were 1:16 in 29 birds, 1:64 in three birds, and 1:256 in two birds. The fact that most birds (85.3%) had low serological titers may be related to the presence of an older infection (BONNA et al. 2006). The results of the titration of seropositive samples from pigeons are shown in TABLE 02.

Table 02 - Titration of positive samples in the MAD test performed on sera of pigeons (*Columba livia*) caught in the region of Goiânia, Goiás between January 10 and November 30, 2012.

Titration MAD	**N**	**Percentage**
1:16	29	85.3%
1:64	3	8.8%
1:256	2	5.9%
Total	34	100.00%

The prevalence of seropositivity in this study was higher than that reported in other studies conducted in different regions. Waap et al. (2008) carried out a study that assessed the prevalence

of *T. gondii* in urban pigeons (*Columba livia*) from different areas of Lisbon, Portugal, and found a prevalence of 4.6%. Yan et al. (2011) studied the occurrence of *T. gondii* in 275 pigeons in southern China and detected a positivity rate of 8.7% using the MAD test.

Salant et al. (2009) conducted a cross-sectional study on the prevalence of *T. gondii* infection in pigeons (*Columba livia*) from various locations in Israel and detected seropositivity of 4% (20/495), reinforcing the importance of these birds in the epidemiology of diseases, especially as they are birds that can be used as sentinels.

In 2004, 665 blood serum samples from pigeons (*Columba livia*) were collected from 44 farms in 11 Taiwanese municipalities. The samples were tested for *T. gondii* antibodies, and 31 (4.7%) birds were tested positive (TSAI et al., 2006). In a study conducted in Mexico, anti-*T. gondii* antibodies were found in seven (1.3%) of 521 pigeons (*Columba livia*) (ALVARADO-ESQUIVEL et al., 2011), showing a much lower prevalence than that found in this study.

Studies conducted in Brazil have also detected a smaller number of positive samples than those found in this study. In a study conducted in São Paulo, serum samples of 238 pigeons were analyzed using MAD, and 12 (5%) were positive (LIMA et al., 2011). In Jaboticabal, SP, Marciano (2004) tested 126 urban pigeons for *T. gondii* using RIFI and found a low positivity rate of 0.8%. GODOI et al. (2010) studied the natural infection of *T. gondii* in free-living pigeons (*Columba livia*) in Ibiúna and Sorocaba, São Paulo, Brazil. The 126 pigeons were tested negative for anti-*T. gondii* antibodies, as tested using MAD. Bioassays were performed in mice using tissues from all captured birds, and using this technique, *T. gondii* was not isolated from any bird.

These results suggest that the prevalence of *T. gondii* infection in pigeons is low, despite their susceptibility to contamination in urban environments. In Goiânia, Goiás, no study has investigated this parasite in pigeons, thus the lack of comparison of birds from the same region. Of the 150 brain samples analyzed, only three (2%) were positive for *T. gondii* DNA and none of the 150 heart samples generated bands detectable by PCR. The PCR results for the detection of *T. gondii* DNA in pigeons are shown in Table 3.

Table 03 - Results of the PCR test performed on the brains and hearts of pigeons (*Columba livia*) caught in the region of Goiânia, Goiás, between January 10 and November 30, 2012.

PCR results	Brain	Hearts
Positive	3 (2%)	0 (0%)
Negative	147 (98%)	150 (100%)
Total	150 (100%)	150 (100%)

Of the three samples that were tested positive by PCR, only one was positive by serology. This difference may be related to the low number of cysts present in the viscera analyzed or

because only a single fragment of the birds' organs was extracted. There may also have been no formation of *T. gondii* cysts in the pigeons analyzed, even after contact with the oocyst of the parasite.

In a study conducted in São Paulo, Lima et al. (2011) investigated the presence of *T. gondii* in pigeon samples and found that no birds were positive by PCR or by isolation from mice, with only 5% of the samples positive by serology.

An important aspect to highlight is the presence of serology-positive and PCR-negative birds, which can be explained by the immunological reaction that birds show when they come into contact with the parasite but without the formation of cysts in their muscles (DUBEY, 2010). Another possibility is that the fragment analyzed does not contain the parasite, generating a false-negative result for the parasite; that is, although antibodies are present, there may be no parasite cysts in the sample analyzed (AIGNER, 2008; TENTER, 2009; DUBEY, 2010; CASARTELLI-ALVES et al., 2012).

Comparative analysis using the Kappa test between the two diagnostic methods used (serology and PCR) showed that there was no agreement between the two tests and that the results in this study were not comparable. This may have been due to the low number of positive birds in the PCR test, due to the absence of *T. gondii* cysts in these birds, or the extraction of DNA from only one fragment of each viscera, which may have resulted in false negatives.

Serological diagnostic methods have a low accuracy in the diagnosis of infections in birds, and the agent in serologically negative individuals is frequently isolated, as reported by Dubey et al. (2005) and Dubey et al. (2007), who observed that cats fed tissues from seronegative chickens eliminated *T. gondii* oocysts approximately 7 days after infection.

Studies on the pathogenesis, serological diagnosis, and epidemiology of toxoplasmosis in pigeons are limited, making it difficult to assess the involvement and importance of this species in terms of public health (BIANCIFIORI et al., 1986; DUBEY, 2010). Studying the occurrence of *T. gondii* in pigeons can be useful in assessing environmental contamination and verifying possible risks to humans (SICK, 1997; GODOI et al., 2010).

CONCLUSIONS

The presence of anti-*T. gondii* antibodies in pigeons captured in the Goiânia region is high, while the detection of DNA in the brain and heart by PCR is low. There is no agreement between MAD and PCR in the detection of *T. gondii*..

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