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Sensitivity and specificity of serological tests, histopathology and immunohistochemistry for detection of *Toxoplasma gondii* infection in domestic chickens

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ABSTRACT

Since free-range chickens are important for the epidemiology of toxoplasmosis, this study evaluated the sensitivity and specificity of different laboratory techniques for the diagnosis of *Toxoplasma gondii* in these animals. Serum samples from 135 adult domestic chickens were tested for anti-*T. gondii* antibodies by the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), modified agglutination test (MAT), and indirect hemagglutination test (IHAT). Tissue samples from all animals were analyzed by histopathology, immunohistochemistry and mouse bioassay (gold standard). Fifty-four chickens were positive for *T. gondii* in the bioassay. The sensitivity and specificity of the different tests were, respectively, 85% and 56% for ELISA; 80% and 52% for IFAT; 76% and 68% for MAT; 61% and 80% for IHAT; 7% and 98% for immunohistochemistry, and 6% and 98% for histopathology. The MAT was the most effective method for the diagnosis of *T. gondii* infection in chickens, followed by ELISA. Histopathology and immunohistochemistry are useful tools for the diagnosis of *T. gondii* infection in chickens due to their specificity.

1. Introduction

Toxoplasmosis is a worldwide zoonosis caused by the protozoan *Toxoplasma gondii* which affects mammals and birds. Free-range chickens are good indicators of environmental contamination since they feed from the ground, exposing them to infection with *T. gondii* oocysts (Dubey et al., 2012). Another fact of epidemiological importance is that raw or undercooked chicken meat is an important source of infection for humans and other animals when it is consumed or handled without good hygiene (Casartelli-Alves et al., 2012; Dubey et al., 2012; Hill and Dubey, 2013).

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Serological and molecular techniques, histopathology, immunohistochemistry and bioassays in mice or cats (gold standard) are used for the definitive diagnosis of *T. gondii* infection in chickens (Dubey et al., 2007; Yan et al., 2010; Dubey, 2010). Serological tests are frequently employed in epidemiological surveys and, like the polymerase chain reaction (PCR), are sensitive methods for the diagnosis of *T. gondii* infection in chickens (Dubey, 2010; Yan et al., 2010). The modified agglutination test (MAT) is the most commonly used method, whereas techniques such as the enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and indirect hemagglutination test (IHAT) are used less frequently (Dubey, 2010). Although less sensitive, immunohistochemistry and histopathology permit visualization of the parasite and its correlation with histological alterations, which is not possible with serological tests or PCR.

The sensitivity and specificity of diagnostic methods for *T. gondii* infection using the mouse bioassay as the gold standard have been investigated in pigs (Dubey et al., 1995), but not in domestic chickens. In view of the epidemiological role of free-range chickens, an accurate diagnosis of *T. gondii* infection in these animals is very important. The more accurate this diagnosis, the more precise will be the evaluation of environmental contamination with *T. gondii* in the area studied. Therefore, the objective of the present study was to evaluate the sensitivity, specificity and accuracy of IFAT, ELISA, MAT, IHAT, immunohistochemistry and histopathology for the diagnosis of *T. gondii* infection in free-range chickens (*Gallus gallus domesticus*).

2. Materials and methods

2.1. Samples

A non-probabilistic sample of 54 chickens that tested positive for *T. gondii* in the mouse bioassay used as the gold standard was selected for this study. Sample size calculation was not possible due to the wide variety of diagnostic techniques used, the absence of suitable sensitivity values in the literature, costs, and technical feasibility. However, the sample size was sufficient to guarantee an expected mean sensitivity of the serological tests of 70%, an absolute error of 10% at this proportion (range 60%–80%), and a level of significance of 11%. At the end of the study, the power of the sample (54 positive chickens) to calculate the difference in sensitivity between diagnostic techniques was evaluated considering the same level of significance as the sample size (11%). To obtain these 54 chickens, 135 chickens were necropsied and their tissues were tested by mouse bioassay between April 2009 and July 2011. All of these 135 chickens were clinically healthy.

The 135 adult free-range chickens used in this study were raised on 48 farms in the state of Rio de Janeiro, Brazil. Blood was collected from these animals by puncture of the axillary vein for IFAT, ELISA, MAT and IHAT. Next, the animals were euthanized by cervical dislocation and necropsied. After coagulation, the blood samples were centrifuged at $1125 \times g$ and the serum was separated and stored at $-20\,^{\circ}C$ until the time of analysis. During necropsy, samples were collected from the encephalon (cerebrum, cerebellum and brainstem), heart and thigh muscle for histopathology, immunohistochemistry and mouse bioassay. The tissue samples used for histopathology and immunohistochemistry were fixed in 10% buffered formalin and processed for embedding in paraffin (Carson and Hladick, 2009). Fresh tissue samples were used for the mouse bioassay.

2.2. Indirect fluorescent antibody test

The IFAT for detection of anti-*T. gondii* IgG antibodies was performed as described previously (Camargo, 1996) using *T. gondii* RH strain tachyzoites as antigen. Rabbit anti-chicken IgY (IgG) conjugated to fluorescein isothiocyanate (FITC) (Sigma–Aldrich, Brazil) was used. The sera were diluted to 1:16, 1:64, 1:256, 1:1024, and 1:4096 in 0.01 M PBS (pH 7.2). The slides were examined under an Y-FL epifluorescence microscope (Nikon®, USA). Positive and negative controls and a conjugate control were included in each battery of slides. Titers $\geq$16 were defined as positive.

2.3. Enzyme-linked immunosorbent assay

ELISA for detection of anti-*T. gondii* IgG antibodies was performed with the BIOLISA Toxoplasmosis IgG® kit (Bioclin, Quibasa Química Básica, Brazil) according to manufacturer instructions. However, since this kit has been developed for detection of anti-*T. gondii* IgG antibodies in human serum or plasma, some adaptations were made for the diagnosis in chicken serum. For adaptation, the conjugate of the kit was replaced with anti-chicken IgG (whole molecule) peroxidase conjugate® (Sigma–Aldrich, Brazil) using a dilution of 1:30,000. Serum of chickens experimentally infected with *T. gondii* and serum of chickens positive by in-house ELISA (Millar et al., 2012) were used as positive controls. Serum of chickens that tested negative by in-house ELISA, IFAT and IHAT served as negative control. Readings equal to or higher than the cut-off value of the plate were considered to be positive.

2.4. Modified agglutination test

The MAT for detection of anti-*T. gondii* IgG antibodies was performed according to a previously described protocol (Desmonts and Remington, 1980). A suspension of *T. gondii* RH strain tachyzoites fixed in 6% formalin was used as antigen (Silva et al., 2002). The antigen was diluted to 1:16, 1:64, 1:256, 1:1024 and 1:4096. Positive and negative controls were included in each microplate. Titers $\geq$16 were defined as positive.

2.5. Indirect hemagglutination test

The IHAT for detection of anti-*T. gondii* IgG antibodies in chicken serum and in serum of mice used in the bioassay was performed with the Imuno-HAI Toxoplasmose® kit (Wama Diagnóstica, Brazil) according to manufacturer instructions. Positive and negative controls were included in each plate and the cut-off titer was 16.

2.6. Histopathology and immunohistochemistry

For histopathology, two serial 5-μm sections per encephalon, heart and thigh muscle tissue were cut from paraffin blocks, stained with hematoxylin–eosin (Carson and Hladick, 2009), and examined. For immunohistochemistry, one 5-μm section per encephalon, heart and thigh muscle tissue was cut from paraffin blocks, mounted on silanized slides, deparaffinized in xylene, and rehydrated in decreasing ethanol concentrations. Endogenous peroxidase was blocked by incubating the slides in a solution of 30% hydrogen peroxide and methanol (45 ml hydrogen peroxide and 55 ml methanol) for 40 min at room temperature. For antigen retrieval, the slides were incubated in citrate buffer, pH 6.0, at 98 °C for 30 min in a water bath. The Novo Link Max Polymer Detection System® (Novocastra, UK) was used for the detection of *T. gondii* according to manufacturer instructions. The slides were incubated with the primary polyclonal anti-*T. gondii* antibody (rabbit) (Cell Marque, USA) diluted to 1:50, overnight at 4 °C. Histological sections containing tissue cysts and *T. gondii* tachyzoites were incubated with 1.5% bovine serum albumin as negative control and with polyclonal anti-*T. gondii* antibody (rabbit) as positive control.

2.7. Mouse bioassay

The mouse bioassay was performed according to a previously described protocol (Dubey, 1998). A pool of 20 g of brain, heart and thigh muscle collected from each chicken was triturated, homogenized, and digested with an acid pepsin solution (pH 1.1–1.2). Five adult specific pathogen-free female Swiss Webster mice were inoculated intraperitoneally with 1 ml of this homogenate obtained from each chicken (Dubey et al., 2006). A total of 675 mice weighing 20–35 g were inoculated. The control group consisting of one mouse per chicken (total of 135 mice) was inoculated intraperitoneally with 1 ml 0.9% sterile saline.

After inoculation, the mice were monitored daily over a period of 45–50 days. Animals showing clinical signs of toxoplasmosis and those that were still alive at the end of the observation period were euthanized by intraperitoneal injection of an overdose of thiopental sodium and blood was collected by intracardiac puncture for the IHAT. Next, these mice were submitted to necropsy for microscopic tissue analysis (Dubey et al., 2006). The bioassay was defined as positive when microscopic analysis revealed the presence of protozoa that were consistent with *T. gondii* in tissues (tachyzoites or cysts) or in peritoneal exudate (tachyzoites), or when anti-*T. gondii* antibody titers were detected in serum by IHAT in at least one of five mice tested per chicken.

2.8. Statistical analysis

The data were entered using the EpiData 3.1 software and analyzed using the Statistical Package for the Social Sciences, version 16. The sensitivity, specificity and accuracy of the diagnostic techniques were calculated using the results of the mouse bioassay as the gold standard. The sensitivity, specificity and accuracy of the IFAT and MAT according to the different antibody titers used were compared to the gold standard to determine the best cut-off titer for the diagnosis of *T. gondii* infection in chickens. An 89% confidence interval was considered in all analyses.

2.9. Ethical approval

The study was approved by the Ethics Committee for Animal Use of FIOCRUZ (permit no. L-012/09).

3. Results

Fifty-four (40%) of the 135 chickens studied were positive for *T. gondii* in the mouse bioassay. Using the mouse bioassay, the parasite was detected in mouse tissues for 39 (72%) of these 54 positive chickens. In 15 (28%) of the 54 positive chickens submitted to the mouse bioassay, the parasite could not be demonstrated in mouse tissues, but mice were seropositive for *T. gondii* by the IHAT. In the mouse bioassay of the 54 positive chickens, the number of positive mice among the five animals tested was one in the samples of 16 (30%) chickens, two in the samples of four (7%) chickens, three in the samples of eight (15%) chickens, four in the samples of 12 (22%) chickens and five in the samples of 14 (26%) chickens. Neither *T. gondii* nor anti-*T. gondii* antibodies were detected in control animals of the mouse bioassays. Five (9%) chickens with a positive bioassay result were negative in all serological and histological tests.

The power of the sample for comparing the sensitivity of the diagnostic techniques, considering 54 chickens with a positive bioassay result, was 99% for histopathology and immunohistochemistry compared to the serological techniques; 87% for ELISA compared to IHAT; 68% for IFAT compared to IHAT; 45% for MAT compared to IHAT; 25% for MAT compared to ELISA; 12% for ELISA compared to IFAT; 11% for MAT compared to IFAT; and 5% for histopathology compared to immunohistochemistry.

Table 1 shows the sensitivity, specificity and accuracy of the different techniques used for the diagnosis of *T. gondii* in chickens. The sensitivity, specificity and accuracy of IFAT and MAT according to the different antibody titers used for the diagnosis of *T. gondii* infection in chickens are shown in Table 2.

Considering the 54 chickens with a positive result in the mouse bioassay, the false-positive rate was 48% (39/81) for IFAT, 44% (35/80) for ELISA, 32% (26/81) for MAT, 20% (16/79) for IHAT, 2% (2/81) for histopathology, and 2% (2/80) for immunohistochemistry. The false-negative rate was 94% (51/54) for histopathology, 93% (50/54) for immunohistochemistry, 39% (21/54) for IHAT, 24% (13/54) for MAT, 20% (11/54) for IFAT, and 15% (8/54) for ELISA.

Eight chickens were positive for *T. gondii* when the results of immunohistochemistry and histopathology were combined. Only tissue cysts were observed. Five of these animals had tissue cysts only in the heart (Fig. 1A and B), one only in the cerebellum, one only in the cerebrum, and another in the cerebrum and brainstem. There were no histological alterations associated with the presence of cysts. Three of the eight chickens with a positive histological result were negative in the mouse bioassay.

Table 1
Sensitivity, specificity and accuracy of ELISA, IFAT, MAT, IHAT, immunohistochemistry and histopathology for the diagnosis of *T. gondii* infection in 135 chickens using a mouse bioassay as the gold standard.

Technique	% Sensitivity (89% CI)	% Specificity (89% CI)	% Accuracy (89% CI)
ELISA	85 (80–90)	56 (49–63)	68 (61–74)
IFAT	80 (74–85)	52 (45–59)	63 (56–70)
MAT	76 (70–82)	68 (61–74)	71 (65–77)
IHAT	61 (54–68)	80 (74–85)	72 (66–78)
Immunohistochemistry	7 (4–11)	98 (95–100)	61 (55–68)
Histopathology	6 (2–9)	98 (95–100)	61 (54–67)

ELISA: enzyme-linked immunosorbent assay; IFAT: indirect fluorescence antibody test; MAT: modified agglutination test; IHAT: indirect hemagglutination test; 89% CI: 89% confidence interval.

Table 2
Sensitivity, specificity and accuracy of the IFAT and MAT according to the different antibody titers used for the diagnosis of *T. gondii* infection in 135 chickens using a mouse bioassay as the gold standard.

Titer	IFAT			MAT		
	% Se (89% CI)	% Sp (89% CI)	%A (89% CI)	% Se (89% CI)	% Sp (89% CI)	%A (89% CI)
16	80 (74–85)	52 (45–59)	63 (56–70)	76 (70–82)	68 (61–74)	71 (65–77)
64	69 (62–75)	74 (68–80)	72 (66–78)	50 (43–57)	94 (91–97)	76 (70–82)
256	37 (30–44)	91 (87–95)	70 (63–76)	20 (15–26)	96 (94–99)	66 (59–72)
1024	9 (5–13)	99 (97–100)	63 (56–70)	2 (0–4)	99 (97–100)	60 (53–67)

Se: sensitivity; Sp: specificity; A: accuracy; IFAT: indirect fluorescence antibody test; MAT: modified agglutination test; 89% CI: 89% confidence interval.

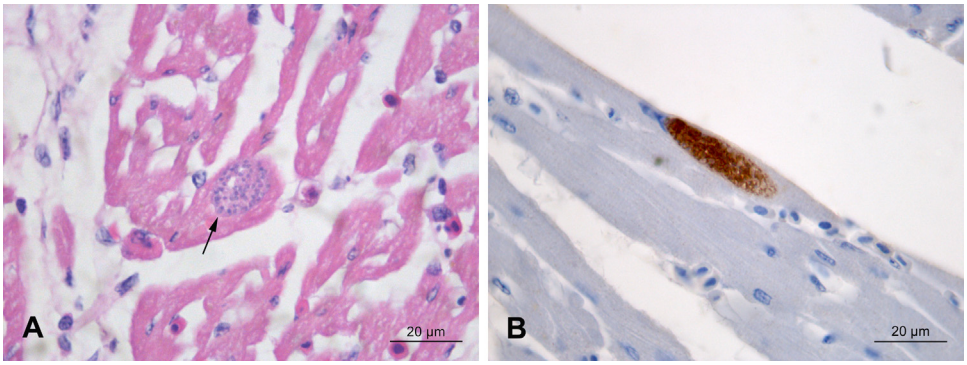

Fig. 1. (A) Histological section of the heart of a chicken showing a *Toxoplasma gondii* cyst (arrow); HE. (B) Immunohistochemistry of the heart of a chicken showing a *Toxoplasma gondii* cyst stained dark brown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Considering not only the mouse bioassay as the gold standard but also the histological tests, the sensitivity and specificity of the different serological tests evaluated were, respectively, 86% and 58% for ELISA, 81% and 54% for IFAT, 77% and 71% for MAT, and 63% and 83% for IHAT.

4. Discussion

Individual analysis of the diagnostic techniques showed that ELISA, followed by the IFAT, was the most sensitive method and IHAT was the most specific method for the serological diagnosis of *T. gondii* infection in chickens. Although the sensitivity of the MAT was lower than that of ELISA and IFAT, its accuracy was higher and very similar to that of IHAT. In addition, the MAT is a simple, low-cost method that does not use a specific conjugate, whereas IFAT and ELISA require the use of a species-specific conjugate and the interpretation of the IFAT result is subjective (Cañón-Franco et al., 2003; Millar et al., 2012). Therefore, the MAT is the most indicated method among those tested for the diagnosis of *T. gondii* infection in chickens in epidemiological surveys designed to detect areas of environmental contamination. These results confirm the efficacy of the MAT in the diagnosis of *T. gondii* infection in chickens demonstrated by other investigators (Silva et al., 2003; Dubey et al., 2006; Dubey, 2010; Beltrame et al., 2012). Although the most sensitive method, ELISA would be a second option since its specificity was one of the lowest among the diagnostic techniques studied. The IFAT was the second most sensitive diagnostic technique, but the least specific. Other investigators (Brandão et al., 2006) also reported limited specificity of the IFAT in detecting anti-*T. gondii* antibodies in chicken serum. The IHAT was found to be a sensitive and specific method in the present study, in agreement with other authors (Beltrame et al., 2012) who observed a sensitivity of 78% and specificity of 83% in relation to the MAT. In contrast, another study (Frenkel, 1981) reported low sensitivity and specificity of the IHAT (46% and 25%, respectively) for the diagnosis of *T. gondii* in birds. The higher sensitivity and specificity of the IHAT observed here and in another study (Beltrame et al., 2012) are probably related to the fact that the same kit

and cut-off value were used in those studies, whereas a different kit and cut-off value were adopted in the other study (Frenkel, 1981). Therefore, as suggested previously (Beltrame et al., 2012), the IHAT using the kit of the present study is an option for the diagnosis of *T. gondii* infection in chickens, particularly in situations in which the MAT, ELISA or IFAT cannot be carried out.

In the present study, all serological methods tested showed limited sensitivity and specificity in chickens. These values, except for the sensitivity of ELISA, were lower than those reported in another study (Dubey et al., 1995) for the serological diagnosis of *T. gondii* in sows. In that study, sensitivity and specificity were 82.9% and 90.2%, respectively, for the MAT, the most sensitive method, and specificity was 98.3% for the IHAT, the most specific method. These differences might be related to the different animal species studied and to the fact that the other authors (Dubey et al., 1995) used bioassays in mice and cats as the gold standard, with the latter being more sensitive. However, comparison of the serological tests should be done with caution because of the limited power of the sample. Therefore, further studies involving a larger sample are needed for a more precise comparison of the different serological tests used for the diagnosis of *T. gondii* in chickens.

The occurrence of false-negative results of *T. gondii* infection in the serological tests evaluated here might be explained by the possibility of recent infection of these chickens when antibody titers are still not detectable. Chickens experimentally infected with *T. gondii* develop antibody titers detectable by MAT and IHAT on day 7 post-infection and by ELISA and IFAT on day 14 post-infection (Dubey et al., 1993; Sedlák et al., 2000; Yan et al., 2010). Another explanation would be a decline in the antibody titer of these animals to undetectable levels during chronic infection. The latter hypothesis, which has also been raised by other investigators for sows (Dubey et al., 1995), is more likely due to the fact that only adult birds were studied which could have been infected for many months or years. Other authors using the MAT (Silva et al., 2003) and IFAT (Brandão et al., 2006) also reported positive bioassay results in serologically negative chickens.

There were several cases of false-positive results of the serological tests. One possible explanation is cross-reactivity with other parasites of the phylum Apicomplexa, such as *Sarcocystis* or *Neospora* which naturally infect free-range chickens (Munday et al., 1977; Martins et al., 2011). Cross-reactivity in serological tests has been observed between *T. gondii* and *Neospora caninum* in dogs (Silva et al., 2007) and between *T. gondii* and *Sarcocystis* in pigs (Moon, 1987; Lind et al., 1997). However, further studies on chickens are needed to confirm this hypothesis. Another explanation of the false-positive serological results in relation to the bioassay might be explained by failure of the latter technique. False-negative bioassay results can be due to the fact that the tissue samples of infected chickens used for this assay were not parasitized, contained non-viable parasites, or had a low parasite burden and were therefore unable to infect mice, a fact also reported for pigs (Dubey et al., 1995). Additionally, the tissue digestion step of the bioassay may reduce *T. gondii* viability (Dubey et al., 1995). The three cases of chickens with a negative bioassay result in which *T. gondii* cysts were detected by histology confirm that the mouse bioassay is not 100% sensitive. As a consequence of this limited sensitivity of the mouse bioassay, the sensitivity and specificity of the serological tests evaluated were slightly underestimated. These underestimated values were demonstrated by the slight increase in the sensitivity and specificity of the serological tests evaluated when the histological tests were used as gold standard in addition to the mouse bioassay.

The sensitivity and specificity of the IFAT and MAT were also analyzed in the present study according to the different antibody titers used, since different cut-off values ranging from 8 to 40 for the IFAT and ≥5 for the MAT (Dubey, 2010) have been reported for the diagnosis of *T. gondii* infection in chickens. A cut-off value ≥16 used in the two tests showed the highest sensitivity in the present study and is recommended for the diagnosis of *T. gondii* infection in chickens.

In contrast to the serological techniques studied, histopathology and immunohistochemistry showed very low sensitivity and high specificity. It should be noted that the power of the sample for comparison of the histopathological and immunohistochemical results with the serological results was highly satisfactory. However, despite the similar specificity and sensitivity of histopathology and immunohistochemistry, the power of the sample was very low to confirm this similarity. The low sensitivity of histopathology and immunohistochemistry might be related to broad distribution of the protozoan in tissues (Tenter et al., 2000), a low parasite burden, and the small size of the tissue sample examined (5-μm sections). In contrast to the present study, *T. gondii* was not detected by histopathology in muscle or brain of infected pigs (Garcia et al., 2006). On the other hand, *T. gondii* was detected by immunohistochemistry in at least one organ (brain, liver, or heart) of 46 seropositive sheep (Silva et al., 2013). These results demonstrate that the concentration of tissue cysts of *T. gondii* per gram of brain or heart tissue in infected chickens may be higher than in infected pigs and lower than in infected sheep.

5. Conclusions

The MAT was the most effective method for the serological diagnosis of *T. gondii* infection in chickens. ELISA was also a sensitive method and could be a second option for use in serological surveys of chickens. Cut-off titers ≥16 in both the MAT and IFAT were found to be effective for the diagnosis of *T. gondii* infection in chickens. Histopathology and immunohistochemistry are useful tools for the diagnosis of *T. gondii* infection in chickens due to their high specificity.

Conflict of interest statement

There is no conflict of interest.

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