ORIGINAL ARTICLE

INFECTION RATE BY *Toxoplasma gondii* IN FREE-RANGE AND BROILER CHICKENS IN THE STATES OF RIO GRANDE DO NORTE AND PARAÍBA, BRAZIL

Maria Cecília Farias dos Santos[1], Ramayana Morais de Medeiros Brito[1,3], Ricardo Wagner de Almeida Vitor[2,] and Valter Ferreira de Andrade-Neto[1]

ABSTRACT

Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii*, a protozoan that has a cosmopolitan geographic distribution and low specificity for intermediate hosts. Domestic chickens are among the most important hosts in toxoplasmosis epidemiology, since they are potential sources of infection for humans, in addition to indicating environmental contamination by protozoa. In this work, we studied the prevalence of *T. gondii* infection in chickens in different breeding systems from distinct mesoregions of Rio Grande do Norte and Paraiba States: broiler chickens from commercial farms and free-range chickens from small farms. Indirect fluorescent antibody test (IFAT) and ELISA techniques were used for detecting specific antibodies in blood samples from the birds. There were no seropositive samples among the broilers tested, indicating that intensive management limited the chances of infection for these animals. Among the free-range chickens, the frequency of IgG anti-*T. gondii* detected by IFAT and ELISA were 37.9% and 40.4% respectively. Among the seropositive samples by IFAT, 33 (27.1%) were positive at a dilution of 1:16; in 1:32, 31 (25.4%); in 1:64, 24 (19.7%); 15 (12.3%) in 1:128, and 19 presented titers greater than or equal to 1:256 (15.6%). The substantial concordance observed between IFAT and ELISA signifies these methods are effective methodologies for the diagnosis of avian toxoplasmosis. The high prevalence of specific antibodies among poultry in the studied region highlights the potential risk of *T. gondii* transmission to humans through consumption of infected meat.

KEY WORDS: *Toxoplasma gondii*; chicken infection; toxoplasmosis transmission; seroprevalence.

1. Federal University of Rio Grande do Norte, Biosciences Center, Department of Microbiology and Parasitology, Natal, Rio Grande do Norte, Brazil.
2. Federal University of Minas Gerais, Institute of Biological Sciences, Department of Parasitology, Belo Horizonte, Minas Gerais, Brazil.
3. Graduate Program in Parasitary Biology, Departament of Microbiology and Parasitology, Federal University of Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil.

Maria Cecília Farias dos Santos – https://orcid.org/0000-0002-9052-5761; Ramayana Morais de Medeiros Brito: Orcid.org/0000-0003-0611-6859; Ricardo Wagner de Almeida Vitor: Orcid.org/0000-0002-8599-0474; Valter Ferreira de Andrade Neto: Orcid.org/0000-0002-9986-0865

Corresponding author: Valter Ferreira de Andrade-Neto. Department of Microbiology and Parasitology, Biosciences Center, Federal University of Rio Grande do Norte, Av. Senador Salgado Filho 3000, CEP 59078-970, Natal, Rio Grande do Norte, Brazil. E-mail: vfan.aneto@gmail.com and/or aneto@cb.ufrn.br

INTRODUCTION

Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii*, an obligate intracellular parasite from the Alveolata group (Adl et al., 2012) causing severe symptoms in congenitally infected newborns and in immunosuppressed individuals (Furtado et al., 2011; McAuley, 2014). Over the past 50 years, The Americas have been responsible for 73.5% of human toxoplasmosis outbreaks with Brazil accounting for 35.3% of the published outbreaks. In addition, the source of infection was mainly consumption of contaminated meat and its derivatives (47.1%) followed by ingestion of oocysts (44.1%) (Pinto-Ferreira et al., 2019). Close contact with domestic animals plays an important role in the epidemiology of toxoplasmosis an indicator of environmental contamination with *T. gondii* oocysts acting as potential sources of infection (Shapiro et al., 2019).

An important aspect related to the prevalence of this protozoan infection in birds is the role these animals play in transmitting *T. gondii* to carnivores through the consumption of infected meat (Nardoni et al., 2019; Khan et al., 2020). Almost twenty years ago, Literak & Hejlicek (1993) had already drawn attention to the fact that chickens from small farms could contain *T. gondii* tissue cysts, risking human infection. Two years later, Camargo et al. (1995) observed that the proportion of individuals who had contact with chickens raised in their backyards was statistically higher in the group with reactive serology than in the group with non-reactive serology, suggesting a relationship between contact with domestic chickens and the increased chances of acquiring infection. Thus, domestic chickens can be reservoirs, playing a crucial role in the epidemiology of toxoplasmosis, as a source of infection to humans (Dubey, 2010).

Chickens reared intensively present low infection rates due to correct management, restriction and hygiene procedures that reduce or even prevent contact with *T. gondii* infection sources (Dubey, 2010; Khan et al., 2020). Recently, consumers have become more concerned about animal welfare, leading to an inflated demand for production systems that reduce animal suffering, such as free-range and organic systems. These methods have spread rapidly throughout the world (Cobanoglu et al. 2014), however, evidence suggests that the health of animals raised on free and organic farms is not always better than that observed in animals raised in conventional livestock production systems (Millar et al., 2012; Tomza-Marciniak et al., 2014; Ferdushy et al., 2016). Seroprevalence analysis of *T. gondii* infection in birds revealed a higher prevalence in free-range chickens than in broilers (Khan et al., 2020). Previous studies conducted in Brazil reported *T. gondii* infection prevalence ranging from 27.9% up to 48.9% in free-range chickens (Sá et al., 2017; Braz et al., 2020; Silva et al., 2020).

Many techniques are utilized for the diagnosis of avian *T. gondii* infection, mainly based on DNA detection and investigation of antibodies against the parasite through serological testing (Liu et al., 2015; Nunes et al.; 2015; Khan et al., 2020). The importance of this diagnosis implies the adoption of specific criteria in the choice of an efficient method, capable of providing reliable results. Regarding animal infection, indirect fluorescent antibody test (IFAT), modified agglutination test (MAT), indirect haemagglutination assay (IHA) and enzyme-linked immunosorbent assay (ELISA) are the most used techniques for routine diagnosis and serological surveys (Casartelli-Alves et al., 2014; Dard et al., 2016; Schares et al., 2018; Sharma et al., 2019). However, the lack of standardized guidelines and inexistence of a gold standard for the diagnosis of avian toxoplasmosis hamper calculation of parameters such as sensitivity and specificity of serological techniques employed to obtain accurate results. Therefore, the main purpose of this study was to determine the occurrence of anti-*T. gondii* antibodies in chickens raised under different systems (free-range and confined chickens) and compare the efficiency of ELISA and IFAT as diagnostic tools.

MATERIAL AND METHODS

Study design

Five hundred twenty two chickens were evaluated, 322 free-range chickens (of which 76 were young chickens from the East Potiguar Mesoregions) and 200 confined broilers. The animals were randomly selected and the sampling locations were East and West Potiguar Mesoregions in the State of Rio Grande do Norte, and Agreste (semi-arid transition region) and Borborema (plateau area) regions in the State of Paraíba, Northeast of Brazil. The samples were collected from November 2018 to November 2019. This work is part of a research project on animal toxoplasmosis carried out over the last eight years.

Free-range chicken samples were collected from 15 rural properties in the following municipalities: Macaíba, Martins and Felipe Guerra, in the State of Rio Grande do Norte; and Pocinhos, Gado Bravo, Boqueirão, Massaranduba, Queimadas and Campina Grande, in the State of Paraíba. Confined chicken samples were collected from three intensive breeding (broiler) commercial farms in the State of Paraíba in the municipalities of Pocinhos, Soledade and Puxinanã (Figure 1). The properties selected for the study were based on non-probabilistic sampling for convenience, in view of the lack of registration lists of the flocks of domestic chickens bred in those States.
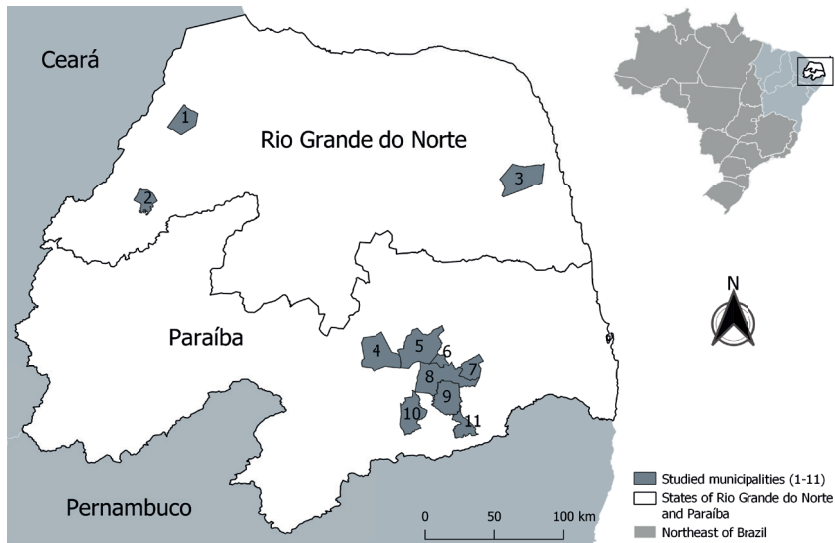
Figure 1. Map of Rio Grande do Norte and Paraíba states, in the Northeast of Brazil, evidencing the municipalities where the samples were collected.
1- Felipe Guerra (05°36'09''S, 37°41'19''W); 2- Martins (06°05'16''S, 37°54'40''W); 3- Macaíba (05°51'30''S, 35°21'14''W); 4- Soledade (07°03'26''S, 36°21'46''W); 5- Pocinhos (07°04'36''S, 36°03'40''W); 6- Puxinanã (07°09'40''S, 35°57'38''W); 7- Massaranduba (07°12'01''S, 35°47'21''W); 8- Campina Grande (07°13'50''S, 35°52'52''W); 9- Queimadas (07°21'30''S, 35°53'54''W); 10- Boqueirão (07°28'54''S, 36°08'06''W); 11- Gado Bravo (07°35'00''S, 35°47'27''W).

Chickens and serum samples

All procedures used in this study were authorized for publication following approval by the Research Ethics Committee of the Federal University of Rio Grande do Norte (CEUA/UFRN 76/2015).

Blood samples were obtained by puncturing the brachial vein of the birds (approximately 0.8 mL/bird) and kept at room temperature for clot retraction. Sera were transported in containers to the Laboratory of Malaria Biology and Toxoplasmosis, Biosciences Center of the Federal University of Rio Grande do Norte. The serum samples were obtained by centrifugation at 2.500 g for 15 minutes, aliquoted into Eppendorf microtubes and stored at -20 °C until serological tests were performed.

Seroprevalence using indirect fluorescence antibody test (IFAT)

IFAT was performed according to the method described by Chiari et al. (1987), with modifications. The antigen used here was prepared with tachyzoites of *T. gondii* RH strain, obtained by peritoneal cavity wash with phosphate-buffered saline (PBS, pH 7.2) of infected Swiss mice. The suspension obtained was transferred to labeled slides and the formalized tachyzoites fixed through drying at room temperature. The prepared slides were stored in appropriate boxes and kept at -20 °C until use.

For IFAT, sera were diluted from 1:16 to 1:256 in phosphate buffered saline (PBS) pH 7.2 to detect specific IgG antibodies. The diluted samples were transferred to previously prepared antigen-sensitized slides, in duplicate. One positive and two negative control sera were used from *T. gondii* infected and non-infected chickens respectively. The slides were coverslipped and examined through a fluorescent microscope (Olympus IX70-FLA; excitation filter BP460-490; barrier filter BA515IF). The samples were considered reactive when the tachyzoites exhibited complete fluorescence. Sera with titers equal to or greater than 1:16 were considered positive, according to Chumpolbanchorn et al. (2009).

Serological frequency using ELISA assay

ELISA was performed according to the technique described by Voller et al. (1976), with modifications described by Clementino et al. (2007). The antigen used here was obtained, as previously described (Clementino et al., 2007), in a concentration of 0.05 μg/well. For the preparation of the antigen, peritoneal exudate was collected from mice, previously inoculated with the RH strain of *T. gondii*. The material was centrifuged twice in PBS pH 7.2 and 10 mL of PBS pH 7.2 were added to the remaining pellet. Then the parasites were counted in a Neubauer chamber and the final concentration adjusted to 1×10^9 tachyzoites/mL. This suspension was processed by ultrasound in five cycles of 40 hertz (in an ice bath), for one minute with one-minute intervals between each cycle. The breaking of the parasites was monitored under an optical microscope. After sonication, the material was centrifuged at 15.000 g at 4ºC for 30 minutes. The supernatant was stored at -20ºC until use. The protein concentration was determined by the Lowry method (1951). Nine control sera were used: three positive and six negative chickens previously tested by IFAT and/or ELISA. Sera were diluted at 1:100 and the conjugate (anti-chicken IgG peroxidase antibody) (Sigma Chemical Company) diluted at 1:4000 followed by the addition of o-phenylenediamine in citric acid solution plus H_2O_2. Absorbance was read at 492 nm using an ELISA reader (BIO-RAD Laboratories).

The cut-off point for the ELISA was the mean absorbance of six *T. gondii* negative chicken serum samples plus three standard deviations tested on each plate. The average of the sera tested in duplicate was divided by the value of the plate cut-off point, in order to determine the reactivity index (RI). The sera with RI values equal to or greater than 1 were considered positive (Altcheh et al., 2006). To ensure the reproducibility of the test and the reliability of the results, ELISA was repeated at least three times.

Statistical analysis

For data analysis, the frequency of positive/negative samples and the estimated prevalence of *T. gondii* infection were evaluated for the serological techniques used with estimates of 95% confidence intervals (CI). The correspondence analysis was performed to evaluate the correlation between the number of positive and negative samples by the two serological techniques. In order to compare IFAT and ELISA, the McNemar test was used to evaluate the equality between the positivity proportions generated by each of the techniques.

The concordance between tests was verified by the Kappa index, where levels of comparison to assess the index were: >0.81 (almost perfect); 0.61 to 0.8 (substantial concordance); 0.41 to 0.6 (moderate concordance); 0.21 to 0.4 (reasonable concordance); 0 to 0.2 (weak concordance); 0 (poor concordance) (Landis & Koch, 1977); the determination of concordance values, co-positivity (PPV – Positive Predictive Value) and co-negativity (NPV – Negative Predictive Value), and prevalence were made through a 2x2 contingency table. The basis of this approach is that the correlation between the methods is evidence of validity, while the non-concordance suggests that the tests are not reliable (Watson & Petrie, 2010).

The Chi-squared test and multifactorial ANOVA analyses were performed to compare seropositivity and the variables related to the characteristics of the animals, using the Minitab 15 software. P values <0.05 were considered significant.

RESULTS

The distribution of the positive samples according to the municipalities are in Table 1. The age distribution was 76 (23.6%) young animals and 246 (76.4%) adult animals. From the total of adult animals, 22 (8.9%) birds were male and 224 (91.1%) female.

Table 1. Frequency of anti-*Toxoplasma gondii* antibodies in the serum of free-range chickens naturally infected, stratified according to the municipality of each sample.

Municipality	Number of properties	Examined samples	Positive samples IFAT (%) Number of positives for each titer (%)					Positive Totals (%)	ELISA (%)
			1:16	1:32	1:64	1:128	≥1:256		
Boqueirão	1	24	-	5 (29.4)	4 (23.5)	1 (5.8)	7 (41.1)	17 (70.8)	15 (65.5)
Campina Grande	2	29	3 (15.8)	3 (15.8)	3 (15.8)	3 (15.8)	7 (36.8)	19 (65.5)	22 (75.9)
Felipe Guerra	4	49	8 (33.3)	5 (20.8)	4 (16.7)	5 (20.8)	2 (8.3)	24 (48.9)	27 (55.1)
Gado Bravo	1	4	-	-	1 (100)	-	-	1 (25)	1 (25)
Macaíba	1	76	-	-	-	-	-	0	0
Martins	2	30	6 (37.5)	5 (31.2)	2 (12.5)	2 (12.5)	1 (6.1)	16 (53.3)	13 (43.3)
Massaranduba	1	22	6 (54.5)	2 (18.2)	1 (9.1)	-	2 (18.2)	11 (50)	6 (27.3)
Pocinhos	2	60	8 (25.8)	10 (32.3)	9 (29)	4 (12.9)	-	31 (51.7)	38 (63.3)
Queimadas	1	28	2 (66.7)	1 (33.3)	-	-	-	3 (10.7)	8 (28.6)
Total	15	322	33 (27) (43.4 - 55.8)[1]	31 (25.4) (18.5 - 33.8)[1]	24 (19.7) (13.6 - 27.6)[1]	15 (12.3) (7.6 - 19.3)[1]	19 (15.6) (10.2 - 23.1)[1]	122 (37.9) (43.40 - 55.80)[1]	130 (40.4) (35.16 - 45.81)[1]

[1] Confidence Interval (CI) 95%. IFAT: indirect fluorescent antibody test.

From the total number of free-range chickens analyzed, 155 were reactive (48.1%, CI 95% 42.56-53.74) and 167 (51.9%, CI 95% 46.25-57.43) non-reactive in at least one of the two methodologies studied. Analyzing each technique individually, 122 samples (37.9%, 95% CI 32.76-43.30) were reactive for the presence of anti-*T. gondii* antibodies by IFAT and 130 samples were reactive by ELISA (40.4%; 95% CI: 35.16-45.81) (Table 1). All the 200 samples from confined chickens were non-reactive for anti-*T. gondii* antibodies in both tests.

In the IFAT titration, the highest number of reactive sera was observed at 1:16 dilution (Table 1). Regarding the ELISA, the animals with positive sera were those with RI values greater than or equal to 1, and the concentration of the highest number of reactive birds exhibited RI ≥1 and <2 (Figure 2).
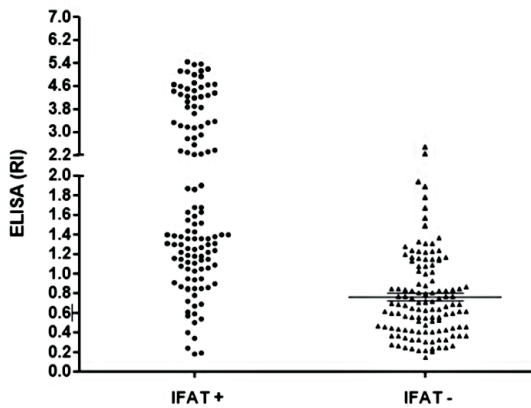

Figure 2. Representative data of the reactivity index (RI) values of each analyzed sample of adult free-range chickens by ELISA, grouped according to the positive or negative results from indirect fluorescent antibody test (IFAT).

The highest values of seropositivity were observed in the municipalities of Campina Grande and Boqueirão, according to the data presented by IFAT and ELISA (Table 1). The proportion of positive farms was 100% for all regions except East Potiguar, and the region with the highest proportion of positive samples was Borborema with a positivity of 70.8% and 62.5% for IFAT and ELISA, respectively (Table 2).

No positive samples were identified in the East Potiguar Mesoregion, therefore excluding the young birds from the analysis of gender and age. When evaluating the seropositivity of these animals according to gender, 40.9% of the males and 50.4% of females reacting to *T. gondii* were detected by IFAT, whereas using ELISA seropositivity was found in 31.8% and 54.9%, respectively. However, the Chi-squared test showed significant differences only for ELISA (Table 3).

According to the McNemar test, there was no significant difference between the positivity ratios between the two serological techniques (X^2 = 1.1034; p = 0.2935). When IFAT test was compared to ELISA, a co-positivity value of 74.6% (PPV=0.746) and co-negativity of 86.9% (NPV=0.869) was observed. In this comparison, a prevalence of 37.8% (CI95% 32.76–43.30) was noted. Likewise, when comparing ELISA to IFAT, similar results were obtained. The co-positivity analysis revealed a value of 79.5% (PPV=0.795) and co-negativity of 83.5% (NPV=0.835). Here, the prevalence obtained was 40.3% (CI95% 35.16–45.81). These comparisons revealed an observed concordance of 82% and a kappa index of 0.62, which indicates a substantial concordance between the analyzed methodologies.

Table 2. Frequency of anti-*Toxoplasma gondii* antibodies in the serum of free-range chickens naturally infected, stratified according to the mesorregion of each sample.

Mesoregion	Examined samples	Positive samples	
		IFAT (%)	ELISA (%)
West Potiguar	79	40 (50.63)	40 (50.63)
East Potiguar	76	0	0
Agreste Paraibano	143	65 (45.45)	75 (52.45)
Borborema	24	17 (70.83)	15 (62.50)
Total	322	122 (37.88) (32.76 - 43.30)[1]	130 (40.37) (35.16 - 45.81)[1]

[1] Confidence Interval (CI) 95%. IFAT: indirect fluorescent antibody test.

Table 3. Frequency of anti-*Toxoplasma gondii* antibodies in the serum of free-range chickens naturally infected, stratified according to age and gender of each animal.

Variables		Examined samples	Positive samples							
			IFAT				ELISA			
			n	%	X^2	p	n	%	X^2	p
Age	Young	76	0				0			
	Adult	246	122	49.59			130	52.84		
Gender[1]	Male	22	9	40.90			7	31.81		
	Female	224	113	50.44	0.7993	0.3713	123	54.91	4.2869	0.0384

[1] Confidence Interval (CI) 95%; evaluating only 246 adult animals. IFAT: indirect fluorescent antibody test.

DISCUSSION

In the present study, a high seroprelavence of *Toxoplasma gondii* infection was detected in adult free-range chickens in the states of Rio Grande do Norte and Paraíba, Northeast of Brazil, showing that 48.1% of the analyzed birds were reactive for anti-*T. gondii* specific antibodies in at least one of the two applied methodologies. The prevalence of *T. gondii* infection in adult birds can be the result of longer exposure to the parasite when compared to young birds. This fact is in accordance with data shown by Moré et al. (2012), which presented a difference of infection positivity between young and adult chickens, varying from 3% to 50%, respectively, on six farms in Argentina. On the other hand, it is important to highlight that different factors may also be related to the seronegativity of young free-range chickens, such as management procedures and less exposure to risk factors. In addition, in the present study, the population of young animals surveyed originated from only one rural property, in the East Potiguar Mesoregion, totaling the entire sample. There is a possibility that animals from the same herd or flock of birds, react homogeneously when they are kept in similar conditions, while the dispersion of toxoplasmosis can vary in animals of different origins (Chiari et al., 1987).

In general, looking at the adult bird population, the seroprevalence found here was similar to that observed by other researchers also studying free-range chickens (Brandão et al., 2006; Dubey et al., 2002; Dubey et al., 2003a; Dubey et al., 2007a; De Oliveira et al., 2009; Dubey et al., 2010). As in Brazil, reports in the world literature on the research of *T. gondii* antibodies in chickens have differing prevalences (Dubey et al., 2003b; Dubey et al., 2003c; Dubey et al., 2004; Dubey et al., 2005; Dubey et al., 2006a; Dubey et al., 2006b; Dubey et al., 2006c; Dubey et al., 2007b; Dubey et al., 2008), which may reflect the varying levels of infectivity in the studied ecosystems or variations in the sensitivity of applied techniques (Garcia et al., 2000).

Regarding the gender of the free-range chickens evaluated in this work, the sample consisted of 224 females and 22 males, considering only adult birds. This difference in gender proportion is justified, as on small farms the sex ratio of males is usually lower, due to reproductive aspects and the welfare of the birds. Although the statistical analysis of the data showed a significant difference between the prevalence of anti-*T. gondii* antibodies in males and females only by ELISA, the gender role in this proportion can be questionable, due to the low number of males evaluated. Ueno et al. (2009) verified the prevalence of *T. gondii* infection in sheep and argued that the divergence of results between genders does not obey a coherent biological explanation and, therefore, this fact would be due to chance.

Analyzing the reactivity index (RI) of the samples tested by ELISA, the distribution of positive samples was concentrated between RI values ≥1 and <2. On none of the properties did the chickens have excessively high titers, as observed in previous studies (Holsback et al., 2012; Vieira et al., 2018). Through experimental infection of domestic chickens, a progressive decrease in antibody titers after reaching a peak at 35 days after infection with antibodies still detectable by direct agglutination at 63 days after infection (Galli et al., 2008).

In general, *T. gondii* seropositivity in animals raised for human consumption does not necessarily reflect the risk of infection to humans, due to variations in the organotropism of the parasite in different host species (Dubey & Jones, 2008; Cenci-Goga et al., 2011). However, cultural eating habits can be considered an infection risk factor. In Brazilian cuisine, the chicken heart is traditionally used in local gastronomy, usually served as a starter or "appetizer", or even in the form of skewers. In such cases, the cooking may not occur homogeneously, favoring the viability of possible tissue cysts present in the bird's cardiac musculature (Dubey et al., 1993; Kaneto et al., 1997), posing a serious infection risk.

Our results demonstrate that the absence of seropositivity among the broilers analyzed highlights the little epidemiological importance of these animals in the transmission of *T. gondii*, indicating that adequate management, in this breeding system, contributes enormously to animal protection. However, the high seropositivity of free-range chickens indicates that the protozoan is widespread in the states of Rio Grande do Norte and Paraíba, indicating that these animals may be bioindicators of environmental contamination by oocysts. Last but not least, the cultural custom in Brazilian cuisine of eating undercooked chicken hearts can be a source of infection for humans, serving as a maintainer of human infection. The concordance observed in the IFAT and ELISA results was considered substantial as well as the seroprevalence noted using both tests; this fact shows the relevance of this study. Finally, these data showed that both techniques are appropriate for carrying out epidemiological surveys of *T. gondii* infection in chickens.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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