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Toxoplasma gondii in free-range chickens from Bahia State: serology, meta-analysis, associated factors and spatial analysis

Toxoplasma gondii em frangos criados de forma extensiva do estado da Bahia: sorologia, meta-análise, fatores associados e análise espacial

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ABSTRACT

Introdução: Galinhas criadas soltas têm sido amplamente estudadas como marcadores de contaminação ambiental por *Toxoplasma gondii*, um coccídeo de felídeos que causa uma importante zoonose e um agente de abortamento em animais de produção. **Objetivo**: Este trabalho teve como objetivo avaliar a frequência de infecção em galinhas criadas em propriedades rurais de Feira de Santana, Bahia, Brasil, e os fatores associados às taxas de positividade. **Métodos:** Um total de 290 amostras de sangue foi obtido de aves de 15 propriedades e examinado pelo teste de aglutinação modificada em diluições iguais ou superiores a 1:25. Dados sobre as condições de criação das aves e o contato com outros animais foram coletados. A coleta de amostras foi realizada entre março e setembro de 2016. **Resultados:** A frequência de soros reagentes (49,7%) em Gallus gallus pode ser considerada similar a outros estudos brasileiros, no entanto, os títulos encontrados foram menores ou iguais a 100 em 90% das aves, indicando potenciais infecções agudas. **Conclusão:** Entre os fatores pesquisados, a área de origem das amostras, a presença de gatos, gatos alimentados com carne crua e a ausência de cães com menos de seis meses foram significativamente associados às taxas de infecção das aves pela regressão logística. A análise de aglomeração espacial mostra uma área de concentração de aves infectadas.

Descriptors: *Toxoplasma gondii*; *Gallus gallus*; galinha caipira; epidemiologia; fatores de risco.

RESUMO

Introdução: Galinhas criadas soltas têm sido amplamente estudadas como marcadores de contaminação ambiental por *Toxoplasma gondii*, um coccídeo de felídeos que causa uma importante zoonose e um agente de abortamento em animais de produção. **Objetivo:** Este trabalho teve como objetivo avaliar a frequência de infecção em galinhas criadas em propriedades rurais de Feira de Santana, Bahia, Brasil, e os fatores associados às taxas de positividade. **Métodos:** Um total de 290 amostras de sangue foi obtido de aves de 15 propriedades e examinado pelo teste de aglutinação modificada em diluições iguais ou superiores a 1:25. Dados sobre as condições de criação das aves e o contato com outros animais foram coletados. A coleta de amostras foi realizada entre março e setembro de 2016. **Resultados:** A frequência de soros reagentes (49,7%) em *Gallus gallus* pode ser considerada similar a outros estudos brasileiros, no entanto, os títulos encontrados foram menores ou iguais a 100 em 90% das aves, indicando potenciais infecções agudas. **Conclusão:** Entre os fatores pesquisados, a área de origem das amostras, a presença de gatos, gatos alimentados com carne crua e a ausência de cães com menos de seis meses foram significativamente associados às taxas de infecção das aves pela regressão logística. A análise de aglomeração espacial mostra uma área de concentração de aves infectadas.

Descritores: *Toxoplasma gondii*; *Gallus gallus*; galinha caipira; epidemiologia; fatores de risco.

INTRODUCTION

Infection by the protozoan parasite *Toxoplasma gondii* is common in animals and humans worldwide[1]. In Brazil, human infection rates range from 50 to 80%, and the congenital disease can affect one in every 1.000 live births[2]. Ingestion of oocysts present in the environment and consumption of meat contaminated with parasitic cysts are the most common forms of infection[3].

Detected in chickens for the first time in Germany in 1939[4], infection by *T. gondii* in these birds gained importance in the study of the biology of the parasite, as it is a relatively

Authors' contributions: LMSMMG conducted the fieldwork and laboratory analyses, VSFF carried out additional data analyses, wrote and reviewed the literature and the final manuscript, JEC performed laboratory analyses and reviewed the final manuscript, ELC assisted with laboratory analyses and reviewed the final manuscript, APS conducted the spatial analysis and reviewed the final manuscript, AVS planned and supervised the fieldwork and laboratory activities, analyzed the epidemiological data, performed the meta-analysis, and reviewed the final manuscript.

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simple source of isolates for phylogenetic and pathogenicity studies[4]. Epidemiological studies determining the factors associated with bird infection. Nevertheless, they are scarce, and most of them address aspects limited to the type of breeding or origin of the birds.

According to Dubey[4], in rural environments, extensively raised birds play an important role in the epidemiology of toxoplasma infection. As they are clinically resistant to infection and live longer than other species preyed upon by felines.

Although the habit of turning over the ground and feeding directly on the ground is an obvious risk for oocyst ingestion and consequent infection in extensively raised birds. Dubey and collaborators[5] monitored 90 sentinel birds during a harsh winter, when the birds could not feed directly on the ground, and observed differences in infection rates on the three properties studied. However, they did not determine which factors would be decisive for bird infection. The main associated factor in most studies is the presence of cats in the environment where the birds are raised[4]. Access of these animals to food or water sources is also associated with infection of birds and other animals in rural environments[4].

Wetter areas allow *T. gondii* oocysts to survive for longer[6], and this factor was associated with a higher frequency of seropositivity in dry lands[7].

Da Silva *et al.*[8] found higher rates of Toxoplasma infection in birds from urban and suburban areas, compared to rural areas. This difference was attributed to the greater space available in rural areas for garbage disposal, reducing the chance of bird infection. This hypothesis was reinforced by the results of Braz *et al.*[9], who also found more positive birds in urban environments. This could be one of the factors significantly associated in the multivariate analysis, in addition to the use of cages, rodent presence and use of food scraps.

This study aimed to evaluate the frequency of anti-*T. gondii* antibodies in birds raised extensively on rural properties in Feira de Santana, Bahia (BA), Brazil, verifying characteristics of the properties and the presence of other animals associated with infection rates.

METHODS

This is a cross-sectional epidemiological study with spatial analysis components to detect anti-*Toxoplasma gondii* antibodies in chicken sera samples; to compare the positivity with the results obtained through meta-analysis, and to investigate potential associations between chicken infection and environmental and behavioral variables on the studied properties. A spatial cluster analysis has added a geographical dimension to the study, helping to identify localized infection patterns. Before the start of sample collections, the project was submitted to the Animal Use Ethics Committee of the State University of Feira de Santana (CEUA/UEFS) and approved by official letter 005/2013.

Study area and sample collection:

The study area for sampling was the Maria Quitéria District (Figure 1), located in Feira de Santana - BA (12°8'56" S. 38°59'21"W), which represents 0.231% of the area of the state. It is the most populated rural area with the highest number of households in the municipality, with 13.903 human inhabitants and 4.345 households[10]. In the absence of a previous survey, the households were indicated by community health agents (CHAs) from the Basic Health Unit of São José, a town in the district. characterizing the sampling as non-probabilistic convenience. The households belonged to two areas monitored by the CHAs.

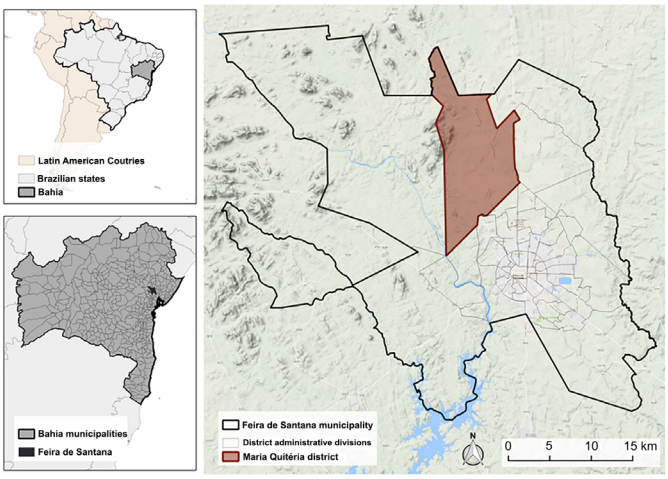

Figure 1. Location of the Maria Quitéria District in Feira de Santana, BA, Brazil.

A total of fifteen rural properties were visited, sampling up to 30 birds per property, for a total of 290 individuals (P1=30, P2=19, P3=16, P4=30, P5=14, P6=12, P7=38, P8=11, P9=13, P10=15, P11=19; P12=22, P13=31, P14=7, P15=13), between march to September 2016. Blood samples from the birds were collected by puncturing the radial vein and stored in 1.5 mL microtubes. These samples were centrifuged at 1600 g for ten minutes; the serum obtained was frozen and kept at -20°C until processing for the anti-*T. gondii* antibody detection tests.

Detection of anti-*Toxoplasma gondii* antibodies:

Antigen used in the serological tests was produced from the RH strain of *T. gondii*, adjusting the final suspension of tachyzoites to $6.0x10^3$ parasites per µL, which was kept at 4ºC until use. Modified direct agglutination technique (MAT) was performed according to Minutti *et al.*[11]. Bird sera were initially diluted 1:25 by adding 10 µl of serum to 240 µl of phosphate-buffered saline solution pH 7.2 (SSTF). and then 100 µl of the diluted serum were transferred to the next well containing 100 µl of SSTF, and so on, obtaining serial dilutions up to 1:800. Afterward, 25 µl of each diluted serum were transferred to 96-well microplates with a "V" bottom, and 25 µl of the antigenic suspension consisting of 2.5 mL of borate buffer pH 8.7, 35 µl of 2-mercaptoethanol, 50 µl of Evans Blue at 2 mg/ml and 150 µl of formalin-inactivated antigen were added. Positive and negative controls. serially diluted from 1:25 to 1:3200, were included in each plate, with the positive control titer being 200.

The microplate was covered with plastic film, shaken at low speed for two minutes, and incubated initially at 37°C for up to 16 hours, and then at 4°C for four to six hours. The reading was carried out, considering samples in which a blue sediment formed at the bottom of the well as negative and samples without sediment as positive.

Collection of epidemiological data and analysis of results:

Data collected from all properties included variables location, farm area (m^2), chicken coop area (m^2), chicken total number, property stocking rate (chicken/m^2), chicken coop stocking rate (chicken/m^2), vaccination, deworming, chicken coop use, presence of dogs, presence of dog younger than six months, access of stray dogs to the property, presence of cats, presence of female cat with kittens, feeding the cat with raw meat, access of stray cats to the property, presence of rodents and presence of pigeons?

Frequencies of reactivity to MAT were tabulated with the epidemiological data from each property and analyzed in contingency Tables using Pearson's χ^2 test with continuity correction. Variables with P values lower than 0.20 were reassessed using logistic regression with the backward stepwise method. The statistical significance of the exclusion of each variable was computed using the likelihood ratio tests adjusted by the Hosmer-Lemeshow test ($P < 0.05$). EpiInfo 7[12] was used in all analyses.

To identify the presence of areas with a higher intensity of animals seroreactivity for *T. gondii*. local cluster analysis was performed using the SatScan® 9.4.2[13]. This method considers the geographic locations of cases (seroreactive for *T. gondii*) and controls (seronegative) of chickens, calculating local rates within scanning circles of various sizes, using the Bernoulli distribution as a statistical method. The circles are centered on each event, with their radius ranging from the shortest distance at which another event is detected to the point where they encompass 50% of the study population. For each cluster, the likelihood ratio test was calculated, comparing the hypothesis that the risk of the disease could be greater inside the circle with the hypothesis that the risk was equal for the areas inside and outside the circle. The circle with the maximum likelihood value was considered the most likely cluster.

Meta-analysis:

The prevalence results from the current study were compared to 18 other Brazilian studies using the same MAT test. These studies were selected based on the following criteria: indexed in PubMed, conducted in Brazil, and using the modified agglutination test (MAT) with a cutoff of 1:25. A random-effects model was employed to assess the prevalence of *Toxoplasma gondii* in chickens. Heterogeneity was evaluated using the I^2 index and Q statistic. A summary prevalence estimated with 95% confidence intervals was calculated, and sensitivity analysis (leave-one-out) was performed. Funnel plot asymmetry and Egger's regression test were used to assess potential publication bias. Data analysis was performed using the metaphor package in R[14].

RESULTS

Anti-*T. gondii* antibodies were detected in 144 (49.7%; 95%CI: 43.8-55.6) of the 290 serum samples examined, with titers of 25 in 50 (34.7%), 50 in 62 (43.0%), 100 in 22 (15.3%), 200 in seven (4.9%), 400 in one (0.7%) and 800 in two (1.4%) samples.

Table 1 presents the frequency of birds that tested seropositive or seronegative for MAT, categorized by epidemiological variables related to their location and management. These variables were included in the first logistic regression model. The variables location of the property, chicken coop area smaller than 28 m^2, total number of birds smaller than 25, stocking rate smaller than 0.004 birds/m^2, and keeping the birds free range were associated with a higher frequency of antibodies in the birds, representing risk factors.

The multivariate analysis showed that only the location of the property in area 8 conditioned a significant association, with a chance of occurrence of positive antibodies 18.7 times greater in this area than in area 7. These areas are contiguous, and the properties are no more than five km apart. They are highly similar in terms of construction types, water collection and storage systems, and vegetation cover.

Table 2 presents the frequency of birds that were seropositive or not to MAT according to the epidemiological variables related to the presence of or contact with dogs, cats, rodents, and pigeons, comprising the second model of analysis of the variables by logistic regression.

Table 1. Absolute and relative frequency of birds positive and negative for the modified agglutination test (MAT) for the presence of anti-Toxoplasma gondii antibodies. according to the management-related variables. Results of univariate and multivariate analysis. Feira de Santana. 2016.

Variables	MAT				Statistics			
	Positive		Negative		Univariate		Multivariate	
	N	%	N	%	*P* Value	OR (CI95% OR)	*P* Value	OR (CI95% OR)
Location								
Area 7	23	21.1	86	78.9	<0.001	0.13 (0.08-0.23)	<0.001	18.7 (7.6-46.0)
Area 8	121	66.8	60	33.2				
Farm area								
< 8712	66	55.0	54	45.0	0.158	1.44 (0.90-2.30)	0.780	0.78 (0.14-4.43)
> 8712	78	45.9	92	54.1				
Chicken coop area (m^2)								
< 28	67	74.4	23	25.6	<0.001	4.65 (2.68-8.09)	0.353	0.62 (0.23-1.69)
> 28	77	38.5	123	61.5				
Chicken total number								
< 25	66	74.2	23	25.8	<0.001	4.52 (2.60-7.87)	0.370	0.46 (0.09-2.48)
> 25	78	38.8	123	61.2				
Property stocking rate (chicken/m^2)								
< 0.004	72	59.5	49	40.5	0.006	1.98 (1.23-3.18)	0.142	3.20 (0.68-15.09)
> 0.004	72	42.6	97	57.4				
Chicken coop stocking rate (chicken/m^2)								
< 1	63	47.0	71	53.0	0.474	0.82 (0.52-1.30)	0.120	0.29 (0.06-1.38)
> 1	81	51.9	75	48.1				
Vaccination								
Yes	29	37.7	48	62.3	0.064	0.58 (0.34-0.99)	0.201	0.31 (0.05-1.86)
No	99	51.0	95	49.0				
Deworming								
Yes	62	48.4	66	51.6	0.799	1.10 (0.68-1.77)	-	-
No	66	46.2	77	53.8				
Chicken coop use								
Confined at night	53	44.2	67	55.8		1		
Always confined	61	50.4	60	49.5	0.044	1.28 (0.77-2.13)	0.624	1.52 (0.28-8.26)
Always free-ranged	32	65.3	17	34.7		2.38 (1.19-4.74)		

Table 2. Absolute and relative frequency of birds positive and negative for the modified agglutination test (MAT) for the presence of anti-Toxoplasma gondii antibodies, according to variables related to the contact with other animals. Results of univariate and multivariate analysis. Feira de Santana, 2016.

Variable	MAT				Statistics			
	Positive		Negative		Univariate		Multivariate	
	N	%	N	%	*P*-value	OR (IC95% OR)	*P*-value	OR (IC95% OR)
Is there a dog?								
Yes	111	45.7	132	54.3	0.004	0.36 (0.18-0.70)	0.684	0.84 (0.38-1.93)
No	33	70.2	14	29.8				
Is there a dog younger than six months?								
Yes	18	26.5	50	73.5	<0.001	0.27 (0.15-0.50)	<0.001	0.28 (0.14-0.58)
No	126	56.8	96	43.2				
Do stray dogs have access to the property?								
Yes	97	53.6	84	46.4	0.022	1.99 (1.13 – 3.51)	0.922	1.04 (0.52-2.06)
No	26	36.6	45	63.4				
Is there a cat on the property?								
Yes	51	61.4	32	38.6	0.016	1.95 (1.16-3.29)	0.035	0.17 (0.03-0.88)
No	93	44.9	114	55.1				
Is there a female cat with kittens?								
Yes	43	62.3	26	37.7	0.023	1.96 (1.13-3.42)	0.172	2.37 (0.69-8.22)
No	101	45.7	120	54.3				
Do you feed the cat raw meat?								
Yes	46	71.9	18	28.1	<0.001	3.34 (1.82-6.11)	<0.001	10.6 (2.81-39.98)
No	98	43.4	128	56.6				
Do you observe stray cats?								
Yes	90	53.9	77	46.1	0.118	1.49 (0.93-2.39)	0.525	0.84 (0.48-1.45)
No	54	43.9	69	56.1				
Is there a presence of rodents?								
Yes	137	49.5	140	50.5	<0.001	0.84 (0.27-2.56)	0.441	0.63 (0.20-2.02)
No	7	53.8	6	46.2				
Is there a presence of pigeons?								
Yes	29	43.9	37	56.1	0.359	0.74 (0.43-1.29)	-	-
No	115	51.3	109	48.7				

The spatial analysis pointed out the existence of two clusters; one of which was statistically significant. This cluster had a radius of 0.43 km around property 13 (p<0.0001). It contained properties 13, 14, 15, 9, 10 and 11, in order of proximity to the center of the cluster (Table 3, Figure 2). The spatial analysis corroborates the data from the multivariate analysis, which indicated a higher frequency of infection on the properties in this area (Table 1), where feeding cats raw meat was most frequently reported. The factor with the highest odds ratio (Table 2) in the association with the reaction to anti-*T. gondii* antibodies in the birds.

Table 3. Identification of clusters of extensively raised chickens reactive to the presence of anti-Toxoplasma gondii antibodies by the modified agglutination test (MAT), associated statistics inside and outside the cluster area. Feira de Santana, Brazil, 2016.

Cluster	Associated statistics	Values of associated statistics	
		Cluster area	Outside cluster area
1	Population	98	192
	Positives	73	64
	Prevalence	74.5%	33.3%
	Expected cases	46.6%	
	RR	2.23	
	Likelihood rate	21.8	
	P Value	<0.0001	
	Radius (Km)	0.43	
2	Population	38	252
	Positives	24	113
	Prevalence	63.2%	44.8%
	Expected cases	17.9%	
	RR	1.34	
	Likelihood rate	2.15	
	P Value	0.6410	
	Radius (Km)	0.15	

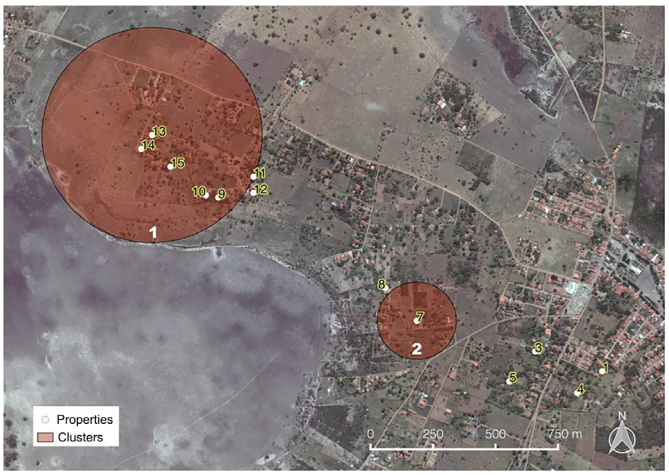

Figure 2. Clusters of extensively raised chickens reactive to the presence of anti-Toxoplasma gondii antibodies by the modified agglutination test (MAT). Feira de Santana, Brazil, 2016.

DISCUSSION

A review of Brazilian studies in which anti-T. *gondii* detection was performed using MAT15, indicating that, among 1,231 chickens examined in Brazil, 495 (40.21%) tested reactive with titers above 20, with an overall weighted prevalence of 52.05% (95% CI: 43.42–60.68). Our study, with a prevalence of 49.70%, is very close to the combined global estimate, indicating that your results are aligned with other Brazilian studies using the same serological test (MAT). Meta-analysis pointed out high heterogeneity between studies (I^2=95.56%), implying that factors such as geographic location, sampling methodology characteristics may contribute to the observed variations. However, the random effects model effectively accounts for this variability.

On the other hand, titers found in the 144 birds examined can be considered relatively low, being less than 100 in 112 (89.30%) birds, considering that in studies carried out in Brazil[6,15], most birds (median of 80.00%) presented titers above 100. Lower titers may indicate acute infections in the birds, since experimental studies demonstrate that up to 15[16] and 28 days after inoculation[17] the birds presented titers below 100 to the MAT. Furthermore, initially seronegative birds, naturally exposed to the parasite and monitored monthly by Dubey et al[5], had titers above 200 that remained for periods of seven months after the beginning of the experiment, indicating a chronic infection.

Based on a review of the results of the examination of 2,066 birds in 19 countries, Dubey et al[6] concluded that MAT is an adequate test for screening birds positive for *T. gondii*, with increased sensitivity of the test according to the titer, verified by the isolation in mice in more than 60.00% of the samples with titers above 40, and also a high predictive value of the negative result, since cats fed with hearts from seronegative birds did not excrete oocysts.

In this study, the presence of cats on the properties was also associated with the infection of birds by *T. gondii*. In a country like Brazil, which has biomes with different climate and land cover profiles, ranging from an equatorial rainforest to the arid regions of the Caatinga, the frequent presence of felids reflects environmental contamination with viable oocysts, even in an environment with unfavorable conditions for the maintenance of parasitic forms for long periods, such as the Caatinga, where the properties studied are located. In the USA, it is estimated that the annual deposition of cat feces leads to an environmental contamination rate of between 10 and 1,500 oocysts per square meter of soil[18]. Similarly, due to the number of cats and the high prevalence of infection; the environment in Brazil must have high contamination rates[19].

Although it was not directly investigated, it is possible that cats on these farms also receive raw chicken meat, which can then be a source of infection for felines, perpetuating the cycle of environmental contamination. There was a strong association between feeding cats raw meat, not necessarily chicken, and infection rates in birds.

Not having a dog under six months of age was significantly associated with the frequencies of serum antibodies in birds by the multivariate model and is a protective factor. The role of dogs in the dispersion of *T. gondii* oocysts in contaminated environments has already been established experimentally[20]. In the case of the farms studied, dogs could act in the mechanical dispersion of oocysts, either through the passage of oocysts through the intestinal tract or by carrying them on their fur.

The presence of seropositive birds and their association with cats on the properties highlight the risk of human infection in these environments, whether through the consumption of contaminated water or food or contact with and subsequent ingestion of contaminated soil."

Several epidemiological variables analyzed in this study did not show statistically significant associations with *Toxoplasma gondii* seropositivity in chickens. Variables such as farm area, property, and chicken coop stocking rates, vaccination status, deworming, the presence of stray cats, rodents, and pigeons did not remain in the final multivariate model. The lack of significance does not necessarily

imply an absence of biological relevance but may indicate that these factors do not exert a direct or dominant influence on the transmission dynamics of *T. gondii* in this specific setting.

For example, while rodents are known intermediate hosts and potential reservoirs of *T. gondii*, their presence alone may not be a strong predictor of transmission in free-range chicken systems, where other environmental factors, such as oocyst contamination from feline feces, play a more critical role. Similarly, the presence of pigeons, often considered potential mechanical carriers of oocysts, did not show a measurable impact, possibly due to differences in their feeding behavior or limited contact with contaminated soil.

Additionally, while stray cats were frequently observed, their role in transmission might be overshadowed by domestic cats with access to raw meat, which was identified as a strong risk factor. These findings highlight the complexity of epidemiological interactions and reinforce the importance of considering multiple environmental and management-related factors when assessing *T. gondii* transmission in extensive poultry production systems.

The findings of this study highlight a significant prevalence of *Toxoplasma gondii* infection in chickens, closely aligning with the overall prevalence estimated in other Brazilian studies using the same MAT test. Although the majority of titers in this study were relatively low, these values may suggest acute infections since lower titers may indicate acute infections in the birds, since experimental studies demonstrate that up to 15[16] and 28 days after inoculation[17], differing from other studies where higher titers were more prevalent, potentially indicating chronic exposure. Risk factors such as the location of the properties, smaller chicken coop areas, free-range systems, and the presence of cats were significantly associated with seropositivity. These findings suggest environmental contamination by oocysts, likely exacerbated by the presence of cats, can play a critical role in the transmission of *T. gondii* in these areas. Moreover, the spatial analysis identified a significant cluster of infection, supporting the multivariate results and emphasizing the importance of localized factors. such as the practice of feeding raw meat to cats, in perpetuating the infection cycle. This reinforces the need for targeted interventions in these areas to mitigate the risks of both animal and human infections.

CONCLUSION

The findings of this study reinforce the role of environmental contamination in the transmission of *Toxoplasma gondii* in free-range poultry systems. The identification of risk and protective factors underscores the complexity of infection dynamics in rural settings. These results enhance the epidemiological understanding of T. gondii and may support strategies to reduce environmental contamination and mitigate risks to both animal and human health."

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