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ORIGINAL PAPER

# First study on seroepidemiology and isolation of *Toxoplasma gondii* in free-range chickens in the semi-arid region of Paraíba state, Brazil

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**Abstract** The aim of this study was to investigate the seroprevalence and isolation of *Toxoplasma gondii* in free-range chickens in the state of Paraíba, northeastern Brazil. For this, blood samples were collected from 483 chickens in five municipalities in the state of Paraíba. The indirect immunofluorescence assay for anti-*T. gondii* antibodies was performed. The seropositive birds were slaughtered, and their brains and hearts were collected in order to perform a bioassay in mice. An epidemiological questionnaire was applied on the smallholdings visited, and univariable and multivariable logistic regressions were used to evaluate risk factors. The prevalence of chickens seropositive for *T. gondii* was found to be 31.5 % (152/483), and 86.1 % (56/65) of the smallholdings were positive. Among the 71 chickens subjected to bioassaying in mice, isolates of *T. gondii* were obtained from 33 (46.5 %). The isolates were named TgCkBrPB1 to 33. It was observed that the higher the chickens' antibody titer was, the greater the chance of isolating the parasite also was. Sixteen of the 33 isolates (48.5 %) were lethal for all the mice inoculated until 30 days post-inoculation. The risk factors for infection with *T. gondii* among these free-range chickens were extensive and semi-extensive rearing systems, smallholdings located in urban areas, and presence of cats. The results indicate that the prevalence of *T. gondii* among chickens in the state of Paraíba is high. Many parasites remained viable in the tissues of the birds studied, and presence of the protozoan was directly related to the management of these birds.

**Keywords** Bioassay · Diagnosis · Toxoplasmosis · Zoonosis

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## Introduction

Toxoplasmosis is an anthropozoonosis caused by *Toxoplasma gondii*, an obligate intracellular parasite of cosmopolitan distribution. This protozoan may affect birds and mammals, including humans, and cats (*Felis catus domesticus*) are the main definitive host. Infection occurs after ingestion of water or food contaminated with oocysts that have been eliminated in the feces of cats, after sporulation in the environment, and also through ingestion of raw or undercooked meat containing cysts of this parasite (Dubey 2010).

This disease can cause many forms of harm to human and animal health. In humans, the risk to pregnant women can be highlighted, since infection by this parasite may cause several disorders in both the mother and the fetus. These may include spontaneous abortion, chorioretinitis, cerebral calcifications, hydrocephalus, and a series of other clinical presentations (Remington et al. 2001). The importance of this protozoan in patients with the AIDS virus can also be emphasized, given the possibility that it may cause toxoplasmic encephalitis in these individuals (Rey 2008). In Brazil, this parasite is widely disseminated and this country presents one of the highest rates of seroprevalence among humans in the world (Gilbert et al. 2008; Dubey 2010).

Toxoplasmosis in chickens follows a predominantly subclinical course and has little clinical importance for this species. However, Dubey et al. (2007b) reported an outbreak of clinical toxoplasmosis among laying chickens and geese on a farm in Illinois, USA. The clinical signs reported were neurological alterations, manifested as torticollis, incapacity to remain standing up, and lying down on their sides. Domestically reared chickens are considered to be important in the epidemiology of this disease, since they are sources of transmission both to humans and to cats. In addition, the presence of *T. gondii* in free-range chickens is one of the best indicators of environmental contamination with oocysts of the parasite, because of the chickens' habit of foraging and feeding from the ground. They are thus considered to be excellent sentinel animals (Dubey et al. 2006; Dubey 2009).

In Brazil, seroprevalence studies have shown that domestically reared chickens have a high rate of positivity for *T. gondii*, ranging from 41 to 80 % (Dubey et al. 2007a; Oliveira et al. 2008; Beltrame et al. 2012; Fernandes et al. 2016). Some studies have also reported high rates of isolation of *T. gondii* from the tissues of free-range chickens, which demonstrate that chickens carry viable cysts that are capable of infecting both humans and animals.

In view of the importance of chickens in transmitting toxoplasmosis to humans and animals, and also of the sparseness of research on this disease in the state of Paraíba, Brazil, the present study had the aims of investigating the seroepidemiological situation of free-range chickens in this state and isolating the parasite, so as to determine the real risk involved in consuming their meat when raw or undercooked, for the human and animal populations.

## Material and methods

### Characterization of the area

The state of Paraíba is located in northeastern Brazil. It presents high temperatures throughout the year, with a range from 20 to 28 °C, with small regional differences. The climate of this state ranges from humid in the coastal zone to semi-arid in the interior, with annual rainfall ranging from 350 to 700 mm (Araújo 2011).

### Chickens

This study used 483 chickens that originated from five municipalities in the state of Paraíba (Fig. 1).

During visits to the localities, blood samples were collected from the brachial vein and the birds were duly identified with numbers corresponding to those of the sample collection tubes. The blood samples were stored in Styrofoam boxes with ice and were sent to the Laboratory of Parasitic Diseases of Domestic Animals (LDPAD) of the Federal University of Campina Grande (UFCG), Patos, PB, for serological tests for *T. gondii* to be performed. After the results from these tests became known, the team returned to the smallholdings to gather the seropositive chickens for subsequent slaughter. Only the birds whose owners agreed to have them slaughtered were gathered in. After these had been slaughtered, their hearts and brains were collected, packed individually in plastic bags, properly identified, stored in Styrofoam boxes with ice, and sent to LDPAD for bioassays to be performed.

### Serological tests and isolation of *T. gondii*

The serum samples from the chickens were examined to investigate the presence of anti-*T. gondii* antibodies by means of the indirect immunofluorescence assay (IFA), as described by Camargo (1974), using tachyzoites of *T. gondii* from the RH reference strain, fixed on a slide, as the antigen. The cutoff point used was 1:16 (Garcia et al. 2000).

The tissues (brain and heart) from the seropositive birds were cut into small pieces and used for bioassays in mice, in accordance with the protocol of Dubey (1998). For each positive bird, three or four 2-month-old albino Swiss mice housed in a single box were subcutaneously inoculated with the homogenates obtained (1 mL per mouse). Parasite numbers in the inoculums were not evaluated.

The mice that died were examined to investigate the presence of *T. gondii* in the tissues (lungs and brains), as previously described by Dubey (2010). Mortality data was based on observation of a 30-day post-inoculation (p.i.) period (Shwab et al. 2016).

The mice that survived for 6 weeks after inoculation were examined serologically to investigate the presence of anti-*T. gondii* antibodies, by means of IFA with a cutoff point of 1:16 (Silva and Langoni 2001). Those that were seropositive remained in the experiment until 2 months after inoculation, when they were sacrificed and their brains examined for *T. gondii*. Those that were seronegative were sacrificed after the result from the serological test and were subjected to the same examination. The mice were considered to be positive when tachyzoites and/or cysts were observed in their tissues.

### Epidemiological questionnaire

The owners were interviewed individually regarding the conditions on their smallholdings, rearing system used, type of feed used, environment in which the animals lived, and presence of cats.

### Statistical analysis

To ascertain the correlation between the antibody titers and the percentage isolation, Pearson's correlation coefficient ($r$) was calculated. To analyze risk factors associated with the frequency of

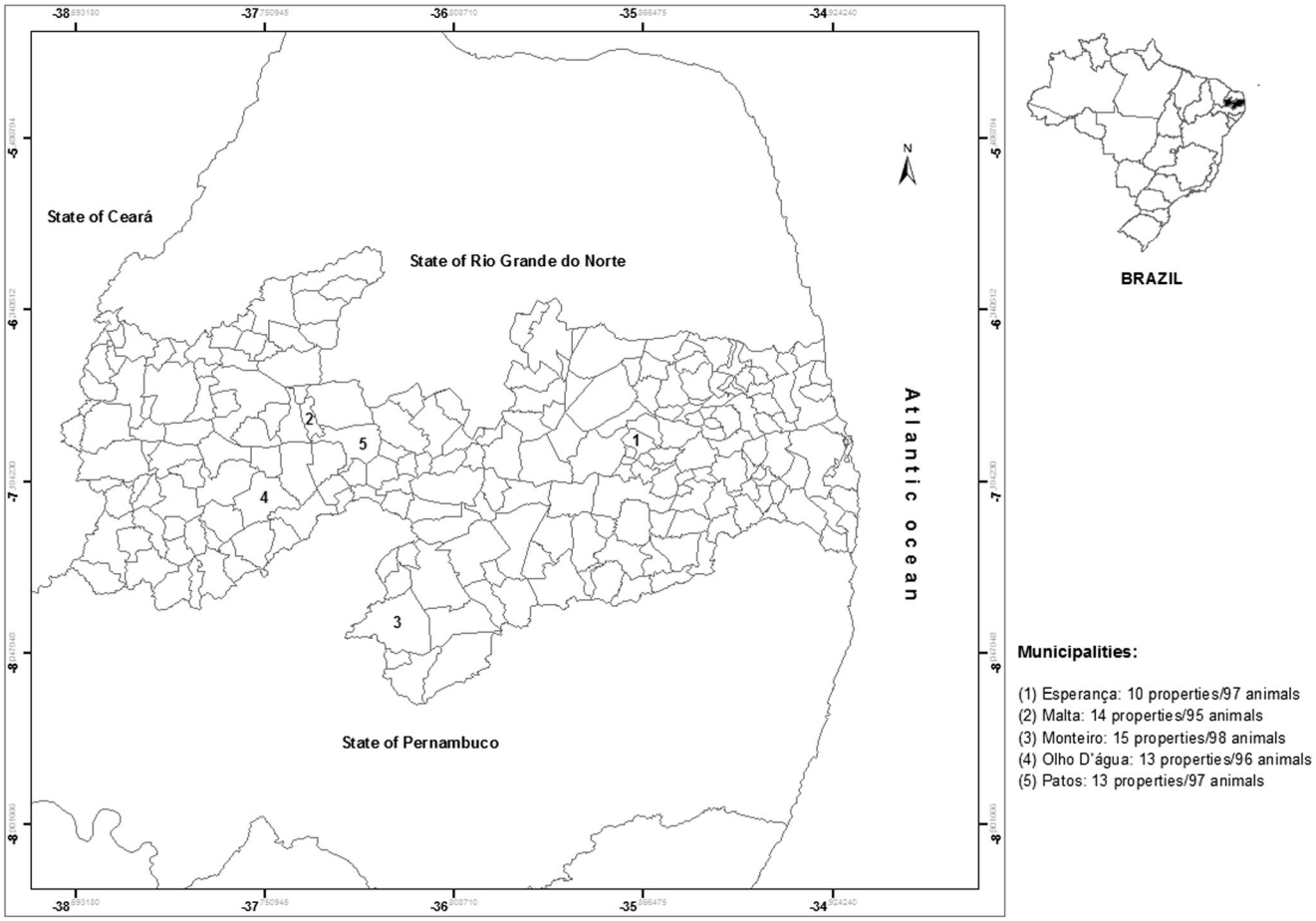


**Fig. 1** Map of Paraíba state, Brazil, showing municipalities where chickens were collected

seropositivity and isolation, the data gathered through the epidemiological questionnaires were used. The risk factor analysis was conducted in two stages: univariable and multivariable analyses. In the univariable analysis, each independent variable was cross-correlated with the dependent variable (seropositivity and isolation), and those that presented $P$ values ≤0.20 through the chi-square test (Zar 1999) were selected for multivariable analysis, using multiple logistic regression (Hosmer and Lemeshow 2000). The significance level used in the multiple analyses was 5 %. All the analyses were performed using the SPSS 20.0 software for Windows.

The Research Ethics Committee of UFCG authorized all the procedures performed in this study (protocol no. 14/2013).

## Results

In this study, 31.5 % (152/483) of the chickens examined were seropositive for *T. gondii* through IFA. Among the 65 smallholdings visited, 56 (86.1 %) presented at least one seropositive chicken. The observed frequencies of birds seropositive for *T. gondii* per municipality ranged from 25 to 36.8 % (Table 1). There were no statistical differences in relation to the prevalence of seropositive animals between the five municipalities studied ($P = 0.284$).

The anti-*T. gondii* antibody titers ranged from 1:16 to 1:4092, and the most frequent titer was 1:64 (44/152). Among the seropositive chickens, 71 were subjected to bioassays for *T. gondii* isolation in mice, from which 33 isolates were obtained. The isolates were named TgCkBrPB1 to 33. It was observed that the percentage of isolation increased as the antibody titer also increased (Table 2), with a positive correlation ($r = 0.88$; 95 % CI = 0.53–0.98; $P = 0.002$).

Considering a 30-day p.i. period, 22 isolates (66.7 %) were lethal to at least one of the mice infected, and these mice died between 16 and 30 days p.i. due to acute toxoplasmosis. Sixteen isolates (48.5 %) were lethal to all the animals infected and, in the cases of six isolates (18.2 %), all the infected mice survived until the end of the experiment, 60 days p.i. Information about *T. gondii* isolates and mouse mortality is showed in Table 3.

The results from the univariable analysis on the risk factors for *T. gondii* are presented in Table 4. The variables of gender ($P = 0.701$) and type of feed used ($P = 0.055$) did not show any significant associations with infection by *T. gondii*. In relation to the environment in which the chickens lived, they

**Table 1** Prevalence of anti-*Toxoplasma gondii* antibodies found through IFA among free-range chickens in the state of Paraíba, Brazil

| Municipalities | Properties | | Chickens | |
|---|---|---|---|---|
| | No. examined | No. positive (%) | No. examined | No. positive (%) |
| Esperança | 10 | 9 (90.0) | 97 | 26 (26.8) |
| Malta | 14 | 13 (92.8) | 95 | 35 (36.8) |
| Monteiro | 15 | 12 (80.0) | 98 | 35 (36.7) |
| Olho D'água | 13 | 11 (84.6) | 96 | 24 (25.0) |
| Patos | 13 | 11 (84.6) | 97 | 32 (33.0) |
| Total | 65 | 56 (86.1) | 483 | 152 (31.5) |

were reared either completely free-range or in fenced-off bare-earth paddocks.

The variables of extensive rearing system (odds ratio 5.41; $P = 0.027$), semi-extensive rearing system (odds ratio 4.81; $P = 0.043$), smallholdings located in an urban area (odds ratio 1.90; $P = 0.002$), and presence of cats (odds ratio 1.95; $P = 0.001$) were considered to be risk factors for infection by *T. gondii*, as shown by the multivariable logistic regression (Table 5).

## Discussion

The seroprevalence of *T. gondii* among free-range chickens found in this study (31.5 %) was considered to be high, and this corroborated the findings from other studies in Brazil, independently of the serological test used. Thus, Oliveira et al. (2008) found that 53.3 % of the chickens were seroreactive in all the states of northeastern Brazil, except Paraíba, which did not have any participation in their study. More recently, Fernandes et al. (2016) reported a seroprevalence rate of 40.6 % (86/212) in the state of Pernambuco, also in northeastern Brazil. Furthermore, Costa et al. (2012) found a higher seroprevalence rate of 80 % (80/100), on the island of Fernando de Noronha, state of Pernambuco. In other regions of Brazil too, these rates are high, with reports of 38.8 % (198/510) in Espírito Santo, in the southeastern region (Beltrame et al. 2012), and 74.4 % (102/137) in Rio Grande do Sul, southern region (Camillo et al. 2015). The results obtained therefore confirm that this agent is widely distributed across Brazil and indirectly show that the soil can be highly contaminated with oocysts of *T. gondii*.

No association between seropositivity for *T. gondii* and the gender of the chickens was found, thus corroborating the findings from other studies conducted among goats, sheep, buffalos, pigs, and wild animals (Ragozo et al. 2009; Correia et al. 2015; Brasil et al. 2015; Feitosa et al. 2014; Pimentel et al. 2009).

Although no statistical difference was found in relation to the type of feed used for the chickens in the present study, the proportion of the seropositive chickens that were only fed with commercially prepared feed was low (8.7 %) in comparison with the proportion that were fed with leftover food (32.5 %). This was because among domestically reared chickens, which were the focus of the present study, the owners had the habit of offering their chickens leftover fruit, meat, and raw viscera that were not used for human consumption. Thus, the chickens were exposed to oocysts and tissue cysts that may have been present in the leftover food. This practice of supplying human food waste to chickens emphasizes these birds' role as

**Table 2** Frequency of isolation of *Toxoplasma gondii* from free-range chickens in the state of Paraíba, Brazil, by means of bioassays in mice, according to the titer of anti-*T. gondii* antibodies

| Titer | No. of seropositive chickens | Isolation | | |
|---|---|---|---|---|
| | | No. of bioassays | No. of isolates | Percent |
| 16 | 19 | 7 | 2 | 28.6 |
| 32 | 24 | 6 | 2 | 33.3 |
| 64 | 44 | 20 | 8 | 40 |
| 128 | 26 | 16 | 6 | 37.5 |
| 256 | 17 | 12 | 6 | 50 |
| 512 | 10 | 6 | 4 | 66.6 |
| 1024 | 7 | 3 | 2 | 66.6 |
| 2048 | 4 | 2 | 2 | 100 |
| 4096 | 1 | 1 | 1 | 100 |
| Total | 152 | 71 | 33 | 46.8 |

**Table 3** Results of the bioassay in mice from chickens from Paraíba state, Brazil

| Municipalities | Property ID | Bioassay in mice[a] | | | |
|---|---|---|---|---|---|
| | | Isolate ID | Day of death | No. of mice died/infected[b] | % mortality |
| Olho D'água | 10 | TgCkBrPB1 | 26, 30 | 2/2 | 100 |
| | 20 | TgCkBrPB31 | Survived | 0/2 | 0 |
| | 24 | TgCkBrPB2 | 17, 20 | 2/3 | 67 |
| | 9 | TgCkBrPB3 | 22, 28 | 2/2 | 100 |
| Patos | 32 | TgCkBrPB4 | 19, 22 | 2/2 | 100 |
| | 32 | TgCkBrPB5 | 22, 24, 29 | 3/3 | 100 |
| | 32 | TgCkBrPB6 | 18, 21, 23 | 3/3 | 100 |
| | 35 | TgCkBrPB7 | 20, 24, 29 | 3/3 | 100 |
| | 35 | TgCkBrPB8 | 23, 23, 26 | 3/3 | 100 |
| | 36 | TgCkBrPB9 | 18, 18, 20 | 3/3 | 100 |
| | 51 | TgCkBrPB28 | 16, 17, 19, 20 | 4/4 | 100 |
| | 51 | TgCkBrPB29 | 16, 21, 26 | 3/4 | 75 |
| | 52 | TgCkBrPB30 | 21, 27, 30, 31 | 3/4 | 75 |
| Monteiro | 37 | TgCkBrPB10 | 17, 26 | 2/4 | 50 |
| | 37 | TgCkBrPB32 | Survived | 0/1 | 0 |
| | 38 | TgCkBrPB11 | 22, 25, 45 | 2/3 | 67 |
| | 38 | TgCkBrPB12 | Survived | 0/1 | 0 |
| | 39 | TgCkBrPB13 | 22, 22 | 2/2 | 100 |
| | 41 | TgCkBrPB14 | Survived | 0/3 | 0 |
| Esperança | 42 | TgCkBrPB15 | 25, 30 | 2/2 | 100 |
| | 43 | TgCkBrPB16 | 31, 34 | 2/3 | 0 |
| | 43 | TgCkBrPB17 | 53 | 1/4 | 0 |
| | 44 | TgCkBrPB18 | 23 | 1/1 | 100 |
| | 44 | TgCkBrPB19 | 38 | 1/3 | 0 |
| Malta | 46 | TgCkBrPB20 | 17, 25 | 2/2 | 100 |
| | 46 | TgCkBrPB21 | 17,18, 19, 22 | 4/4 | 100 |
| | 47 | TgCkBrPB22 | Survived | 0/3 | 0 |
| | 47 | TgCkBrPB23 | 38 | 1/1 | 0 |
| | 48 | TgCkBrPB24 | 23 | 1/2 | 50 |
| | 49 | TgCkBrPB25 | Survived | 0/2 | 0 |
| | 49 | TgCkBrPB26 | 19, 22, 23 | 3/3 | 100 |
| | 50 | TgCkBrPB33 | 20 | 1/1 | 100 |
| | 50 | TgCkBrPB27 | Survived | 0/2 | 0 |

[a] Three or four mice were inoculated. Parasite numbers were not evaluated in the inoculums

[b] Mortality data was based on observation of a 30-day post-inoculation

sentinels, not only regarding environmental contamination but also regarding human exposure to *T. gondii*.

Intensive rearing has been shown to be safer with regard to avoiding infection of chickens by *T. gondii*, in comparison with extensive and semi-extensive rearing. In extensive rearing systems, chickens are subject to contact with infected felids and contaminated earth and water and spend longer periods of time in the same environment, which thus increases the chances of becoming infected by the parasite. Moreover, none of the smallholdings visited had any basic sanitation (sewage disposal system) or any regular garbage collection. Other studies have also noted that the numbers of seropositive chickens in intensive rearing systems are lower than in extensive systems. Xu et al. (2012) found seroprevalences of 5.6 and 18.8 % among chickens in intensive and extensive systems, respectively. Millar et al. (2012) conducted a study in the state of Rio de Janeiro and observed that 14.8 % of the laying chickens that were reared intensively presented anti-*T. gondii* antibodies, while among those that were extensively reared, the proportion of reactive birds was 51.4 %.

**Table 4** Univariable analysis on risk factors associated with seropositivity for *Toxoplasma gondii* among free-range chickens in the state of Paraíba, Brazil

| Variable/category | No. of chickens | No. of seropositive chickens (%) | *P* |
|---|---|---|---|
| Gender | | | |
| Male | 61 | 21 (34.4) | |
| Female | 422 | 131 (31.0) | 0.701 |
| Feed | | | |
| Commercial | 23 | 2 (8.7) | |
| Leftover food | 120 | 39 (32.5) | |
| Commercial + leftovers | 340 | 111 (32.6) | 0.55 |
| Rearing system | | | |
| Intensive | 23 | 2 (8.7) | |
| Semi-extensive | 114 | 35 (30.7) | |
| Extensive | 346 | 115 (33.2) | 0.48 |
| Property location | | | |
| Rural | 302 | 76 (25.2) | |
| Urban | 181 | 76 (42.0) | 0.002 |
| Cat presence | | | |
| No | 225 | 56 (24.9) | |
| Yes | 258 | 96 (37.2) | 0.005 |

The presence of cats is considered to be extremely important with regard to infection by *T. gondii* among extensively reared chickens. This correlation was also reported by other authors such as Bonna et al. (2006) among pigs and chickens reared in Rio de Janeiro, Brazil and Millar et al. (2012) among laying chickens reared within a semi-extensive system.

Chickens kept on smallholdings located in urban areas were more susceptible to infection by *T. gondii* than were animals that were kept on rural smallholdings. This corroborated the findings of Silva et al. (2003), who also observed that the percentage of chickens positive for *T. gondii* was lower in rural areas of southern Brazil. This result can be attributed to greater population density of cats in urban areas, which leads to greater likelihood that chickens will encounter feces from infected cats on the ground, containing oocysts. This will then increase the likelihood of infection among the chickens. Weigel et al. (1995) stated that the number of cats on smallholdings is more important than the presence of cats, given that the larger the number of cats is, the larger the number of oocysts contaminating the environment may also be.

**Table 5** Risk factors associated with seropositivity for *Toxoplasma gondii* among free-range chickens in the state of Paraíba, Brazil, determined by means of multiple logistic regression

| Variable | Odds ratio (95 % CI) | *P* |
|---|---|---|
| Extensively reared chickens | 5.41 (1.21–24.08) | 0.027 |
| Semi-extensively reared chickens | 4.81 (1.05–22.04) | 0.043 |
| Urban areas | 1.90 (1.27–2.85) | 0.002 |
| Cat presence | 1.95 (1.30–2.92) | 0.001 |

It was observed that the greater the antibody titer that the chickens presented was, the greater the percentage isolation of *T. gondii* also was, corroborating the findings of Holsback et al. (2012), Beltrame et al. (2012), and, more recently, Dubey et al. (2016). This association had already been observed in other species such as sheep, goats, and cats (Gebremedhin et al. 2014; Pena et al. 2006).

The percentage of the bioassays that were positive for *T. gondii* was 46.5 % (33/71). This was similar to the proportion reported by Holsback et al. (2012), who obtained an isolation rate of 40.7 % (11/27) by using the brains and hearts of seropositive chickens from the state of Mato Grosso do Sul. However, other researchers have found higher rates, such as Dubey et al. (2007b), who obtained an isolation rate of 100 % in an outbreak of toxoplasmosis in Illinois, USA, and Beltrame et al. (2012), who observed an isolation rate of 78.1 % in Espírito Santo, Brazil. This difference can be explained by the tissues used for isolation, given that these last authors not only used the brains and hearts of seropositive chickens for the bioassays but also their thigh muscles. Although the heart is the organ most parasitized by this protozoan in chickens, Dubey et al. (2007b) stated that it was important to use macerates from different organs, separately or in mixtures, in order to increase the sensitivity of the isolation. They highlighted the thigh muscles, which are strongly parasitized by this protozoan.

Several factors are involved in the virulence of *T. gondii* samples in mice, including the stage of the parasite (among the three infective stages, oocysts are more virulent than bradyzoites or tachyzoites), the infection route, dose, lineage of mice, and genetic characteristics of the sample. In Brazil, the virulence of these isolates tends to be high, and this differs from what is observed in other countries, particularly those in the northern hemisphere, which present a predominance of non-virulent strains of the parasite. In the present study, it was not possible to quantify the parasites in the inoculums, and it could have interfered with results on mouse mortality. Even though the mortality information obtained from the first isolation, from the tissues of chronically infected hosts, at a time when it is not feasible to know what the inoculating dose was should not be neglected. It the present paper, it was observed that most of the isolates [(48.5 % (16/33)] were lethal for all the mice infected. Likewise, more recently, in Brazil, Beltrame et al. (2012) observed that 44 of the 48 isolates obtained from free-range chickens were lethal for all the mice infected. Vitaliano et al. (2014) obtained 15 isolates from wild animals in several Brazilian states and found that all the mice infected with these isolates died due to acute toxoplasmosis.

## Conclusion

The high prevalence of anti-*T. gondii* antibodies among these free-range chickens indicates that great environmental contamination with oocysts of this protozoan exists in the state of Paraíba. Moreover, the meat of these free-range chickens is important with regard to the epidemiology of toxoplasmosis in the region studied, and it may be a source of infection for humans and animals. *T. gondii* infection is directly related to the rearing system used for these chickens, and therefore, the way in which this disease can be controlled depends on the owners' knowledge of the means of transmission.

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