

Antibodies anti-*Toxoplasma gondii* and anti-*Neospora caninum* in backyard pigs from the state of Mato Grosso, Brazil

Anticorpos anti-*Toxoplasma gondii* e anti-*Neospora caninum* em porcos de criações de fundo de quintal do Estado de Mato Grosso, Brasil

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Abstract

To estimate the seroprevalence of *Toxoplasma gondii* and *Neospora caninum*, using an indirect immunofluorescent assay (IFA), and identify the risk factors associated, serum samples were collected from 1,070 pigs from 320 backyard pig farming in the of Mato Grosso state. The animal-level seroprevalence of *T. gondii* and *N. caninum* was 32.48% and 13.49%, respectively, with a herd seroprevalence of 55.63% for *T. gondii* and 27.81% for *N. caninum*. Feeding the animals with leftovers increases the probability of the presence of anti-*T. gondii* antibodies in pigs by 1.09-fold. Unlike to *T. gondii*, feeding with leftovers was found to be negatively associated with *N. caninum* seropositivity in farm-level analysis and in the animal-level model, so decreasing the chances of positivity. Yet, age was considered a risk factor for *N. caninum* seropositivity. Further studies are necessary to evaluate the impact of *T. gondii* infection on backyard pig farming production, and its importance as a source of toxoplasmosis infection in humans in the Mato Grosso state, as well as, the role of domestic pigs in the epidemiology of neosporosis.

Keywords: Toxoplasmosis, neosporosis, seroprevalence, risk factors.

Resumo

Para estimar a soroprevalência de *Toxoplasma gondii* e *Neospora caninum*, utilizando a reação de imunofluorescência indireta (RIFI), e identificar os fatores de risco associados, foram coletadas amostras de soro de 1.070 suínos provenientes de 320 criatórios de fundo de quintal no Estado de Mato Grosso. A soroprevalência para nível animal de *T. gondii* e *N. caninum* foi de 32,48% e 13,49%, respectivamente, com uma soroprevalência de rebanho de 55,63% para *T. gondii* e 27,81% para *N. caninum*. Alimentar os animais com sobras aumenta a probabilidade da presença de anticorpos anti-*T. gondii* em porcos em 1,09 vezes. Ao contrário de *T. gondii*, a alimentação baseada em sobras foi encontrada como negativamente associada à soropositividade para *N. caninum* na análise em nível de fazenda e no modelo em nível animal, diminuindo assim as chances de positividade. Ainda, a idade foi considerada um fator de risco para a soropositividade para *N. caninum*. Mais estudos são necessários para avaliar o impacto da infecção por *T. gondii* na produção de suinocultura de quintal e sua importância como fonte de infecção para toxoplasmose em humanos no Estado de Mato Grosso, bem como o papel dos suínos domésticos na epidemiologia da neosporose.

Palavras-chave: Toxoplasmose, neosporose, soroprevalência, fatores de risco.

Introduction

Toxoplasma gondii is a coccidian parasite with cats as definitive hosts, and warm-blooded animals as intermediate hosts (DUBEY, 2010). Serological surveys have reported *T. gondii* infection to be

prevalent in pigs worldwide (DUBEY, 2009), and up to 90% of pigs surveyed in Brazil had *T. gondii* antibodies (DUBEY et al., 2012). The seroprevalence varied dramatically among the different classes surveyed, such as market pigs versus sows and indoor pigs from bio secure housing systems in contrast with free-range (DUBEY, 2010).

Tissue cysts of *T. gondii* have been found in meat, meat-derived products, or offal (TENTER, 2009), and among the food animals,

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infected pigs and pork products are the most likely meat source of *T. gondii* infection for people in many countries (DUBEY, 2009), including Brazil (DUBEY et al., 2012).

Neospora caninum is a recently recognized protozoan parasite that was misidentified as *T. gondii* until 1988. This coccidian parasite uses canids (dogs, coyotes, dingoes and grey wolves) as definitive hosts and warm-blooded animals as intermediate hosts (DUBEY et al., 2017).

Information on seroprevalence of *N. caninum* in domestic pigs is limited worldwide (DUBEY et al., 2017), with low *N. caninum* seropositivity observed in this species from Brazil (AZEVEDO et al., 2010; FEITOSA et al., 2014).

Mato Grosso state in midwestern Brazil is the fifth largest producer of pigs in the country, with 2,613,925 million animals distributed in 34,093 farms; of this total, 98.78% are backyard pig farming, and make up 16.76% of the total population of pigs from the state (INDEA, 2016). The aim of the study was to evaluate the presence of anti-*T. gondii* and anti-*N. caninum* antibodies in pigs from backyard pig farming establishments in Mato Grosso state, Brazil, and identified the risk factors associated with the seropositivity.

Materials and Methods

Study area, serum collection and epidemiological information

Blood samples had been collected for a field surveillance as part of the National Programme for Control and Eradication of classical swine fever (CSF), by the Mato Grosso Institute for Agricultural Defense (INDEA/MT), in the state of Mato Grosso, midwestern Brazil, between September and December 2014. Then the stored samples were made available for the present study.

Sampling

The sample was carried out following the criteria established in the Manual of standardization seroepidemiological survey in swine farms (BRASIL, 2010). The procedure of selecting sample was done in 2 stages. The first stage (called primary sampling units) involved selecting a predetermined number of random farms.

The procedure of selecting sample was done in 2 stages. The first stage (called primary sampling units) involved selecting a predetermined number of random farms, and the sample size was calculated using the following algebraic expression, previously described (SCHEAFFER et al., 2011):

$$n = \frac{z_{\alpha}^2 p(1-p)N}{(N-1)d^2 + z_{\alpha}^2 p(1-p)} \quad (1)$$

Thus, we considered the total number of farms 3,397, expected proportion of positive farms $p = 0.50$ (due to the absence of prior studies), maximum error of estimate $d = 0.055$, and, $z_{\alpha} = 1.96$ is the value of standard normal distribution table, for a 95%

confidence interval (CI). This resulted in a sample of 320 farms, characteristically with pigs reared for their own consumption, in family-run farms with no industrial features.

Sampling of animals from each farm remained the same presented in field surveillance conducted by INDEA/MT. Concurrent with sampling, epidemiological data were collected through questionnaire survey and used for the risk factor analysis.

Following this, a predetermined number of pigs (called secondary sampling units) aged over 8 months were sampled. For the secondary sampling unit, sample size was pre-established considering the number of pigs older than 8 months on each farm, minimum prevalence of farms and adult animals positives of 1% and 10%, respectively, 95% of probability of detecting a positive farm, and 95% of sensitivity of the herd. Considering the number of animals on each farm, the criteria was as follows: all pigs were sampled from farms with up to 15 pigs; 15 pigs were sampled from farms with 16-20 pigs; 20 from farms with 21-30 pigs; 23 from farms with 31-50 pigs; 26 from farms with 51-80 pigs; and finally, 30 were sampled from farms with over 80 pigs. Thus, total 1,070 serum samples were randomly collected.

Indirect immunofluorescence assay

Serum samples were analyzed individually for anti-*T. gondii* and anti-*N. caninum* IgG antibodies by indirect immunofluorescence assay (IFA), as described by Camargo (1964) and Azevedo et al. (2010), respectively. RH strain and isolate NC-1 tachyzoites of *T. gondii* and *N. caninum*, respectively, maintained in Vero cell cultures and fixed on slides were used as target antigens, along with anti-pig IgG conjugate (Sigma, St Louis, MO, USA), at a 1:100 dilution. Serum samples were considered positive when the majority of fixed tachyzoites (>50%) showed complete peripheral fluorescence at 1:64 (GARCIA et al., 1999) and 1:50 (AZEVEDO et al., 2010) dilutions for *T. gondii* and *N. caninum*, respectively. All the seropositive serum samples were retested using 2-fold serial dilutions. Positive and negative pig sera were used as controls.

Seroprevalence and statistical analysis

We expressed the seroprevalence of *T. gondii* and *N. caninum* on the farms and among the animals in the form of estimated value and confidence intervals, according to Thrusfield (2007). Furthermore, to calculate the seroprevalence of *T. gondii* and *N. caninum* among the animals were used weighted values (DOHOO et al., 2003). The following expression was used to determine the weighted value for each animal:

$$\frac{\text{Total number of } >8 \text{ month-old pigs}}{\text{total of } >8 \text{ month pigs sampled}} * \frac{\text{total of } >8 \text{ month-old pigs on the farm}}{\text{total of } >8 \text{ month-old pigs sampled on the farm}} \quad (2)$$

In the farm-level analysis, to describe the seroprevalence of *T. gondii* and *N. caninum*, each farm was analyzed under the assumption that a farm would be considered positive if at least one animal tested seropositive. The following steps were taken in order to create a multiple logistic regression model. Firstly, for continuous variables, a linearity test was carried out using

Graphs for Log Odds. The continuous variable was categorized if the visual evaluation shown a nonlinear trend. We used the optimization methodology of the R package CatPredi, as described (BARRIO et al., 2017). Secondly, regarding the categorical variables, we used a chi-square or Fisher's exact test on the results, and then we included into the model the various predictor variables, and all the variables with a value of $p < 0.20$. Thirdly, we tested the selected variables for collinearity, including multicollinearity analysis (VATCHEVA et al., 2016). This ensured a mean variance inflation factor (VIF) < 10 , before fitting them into my model. Finally, all the variables selected, as previously described, were included to build the model. Based on the Wald test, we removed the least significant variables, and the logistic regression analysis was repeated. Once repeated, the adjusted model was compared to the previous model using likelihood ratio test to verify for confounding factors. In case of a change greater than 30% in the parameter estimates, we considered the removed variable a confounding factor, and once more included in the model.

The following variables were used to build the herd model for *T. gondii*: farm is in the rural areas or near indigenous reserves (yes or no); farm is in the peri-urban areas or poor communities (yes or no); pigs were raised extensively (yes or no); feeding with leftovers (yes or no); presence of cats (yes or no); farm is located near dumps (yes or no); creates or fattens (yes or no); farm is close to environmental reserves (yes or no); slaughtering on the farm (yes or no).

Herd model for *N. caninum* was building using the following variables: farm is in the rural areas or near indigenous reserves (yes or no); farm is in the peri-urban areas or poor communities (yes or no); pigs were raised extensively (yes or no); feeding with leftovers (yes or no); presence of dogs (yes or no); farm is located near dumps (yes or no); creates or fattens (yes or no); farm is

close to environmental reserves (yes or no); slaughtering on the farm (yes or no).

For animal-level analysis, we followed the same steps presented above to analyze the farms. However, in order to correct the effect of animal selection for which weighted values were used, and described above, we applied a complex analysis to the tests, as previously described (KORN & GRAUBARD, 1999).

The variables used to build the model for *T. gondii* were: sex (male or female); feeding with leftovers (yes or no); presence of cats (yes or no); farm is located near dumps (yes or no). While the following variables were used to construct the model for *N. caninum*: sex (male or female); feeding with leftovers (yes or no); presence of dogs (yes or no); farm is located near dumps (yes or no). R statistical software package (R DEVELOPMENT CORE TEAM, 2013) were used for statistical analysis.

Ethics statement

In this study, all procedures using animals complied with the Ethical Principles in Animal Research adopted by the College of Animal Experimentation (COBEA) and were approved (protocol number 23108.215.014/2017-80) by the Animal Research Ethics Committee of the Federal University of Mato Grosso, Brazil.

Results

Of the 320 farms analyzed, anti-*T. gondii* and anti-*N. caninum* antibodies were detected in at least one animal in 178 and 89 farms (Figure 1), respectively; this resulted in a herd seroprevalence of 55.63% (CI 95%; 50.18%; 61.07%) for *T. gondii* and 27.81% (CI 95%; 22.90%; 32.72%) for *N. caninum*. Moreover, the

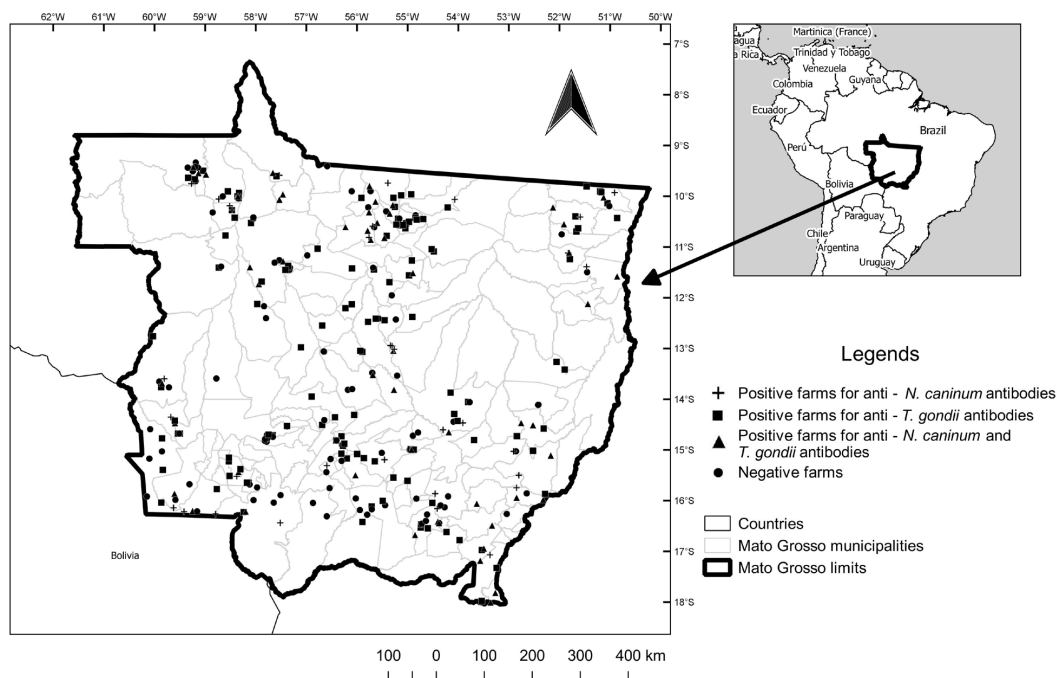


Figure 1. Spatial distribution of the 320 farms sampled for the detection of anti-*Toxoplasma gondii* and anti-*Neospora caninum* of the Mato Grosso state, Brazil, between September and December 2014.

seroprevalence of *T. gondii* in the total population was 32.48% (CI 95%; 28.49%; 36.46%), with titers ranging from 64 to 8,192, while that of *N. caninum* was 13.49% (CI 95%; 10.89%; 16.02%), and titers ranged from 50 to 6,400. Distribution of endpoint titers among the positive animals for *T. gondii* and *N. caninum* are listed in Table 1.

The relationship between the presence of anti-*T. gondii* and anti-*N. caninum* antibodies and the variables of the logistic

model on the 320 farms sampled are summarized in Table 2. In farm-level analysis, no variable was found to be significantly associated with the seroprevalence of *T. gondii* in pigs, after the model of multivariate analysis was applied. However, feeding with leftovers was found to be negatively associated with the presence of *N. caninum* seropositive pigs on farms, since farms that provide feeding with leftovers were 38.13% less likely to have pigs testing positive for *N. caninum* (Table 3).

Table 1. Results of indirect immunofluorescence assay (IFA) endpoint titers obtained among 1,070 pigs (> 8 months) on 320 farms of the state of Mato Grosso, Brazil, between September and December 2014, using *Toxoplasma gondii* and *Neospora caninum* antigens.

Endpoint antibody titers	N°. seroreactive sera to <i>Toxoplasma gondii</i> (%)	Endpoint antibody titers	N°. seroreactive sera to <i>Neospora caninum</i> (%)
64	82 (23.63)	50	65 (45.13)
128	114 (32.85)	100	47 (32.63)
256	79 (22.76)	200	16 (11.11)
512	45 (12.96)	400	11 (7.63)
1,024	8 (2.3)	800	1 (0.69)
2,048	9 (2.59)	1,600	3 (2.08)
4,096	7 (2.01)	3,200	0
8,192	3 (0.86)	6,400	1 (0.69)
Total of seropositives	347		144
Total of seronegatives	723		926

Table 2. Relationship between the presence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies and variables of the logistic model, on the 320 farms sampled in the of Mato Grosso state, Brazil, between September and December 2014.

Associated variable	<i>Toxoplasma gondii</i>		<i>Neospora caninum</i>	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Farm is in the rural areas or near indigenous reserves				
Yes	96 (30)	224 (70)	41 (12.8)	279 (87.2)
No	85 (26.6)	235 (73.4)	48 (15)	272 (85)
Farm is in the peri-urban areas or poor communities				
Yes	27 (8.4)	293 (91.6)	74 (23.2)	246 (76.8)
No	151 (47.2)	169 (52.8)	15 (4.7)	305 (95.3)
Pigs were raised extensively				
Yes	42 (13.1)	278 (86.9)	29 (9.1)	291 (90.9)
No	136 (42.5)	184 (57.5)	60 (18.8)	260 (81.2)
Feeding with leftovers				
Yes	58 (18.1)	262 (81.9)	45 (14.1)	275 (85.9)
No	120 (37.5)	200 (62.5)	44 (13.7)	276 (86.3)
Presence of cats*				
Yes	50 (15.6)	270 (84.4)	n/t***	n/t
No	128 (40)	192 (60)	n/t	n/t
Presence of dogs**				
Yes	n/t	n/t	4 (1.2)	316 (98.2)
No	n/t	n/t	85 (26.6)	235 (73.4)
Farm is located near dumps				
Yes	6 (1.9)	314 (98.1)	6 (1.9)	314 (98.1)
No	172 (53.8)	148 (46.2)	83 (25.9)	237 (74.1)
Farm is close to environmental reserves				
Yes	70 (21.9)	250 (78.1)	43 (13.4)	277 (86.5)
No	108 (33.7)	212 (66.3)	46 (14.3)	274 (85.7)
Slaughtering on the farm				
Yes	12 (3.7)	308 (96.3)	7 (2.2)	313 (97.8)
No	166 (51.9)	154 (48.1)	82 (25.6)	238 (74.4)

*variable used only for *T. gondii*; **variable used only for *N. caninum*; ***n/t = not applicable.

Table 3. Results of the logistical regression model for risk factors associated with finding in farm-level analysis and in the animal-level model for *Toxoplasma gondii* and *Neospora caninum* seropositivity, among 1070 pigs (> 8 months) on 320 farms of the state of Mato Grosso, Brazil, between September and December 2014.

Analysis level	Protozoa species	Estimate	SE*	p-value	OR**	CI*** 95%	
	Associated variable						
Farm-level analysis	<i>Neospora caninum</i>						
	Feeding with leftovers	-0.79	0.03	<0.01	0.61	0.26	0.75
Animal-level analysis	<i>Toxoplasma gondii</i>						
	Feeding with leftovers	0.09	0.02	0.01	1.09	1.01	1.18
	Age	<0.01	<0.01	<0.01	1.0029	1.0008	1.0051
	<i>N. caninum</i>						
	Feeding with leftovers	-0.05	0.01	0.03	0.94	0.89	0.99

*Standard error; **Odds Ratio; ***Confidence interval.

Table 4. Relationship between the presence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies and variables of the logistic model, on the pigs sampled in the of Mato Grosso state, Brazil, between September and December 2014.

Associated variable	<i>Toxoplasma gondii</i>		<i>Neospora caninum</i>	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Sex				
M	138 (12.8%)	932 (87.1%)	46 (4.2%)	1024 (95.7%)
F	210 (19.6%)	860 (80.3%)	98 (9.1%)	972 (90.8%)
Farm next to the dumping ground				
Yes	12 (1.12%)	1.058 (98.8%)	12 (1.12%)	1058 (98.8%)
No	335 (31.3%)	735 (68.9%)	133 (12.4%)	937 (87.5%)
Feeding with leftovers				
Yes	240 (22.4%)	830 (77.5%)	45 (4.2%)	275 (25.7%)
No	107 (10%)	963 (90%)	44 (4.1%)	276 (25.7%)
Presence of cats*				
Yes	103 (9.6%)	967 (90.3%)	n/t	n/t
No	243 (22.7)	827 (77.2%)	n/t	n/t
Presence of dogs**				
Yes	n/t***	n/t	101 (9.43%)	969 (90.5%)
No	n/t	n/t	43 (4.01%)	1027 (95.9%)

*variable used only for *T. gondii*; **variable used only for *N. caninum*; ***n/t = not applicable.

The relationship between the presence of anti-*T. gondii* and anti-*N. caninum* antibodies and the variables of the logistic model of the 1,070 pigs sampled is shown in Table 4. In animal-level model for *T. gondii* seropositivity, one variable (feeding with leftovers) was found to be a risk factor, since pigs that were fed with leftovers were 1.09 times more likely to test positive for anti-*T. gondii* antibodies. In accordance with the farm-level analysis, the variable feeding with leftovers also appeared to be negatively associated, decreasing the chances of positivity, and pigs feeding with leftovers were 5.44% less likely to test positive for *N. caninum* antibodies. Finally, age was a variable significantly associated, considered a risk factor, since in 8-month-old pigs, with each added month of life, the animals were 1.0029 times more likely to be seroreactive to *N. caninum* (Table 3).

Discussion

Here, we provide a serological survey for the presence of antibodies anti-*T. gondii* and anti-*N. caninum* in family-run farms with no technological improvements. We observed that more than half of studied farms (55.63% of herd seroprevalence) had at least one animal testing positive for anti-*T. gondii* antibodies. Moreover, a high rate seroprevalence (32.48%) in the total population of the present study was similarly observed in a previous survey performed in the western Amazon (CAVALCANTE et al., 2006); southern (CADEMARTORI et al., 2014) and northeastern (SAMICO-FERNANDES et al., 2017) regions from Brazil, where pigs were raised in similar conditions.

In addition, we observed a low seroprevalence for *T. gondii* as compared to the previous serological survey performed at 3 commercial farms of Nova Mutum and Diamantino municipalities, state of Mato Grosso in 2010, where Muraro et al. (2010) found that 12.8% of the tested animals were seropositive. Probably, the higher seroprevalence rate was influenced by the management systems, once according to Bezerra et al. (2009), the level of technology is a determining factor for infection by *T. gondii* in pig farms, since higher prevalence of *T. gondii* antibodies are observed in pigs raised extensively in free-range farms than from intensive farms (VAN DER GIESSEN et al., 2007).

Notably, the sampled farms raised pigs for their own consumption, where the animals were slaughtered on the farm without any inspection, so this type of management causes exposure of farmers or people, who slaughter or even consume pork meat, to *T. gondii* infection, as pigs are considered an important source of toxoplasmosis infection in humans (DUBEY, 2010). Indeed, humans may get infected by several routes, and there are numerous reports tracing toxoplasmosis to the consumption of infected meat with some serological surveys incriminating meat more strongly than cats, as a source of human infection (DUBEY, 2010). On the other hand, humans may also get infected during evisceration and handling of the meat (TENTER, 2009).

Feeding the animals with leftovers increases the probability of the presence of anti-*T. gondii* antibodies in pigs by 1.09-fold. This practice has already been associated with risk factors for *T. gondii* seropositivity (ALVARADO-ESQUIVEL et al., 2014; FEITOSA et al., 2014), suggesting that food containing raw or undercooked meat, as well as, unwashed vegetables (ALVARADO-ESQUIVEL et al., 2014; FEITOSA et al., 2014), accounts in part for the *T. gondii* infection in pigs. However, this hypothesis needs evidence. Indeed, the type of food can influence *T. gondii* seropositivity rates, since Dubey et al. (1992) observed that garbage-fed pigs had a higher seroprevalence of *T. gondii* (67.3%) than grain-fed pigs (33.8%), in a study conducted in Hawaii.

The seroprevalence of *N. caninum* is relative higher (13.49%) with animals presenting titers up to 6,400, when compared to previous studies conducted in Brazil using the same diagnostic method (IFA) and cut-off (1:50). The seroprevalence varied from 3.1% to 3.2% in domestic pigs in the country (AZEVEDO et al., 2010; FEITOSA et al., 2014). However, except for these serological evidences, currently there is no credible proof on the actual role of pigs in the epidemiology of *N. caninum*. In fact, only one study demonstrated experimental neosporosis in pigs with transplacental transmission of *N. caninum* (JENSEN et al., 1998), but there has not been any isolation of *N. caninum* from pigs in natural conditions till date.

Unlike to *T. gondii*, feeding with leftovers was negatively associated with *N. caninum* seropositivity in farm-level analysis and in the animal-level model. Although we are not aware of any study in literature reporting such an association, this management (feeding with leftovers) appeared not to be an important route of infection for *N. caninum*. How pigs acquire *N. caninum* infections in nature is not fully understood. Probably, horizontal transmission by ingestion of sporulated oocysts from food and water contaminated with dog feces represents the main route of infection, since differently as observed for *T. gondii*, the number

of *N. caninum* in tissues of chronically infected animals is low (DUBEY et al., 2017), therefore, the route of infection by ingested bradyzoite in the encysted stage of the parasite in tissues seems to be not important. However, further studies are needed to prove this statement.

Finally, *N. caninum* seroprevalence rates increased with age. Though this variable was never found to be associated with *N. caninum* seropositivity in pigs (CERQUEIRA-CÉZAR et al., 2017), several studies demonstrated that age was a statistically significant factor, probably due to the increase in probability of horizontal transmission by ingestion of sporulated oocysts. We believe that management facilities allow greater exposure of contaminated environments, since most farms (69.68%) recorded the presence of dogs, a known definite host for *N. caninum*.

Conclusion

We identified a high seroprevalence of *T. gondii* and *N. caninum* in pigs raised on family-run farms with no technological improvements. Feeding with leftovers was found to be risk factor for the seroprevalence of *T. gondii* in the animal-level analysis. Unlike to *T. gondii*, feeding with leftovers was negatively associated with *N. caninum* seropositivity in farm-level analysis and in the animal-level model, showing that this route of infection is probably not important for *N. caninum*. Furthermore, *N. caninum* seroprevalence rates increased with age. So, further studies are necessary to evaluate the impact of *T. gondii* on backyard pig farming production, as well as, its importance as a source of toxoplasmosis infection in humans in the Mato Grosso state, and the role of domestic pigs in the epidemiology of neosporosis.

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