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Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in swine from Northeastern Brazil

Prevalência de anticorpos anti-*Toxoplasma gondii* e anti-*Neospora caninum* em suínos do Nordeste do Brasil

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Abstract

A serologic survey was conducted among 130 swine slaughtered in the public slaughterhouse of the city of Patos, Paraíba State, Northeastern Brazil, to determine the prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies, and to verify possible associations between sex of the animals and antibody prevalence. The sera were analyzed by indirect antibody tests, considering 1:64 (*T. gondii*) and 1:50 (*N. caninum*) dilutions as cut-off points. The prevalence of anti-*T. gondii* antibodies was 36.2% (47/130) (95% CI = 27.9 – 45.0%) with reciprocal titers ranging from 64 to 2,048, and of anti-*N. caninum* antibodies was 3.1% (4/130) (95% CI = 0.8 – 7.7%) with reciprocal titers ranging from 50 to 6,400. Three of the four *N. caninum*-positive samples were also positive for *T. gondii* antibodies. All *Neospora* and *Toxoplasma* IFAT-positive animals were also positive for confirmatory immunoblotting techniques using total and purified *N. caninum* and *T. gondii* tachyzoite antigens, i.e., p38 (NcSRS2) and p30 (TgSAG1). There was no association between sex of animals and prevalence of anti-*T. gondii* and anti-*N. caninum* antibodies. This is the first indication of *N. caninum* natural infection in pigs from Brazil.

Keywords: Swine, *Toxoplasma gondii*, *Neospora caninum*, seroprevalence, Northeastern Brazil.

Resumo

Foi conduzido um estudo sorológico em 130 suínos abatidos no matadouro público do município de Patos, Estado da Paraíba, Nordeste do Brasil, com o objetivo de determinar a prevalência de anticorpos anti-*Toxoplasma gondii* e anti-*Neospora caninum*, e verificar possíveis associações entre o sexo dos animais e a prevalência de anticorpos. Os soros foram analisados pelo testes de imunofluorescência indireta, considerando as diluições 1:64 (*T. gondii*) e 1:50 (*N. caninum*) como pontos de corte. A prevalência de anticorpos anti-*T. gondii* foi de 36,2% (47/130) (IC 95% = 27,9 – 45,0%) com títulos variando de 64 a 2.048, e anti-*N. caninum* de 3,1% (4/130) (IC 95% = 0,8 – 7,7%) com títulos variando de 50 a 6.400. Três das quatro amostras positivas para anticorpos anti-*N. caninum* também foram positivas para anticorpos anti-*T. gondii*. Todos os animais positivos na RIFI para *Neospora* e *Toxoplasma* também foram positivos nas técnicas confirmatórias de imunoblotting usando antígenos totais e purificados de taquizoítos de *N. caninum* e *T. gondii*, ou seja, p38 (NcSRS2) e p30 (TgSAG1). Não houve associação entre o sexo dos animais e a prevalência de anticorpos anti-*T. gondii* e anti-*N. caninum*. Essa é a primeira indicação de infecção natural por *N. caninum* em suínos do Brasil.

Palavras-chave: Suínos, *Toxoplasma gondii*, *Neospora caninum*, soroprevalência, Nordeste do Brasil.

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Introduction

The production and productivity indices of swine herds can be influenced by several conditions including genetic, environmental, nutritional, toxic, management, and infectious factors. Among parasitic diseases, toxoplasmosis occupies an important position. Toxoplasmosis is a worldwide zoonosis (ACHA; SZYFRES, 2003), and its control in pigs is important for consumer protection. This infection may also affect the reproduction of sows (DUBEY; URBAN, 1990; GIRALDI et al., 1996; JUNGENSEN et al., 2001) and productivity of pigs in general and thus may play an important role in the swine industry.

It is now accepted that *Neospora caninum* is a significant cause of bovine abortion worldwide and, with the exception of dogs, the disease has rarely been described in other species (DUBEY; LINDSAY, 1996; DUBEY, 1999). Experimental infection of sows led to transplacental transmission of *N. caninum* (JENSEN et al., 1998) but naturally acquired porcine infection has not been described and nothing is known on its possible presence in the pig population of Brazil.

Recent surveys conducted worldwide showed that the prevalence of anti-*N. caninum* antibodies in swine is very low. Using ELISA as screening test and an immunoblotting technique as confirmatory test, one positive animal was detected among 2,041 sows from Hesse, Germany (DAMRIYASA et al., 2004). All 454 breeding sows from England and Wales were negative by IFAT (HELMICK et al., 2002). In Switzerland, seropositive frequencies of 3 and 1% were observed in sows and fattening pigs, respectively, by ELISA, however, no case was confirmed by polymerase chain reaction (PCR) (WYSS et al., 2000).

To date, no published report exists on seropositivity to *T. gondii* and *N. caninum* in pigs in the State of Paraíba. This study was conducted to determine the prevalence of anti-*T. gondii* and anti-*N. caninum* antibodies in swine slaughtered in the public slaughterhouse of Patos municipality, State of Paraíba, Northeastern Brazil.

Material and Methods

1. Field samples

Sera from swine slaughtered in the public slaughterhouse of Patos municipality (7° 1' S and 37° 17' W) were analyzed. The number of samples examined was calculated taking into account the assumed prevalence for anti-*T. gondii* and anti-*N. caninum* antibodies close to 50%, to maximize the sample size, and to obtain a minimal confidence of 95% and a statistical error of 10% (THRUSFIELD, 1995). Calculations were executed using EpiInfo version 6.04, resulting in a sample of 96 swine. As a matter of opportunity a total of 130 blood samples were collected during October and November 2006. In the region, swine are confinement-raised; however, in general the conditions of sanitary management are not appropriate. It was not possible to recover the husbandry/history of the pigs tested, as the animals are slaughtered without identification.

Blood was collected before slaughter from the *Vena cava cranialis* into sterile vacuum tubes, separated after clotting, and the sera were centrifuged and stored at -20 °C until analysis.

2. IFAT

For the detection of anti-*T. gondii* antibodies IFAT (CAMARGO, 1974) was performed considering 1:64 dilution as the cut-off point (GARCIA et al., 1999a), using RH strain tachyzoites as antigen. IFAT for *N. caninum* (PARÉ et al., 1995) was performed using 1:50 dilution as the cut-off point (LINDSAY et al., 1995); NC-1 strain of *N. caninum* cultured in bovine monocytes was used as the antigen. The conjugate used in both tests was anti-pig IgG (whole molecule, SIGMA, St. Louis, MO, USA). Positive and negative pig sera were used as controls and the positive samples were tested until the maximum dilution titer was reached.

3. Immunoblot analyses

The NC-1 strain of *N. caninum* (DUBEY et al., 1988) and the RH strain of *T. gondii* (SABIN, 1941) were maintained in Vero cell cultures and purified as previously described (SCHARES et al., 1998, 1999). Cell-culture-derived tachyzoites were frozen as a pellet at -80 °C until used for immunoblot or antigen purification.

The surface antigens p38 (NcSRS2) and p30 (TgSAG1) of *N. caninum* and *T. gondii*, respectively, were purified by affinity-chromatography (SCHARES et al., 2000) using the monoclonal antibodies mAb 4.15.15 and P30/3 (ISL, UK).

For westernblot, total-cell-culture-derived tachyzoites or purified surface antigens of *N. caninum* NC-1 and *T. gondii* RH were used. The immunoblot was performed as described (SCHARES et al., 1998). Pellets of 4×10^7 tachyzoites or purified p38 (0.05 µg) of *N. caninum* and 2×10^7 tachyzoites or purified p30 (0.05 µg) of *T. gondii* were incubated in non-reducing sample buffer (2% [w/v] sodium dodecyl sulfate [SDS], 10% [v/v] glycerol, 62 mM TrisHCl, pH 6.8) for 1 minute (94 °C), separated in 12% (w/v) SDS polyacrylamide minigels of 60 × 70 × 1 mm size, and transferred to PVDF membranes (Immobilon-P, Millipore). After the transfer, membranes were blocked using PBS-TG (PBS with 0.05% [v/v] Tween 20 (Sigma) and 2% [v/v] liquid fish gelatin [Serva, Germany]) and cut into 50 strips and examined as described below.

To detect antibodies against *N. caninum* tachyzoite antigens, the incubation of westernblot strips was performed as previously described (SCHARES et al., 1998), with a few modifications. Sera were diluted 1:50 in PBS-TG. Serum of an experimentally *N. caninum*-infected swine (2.5×10^6 tachyzoites i.m., isolate SweB1) (JENSEN et al., 1998), kindly provided by Prof. Peter Lind (Department of Veterinary Diagnostics and Research, Section of Immunology and Parasitology, Copenhagen, Denmark), was used as a positive control.

Based on previous studies (reviewed in DUBEY; SCHARES, 2006), only reactions with antigens of 29, 30, 33, and 37 kDa Mr were regarded to be specific for the interpretation of the *N. caninum* immunoblot using total tachyzoite antigens. In the *N. caninum* immunoblot using purified p38 (NcSRS2), the reactivity of sera with a single band of 38 kDa Mr was regarded a positive reaction.

To detect antibodies against *T. gondii* tachyzoite antigens, the incubation of westernblot strips was performed as described for *N. caninum*. Sera were diluted 1:10 in PBS-TG. We used a serum of a swine experimentally infected with 10^4 *T. gondii* Me49, i.v.

(kindly provided by Dr. Christian Bauer, Institute for Parasitology, Justus Liebig University Giessen, Germany) as a positive control. Based on the previous results (HUSKINSON et al., 1989) in this test, only the reactions against three immunodominant antigens of about 20, 30, and 35 kDa were regarded to be specific. In the *T. gondii* immunoblot using purified p30 (TgSAG1), the reactivity of sera with a single band of 30 kDa Mr was regarded as a positive reaction.

In all immunoblots, peroxidase conjugated “Affini Pure” goat anti-swine IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used diluted 1:250 in PBS-TG.

4. Statistics

The prevalence of anti-*T. gondii* and anti-*N. caninum* antibodies was estimated from the ratio of positive results to the total number of swine examined, with the exact binomial confidence interval of 95% (THRUSFIELD, 1995), using the program EpiInfo, version 6.04. Positive associations between sex of the animals and *T. gondii* and *N. caninum* antibody seroprevalence were verified by Chi-square test (χ^2) or by Fisher exact test (ZAR, 1999), with a significance level of 5%.

Results

For *T. gondii*, from 130 tested samples, 47 were positive (IFAT \geq 1:64), resulting in a seroprevalence of 36.2% (95% CI = 27.9 - 45.0%) as presented in Table 1. In male pigs, the seroprevalence was 37.8% (95% CI = 23.8 - 53.5%) and in female 35.3% (95% CI = 25.2 - 46.4%). There was no association between sex and prevalence of anti-*T. gondii* antibodies ($p = 0.92$). Overall, eight (6.2%), seven (5.4%), 10 (7.7%), 16 (12.3%), five (3.8%), and one (0.8%) of the animals were seropositive at reciprocal titers of 64, 128, 256, 512, 1,024, and 2,048, respectively (Table 2).

For *N. caninum*, from 130 tested samples, four were positive (IFAT \geq 1:50), resulting in a seroprevalence of 3.1% (95% CI = 0.8 - 7.7%) (Table 1). In male pigs, the seroprevalence was 2.2% (95% CI = 0.1 - 11.8%) and in female 3.5% (95% CI = 0.7 - 10.0%) and no association between sex and prevalence was observed ($p = 1.00$). Overall, two (1.5%), one (0.8%), and one (0.8%) pigs were seropositive at reciprocal titers of 50, 200, and 6,400, respectively (Table 2). Three of the four *N. caninum*-positive sows were also *T. gondii* positive, all with titers of 1:512.

All the four *N. caninum* IFAT-positive samples and three *N. caninum* IFAT-negative samples (<1:50) were examined by immunoblotting. In contrast to two negative sera (Figure 1a - lanes 5, 6), all positive sera (Figure 1a - lanes 1, 2, 3, 4) recognized immunodominant antigens in the *N. caninum* total tachyzoite antigen. Two *N. caninum* IFAT-positive sera reacted with three immunodominant antigens of 29, 33, and 37 kDa; two other *N. caninum* IFAT-positive sera recognized the immunodominant antigen of 37 kDa. One *N. caninum* IFAT-negative serum showed faint reactions with a 30 kDa *N. caninum* antigen (Figure 1a - lane 7). To confirm the specificity of the reactions, purified p38 antigen of *N. caninum* was also probed. In contrast to the *N. caninum* IFAT-negative sera, all of the *N. caninum* IFAT-positive sera reacted with this antigen (Figure 1b).

Table 1. Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies by IFAT in 130 swine slaughtered in the municipal public slaughterhouse of Patos, Paraíba State, Northeastern Brazil, by sex.

Sex	No. of pigs	Prevalence of anti- <i>T. gondii</i> antibodies			Prevalence of anti- <i>N. caninum</i> antibodies		
		No.	%	95% CI (%)	No.	%	95% CI (%)
Male	45	17	37.8	23.8 - 53.5	1	2.2	0.1 - 11.8
Female	85	30	35.3	25.2 - 46.4	3	3.5	0.7 - 10.0
Total	130	47	36.2	27.9 - 45.0	4	3.1	0.8 - 7.7

CI = Confidence Interval

Table 2. Prevalence of anti-*T. gondii* and anti-*N. caninum* antibodies by IFAT in 130 sera from swine slaughtered in the municipal public slaughterhouse of Patos, Paraíba State, Northeastern Brazil, by serum dilution.

<i>T. gondii</i>			<i>N. caninum</i>		
Reciprocal titer	No. of positive pigs	Prevalence (%)	Reciprocal titer	No. of positive pigs	Prevalence (%)
64	8	6.2	50	2	1.5
128	7	5.4	200	1	0.8
256	10	7.7	6,400	1	0.8
512	16	12.3			
1,024	5	3.8			
2,048	1	0.8			
Total	47	36.2		4	3.1

When all samples were probed with total *T. gondii* antigen the three *N. caninum* and *T. gondii* IFAT-positive samples and one negative control serum reacted with immunodominant antigens of *T. gondii* of 30 kDa (4/4 sera), 20 kDa (2/4 sera), and 35 kDa (2/2 sera) (Figure 1c - lanes 1, 3, 4, 5). All of these sera also reacted with the purified p30 antigen of *T. gondii* tachyzoites (Figure 1d - lanes 1, 3, 4, 5). One of the *N. caninum* IFAT-positive sera was only *N. caninum* but not *T. gondii* positive in the immunoblot analyses as previously determined by IFAT (Figure 1c and 1d - lane 2).

Discussion

This is the first survey to give indication on the presence of *N. caninum* antibodies in pigs in Brazil.

The occurrence of *T. gondii* antibodies of 36.2% is in the range of the values found in other studies from Brazil with adult pigs (8.54 to 90.4%) (GUIMARÃES et al., 1992; GARCIA et al., 1999b; CAVALCANTE et al., 2006; MOURA et al., 2007; MILLAR et al., 2008).

As the samples were obtained from slaughtered animals, the only available information was on the sex of the pigs. No association was observed when sex was compared with seroprevalence of *N. caninum* and *T. gondii* antibodies.

Toxoplasma gondii antibody titers were higher than observed in sows from Germany, where 73% of the reactive samples presented

titers of 1:16, just above the cut-off value used in their assay (DAMRIYASA et al., 2004). However, other Brazilian studies using the same method as the present study (IFAT) also found higher titer values (GARCIA et al., 1999b; CAVALCANTE et al., 2006).

In studies conducted in Hesse, Germany (DAMRIYASA et al., 2004), sows responding to *Sarcocystis miescheriana* antigen were found to be more frequently *T. gondii* positive (21%) than *Sarcocystis*-negative animals (16.9%). There were no studies using sera from Brazilian pigs testing the presence of antibodies to other related coccidian parasites and cross reactivity with *T. gondii*. This might be of epidemiological importance and requires further investigation.

Concerning *N. caninum*, 3.1% (four samples) were positive by IFAT (≥ 50) and three of them were also *T. gondii* positive by IFAT. To confirm the specificity of our IFAT results we employed four western blotting techniques, using total and purified antigens of *N. caninum* and *T. gondii*.

For cattle it is well established that *T. gondii*-infected animals show only limited cross-reactions with non-reduced immunodominant *N. caninum* tachyzoite antigens (STAUBLI et al., 2006) and with the native, immunopurified 38 kDa (NcSRS2) antigen (SCHARES et al., 1999; SCHARES et al., 2000). All *N. caninum* IFAT-positive sera recognized immunodominant

N. caninum antigens and the purified 38 kDa antigen (Figure 1a, 1b - lanes 1-4). However, three of these four *N. caninum*-positive sera also reacted with immunodominant *T. gondii* antigens and with the 30 kDa antigen of *T. gondii* (Figure 1c, 1d - lanes 1, 3, 4). This suggests that three of four pigs were infected with both *N. caninum* and *T. gondii*.

One *N. caninum* IFAT- and immunoblot-negative serum also showed clear reactions with immunodominant *T. gondii* antigens and with the 30 kDa antigen of *T. gondii* (Figure 1c, 1d - lane 5). However, this serum did not react with either *N. caninum* total immunodominant antigens or the 38 kDa antigen, thus confirming the specificity of our IFAT results. When the samples were probed with total *T. gondii* antigen, the sample that was *N. caninum* IFAT-positive and *T. gondii* IFAT-negative was the only negative in the immunoblot analyses (Figure 1c, 1d - lane 2), also confirming the specificity of our IFAT results.

Consequently, similar to the findings of others (DAMRIYASA et al., 2004) our serological results suggest that only low numbers of pigs seem to be naturally infected with *N. caninum*. However, the final proof of natural *N. caninum* infection in pigs is lacking and can only be achieved by isolating viable *N. caninum* from swine. Nevertheless, our results show that further studies are necessary to elucidate a potential effect of *N. caninum* on reproduction in swine.

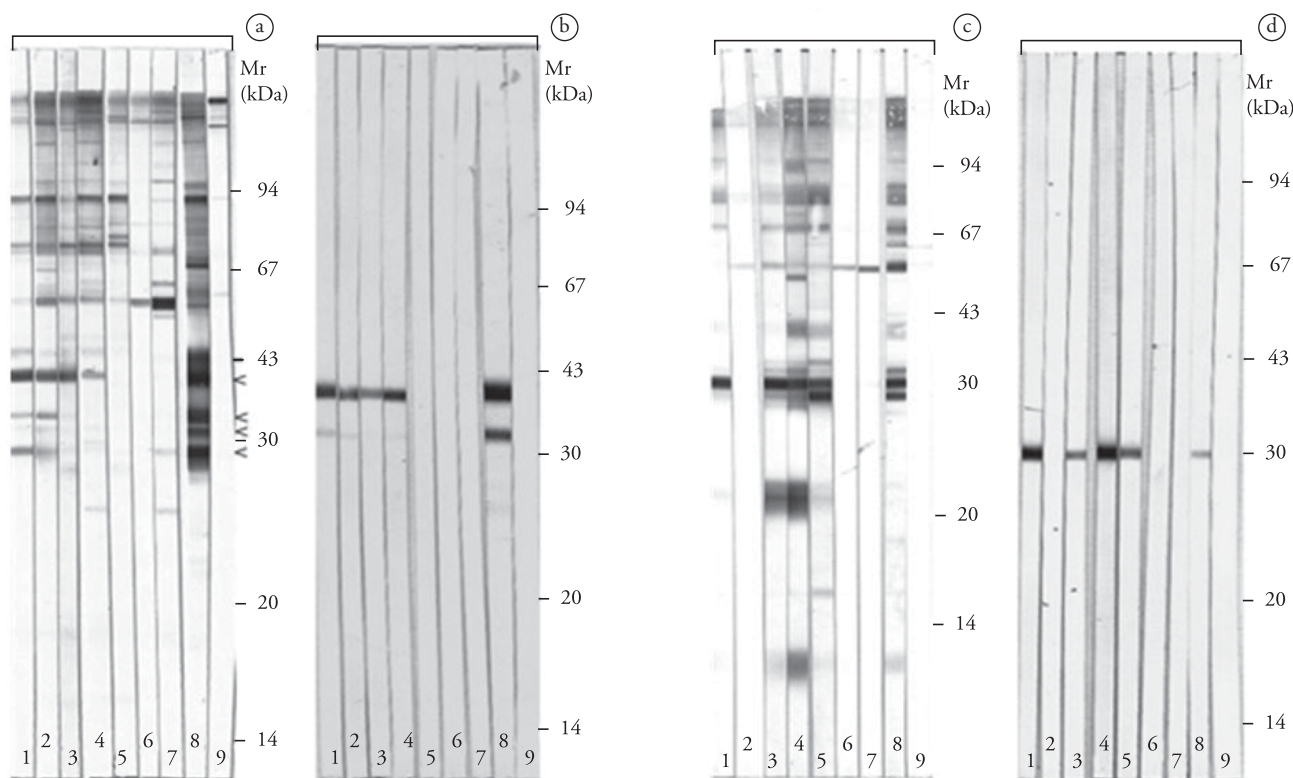


Figure 1. Immunoblotting analyses of four *Neospora caninum*-positive samples (Lanes 1 – 4) with IFAT titers of 1:50 (lanes 1 and 2), 1:200 (lane 3), and 1:6400 (lane 4); and three *N. caninum*-negative samples (lanes 5 – 7), with IFAT titers $<1:50$. Sera were probed with total *N. caninum* antigen (a), purified *N. caninum* p38 (NcSRS2) (b), total *T. gondii* antigen (c), and purified *T. gondii* p30 (tgSAG1) (d). The position of immunodominant antigens is marked by (<). In contrast to two negative sera, all *N. caninum* IFAT-positive sera recognized immunodominant antigens of *N. caninum*. One of these sera was negative for antibodies against *T. gondii* (lane 2). Another serum was positive for *T. gondii* but negative for *N. caninum* (lane 5). *N. caninum* controls: positive (a and b, lane 8), negative (a and b, lane 9). *Toxoplasma gondii* controls: positive (c and d, lane 8), negative (c and d, lane 9).

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