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Research paper

Does *Neospora caninum* cause reproductive problems in pigs?

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ABSTRACT

Neospora caninum is known to cause reproductive disturbances in several animal species, such as cattle, sheep, and goats. However, research on the effects of *N. caninum* on reproduction in pigs is limited. The objective of this study was to verify the transplacental transmission of *N. caninum* in pigs during several gestational stages. Twelve healthy *Toxoplasma gondii* and *N. caninum* seronegative female pigs were selected and separated into four groups of three animals each. Group A was maintained as a control group. Groups B, C, and D were inoculated intravenously with 2.9×10^7 tachyzoites of the *N. caninum* strain Nc1, 30 days before conception and at 45 and 90 days of gestation, respectively. Blood samples were collected from females periodically through IFAT for IgG and IgM screening to confirm the infection. At birth, after blood samples were collected from the piglets, they were then euthanized for the collection of the brain, heart, lung, liver, and diaphragm, which were then subjected to PCR. All inoculated gilts seroconverted (IgG) from the seventh day after inoculation. Nine of the 12 females expelled 24 mummified fetuses at the time of delivery, two in group A (eight), two in group B (four), three in group C (nine), and two in group D (three). Of the 24 mummified fetuses, nine were positive for *N. caninum* (one (25%) fetus of group B, seven (77.8%) of group C, and one (33.3%) of group D). A total of 126 live piglets were born. When the organs of the piglets from the inoculated females were analyzed by PCR for *N. caninum*, 88 (93.61%) were positive. All gilts inoculated produced at least one positive piglet. This demonstrates that there is transplacental transmission of *N. caninum* in all phases of gestation, regardless of the time of infection.

1. Introduction

Of all the protozoans that infect several animal species, *N. caninum* is known to cause damage, especially in cattle. The main clinical signs are related to reproductive disturbances, notably abortions and stillbirths (Cantón et al., 2014; Mcallister, 2016) in ruminants and neuromuscular problems in dogs (Mcallister, 2016).

To date, there have been only a few studies verifying the effects of *N. caninum* infection in pigs. Some research on the occurrence of *N. caninum* has been conducted in several countries. In domestic pigs, the prevalence varies from 3.1 to 18.9% in several parts of the world (Helmick et al., 2002; Bartova and Sedlak, 2011; Sharma et al., 2015; Feitosa et al., 2014; Silva, 2017). The highest prevalence so far was detected in pigs in the state of Santa Catarina, Brazil, the largest producer state in the country. However, when the prevalence in wild pigs was evaluated, it varied from 4.9 to 58.3% (Kamga-Waladjo et al., 2009; Cerqueira-Cezar et al., 2016; Soares et al., 2016; Lopes et al., 2018). The pathogenesis of the disease and its consequences in the swine species remain unclear.

The main problems in swine matrices are reproductive disorders. The drop in piglets/sow/year results in economic losses (Bortoletto et al., 2014). A variety of pathogens are responsible for reproductive disorders in pigs. These pathogens may be viruses, bacteria (Bortoletto et al., 2014), or protozoans such as *T. gondii* (Basso et al., 2015).

The correct diagnosis of the causes of abortion is important for the adoption of appropriate treatment, control, and prevention. As the natural infection by *N. caninum* has not yet been correlated with reproductive disorders in pigs, the spread of the parasite may be facilitated due to lack of control and prevention measures.

Therefore, our objective is to evaluate the effect of the experimental infection with tachyzoites of the *N. caninum* strain Nc1 in swine matrices at different stages of pregnancy.

2. Material and methods

2.1. Ethics committee on animal experimentation

The present study was approved by the Committee on Ethics in the

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Use of Animals (CEUA) of the CAV/UDESC, under protocol number 7997170717, on August 21, 2017.

2.2. Selection of animals

Twelve gilts, primiparous, approximately 10 months old and 130 kg gilts of the Agroceres Pic Camborough commercial strain were selected. The selection criterion included seronegativity (IFAT) for *T. gondii* and *N. caninum*.

The animals were kept in individual stalls in the swine sector of the Agroveterinary Sciences Center (CAV), State University of Santa Catarina (UDESC), where they received balanced rations, water, and routine management.

The gilts and the male donor semen were given two doses of Parvovirus, Erysipelas, Leptospira (Venco® Part. 003116, Fab. Out/16, Val. Out/18), and Circovirus (Venco® Part 005116, Nov/16, Val. Nov/18), 60 days before conception.

Fertile gilts period was detected by daily contact with a male. After detection of estrus, intrauterine insemination with fresh diluted semen, stored for a maximum of 72 h between 15 °C and 18 °C, occurred every 24 h (until male acceptance).

Semen was collected from a male with previously known fertility. The animal was kept in an individual bay in the swine sector of the Agroveterinary Sciences Center (CAV) of the State University of Santa Catarina (UDESC). The ejaculate was collected using the hand-gloved method, with a plastic cup and filter, to separate the fraction of the gel-rich ejaculate for later disposal. After collection, the semen was diluted 1:1 with DiluPork BTS + ®.

2.3. Inoculation

The gilts were separated into four groups of three animals each. The animals of group A were maintained as controls (PBS). The animals of groups B, C, and D were inoculated 30 days before conception and at 45 and 90 days of gestation, respectively.

The inoculum consisted of 2.9×10^7 tachyzoites of the *N. caninum* Nc1 strain intravenously obtained through cell culture, using VERO cell enriched with fetal bovine serum.

Gestation was confirmed using transabdominal ultrasonography, performed at days 45 and 90, to evaluate the maintenance of pregnancy and fetal viability.

2.4. Pre-delivery

In the post-insemination period, i.e. up to day 110 of gestation, gilts were kept in individual stalls and observed three times a day for the possibility of a miscarriage. On day 110 of gestation, the females were transferred to maternity cages for delivery.

On day 114 of gestation, labor was intramuscularly induced with 0.7 mL of Cloprostenol (Sincrocio®) (Part. 0009/17, Fab. Set/17, Val. Set/19) 24 h prior to delivery.

2.5. Sampling

2.5.1. Gilts

Samples of blood were collected from two days prior to inoculation until the day of calving; thus, the animals in group B were collected on days -2, 2, 5, 7, 14, 21, 28, 35, 42, 55, 70, 85, 100, 115, and 130 days post-inoculation (DPI). Those in group C on days -2, 5, 7, 14, 21, 28, 35, 42, and 55 DPI, and those in group D, -2, 2, 5, 7, 14, and 21 DPI. The animals in group A (control) were sampled on the same days as the animals in group C.

Serum samples were tested for IgG and IgM against *N. caninum* using Indirect Immunofluorescence-IFAT reaction while PCR was used to detect parasitemia.

During delivery, amniotic fluid sample was collected for PCR and IFAT (IgG and IgM).

2.5.2. Piglets

From term-born piglets, blood was collected immediately after birth (pre-colostral) (time 0) for the detection of IgG and IgM antibodies (IFAT) and parasite DNA (parasitemia) by PCR (Buffy Coat).

In addition, saliva was collected using swabs, which were placed in conical tubes containing 200 µl of PBS. The conical tubes were kept at rest for 1 h and then centrifuged at 1700 × g for 3 min. The obtained samples were stored (−20 °C) until processing. The samples were then subjected to PCR and IFAT.

Immediately after blood sampling, the piglets were weighed and measured and then placed with the mother for colostrum ingestion for 24 h.

After 24 horas (time 24) ingesting breast milk, blood (serum) and saliva from piglets were collected and then euthanized.

Euthanasia was carried out according to the 2017 modified 'Brazilian Guide to Best Practices for Animal Euthanasia' which recommends the use of 70% CO_2.

All the necropsied piglets and fragments of the central nervous system (CNS), lung, liver, heart, and diaphragm, in addition to the placentas, were collected for PCR analysis.

Mummified fetuses were measured using a tape measure to estimate the age of death. The estimation of gestational age was performed using the formula [(21 + (3 × fetal measure) (cm)] (Pescador et al., 2010). We also collected CNS, heart, and lung fragments for PCR.

2.6. Indirect immunofluorescence reaction (IFAT)

IgG and IgM antibodies against *N. caninum* were obtained in all serum, plasma, milk, amniotic fluid, and saliva samples obtained from the animals (sows and piglets). The cut-off point used was 1:50 for serum, plasma, milk, and amniotic fluid (Paré et al., 1995) and 1:25 for saliva (Azevedo et al., 2010).

2.7. Polymerase chain reaction (PCR)

For PCR, organs/tissues harvested during necropsy were washed with PBS (pH 7.2) at the time of collection and stored at −20 °C until processing.

All the harvested organs were individually macerated and DNA was extracted using the phenol-chloroform technique (organs, leukocyte layer, saliva, milk, amniotic fluid) according to Cavalcante (2010).

For PCR, the Nc5 region (5,10) was selected as the target sequence for DNA amplification. For this purpose, the primers Np21/Np6 (5'-CCCAGTGCGTCCAATCCTGTA-3')/(5'-CTCGCCAGTCAACCTACGTCT TCT-3') (Muller et al., 1996) were used. The reaction was carried out in a final volume of 50 µL containing 10× buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 20 µM of each primer, 1.25 U Taq. The following PCR cycle was used; initial denaturation at 95 °C – 5 min, 40 cycles at 94 °C – 1 min/63 °C – 1 min/74 °C – 3.5 min and a final extension at 74 °C – 10 min. The amplified samples were submitted to agarose gel electrophoresis (1.5%) for visualization. As positive and negative controls, tachyzoites of the *N. caninum* strain Nc1 and autoclaved ultrapure water were used, respectively.

2.8. Bioassay in dogs

Three non-defined dogs at approximately 45 days old and seronegative (IFAT, IgG) for *T. gondii* and *N. caninum* were selected.

Dog 1, male, was attached to the group B; female dogs 2 and 3 were bound to groups of C and D, respectively.

All dogs were vermifugated (5 mg/kg praziquantel, 14.5 mg/kg pyrantel, and 15 mg/kg febantel) 15 days before inoculation and kept in individual cages with ad libitum food and water.

For inoculation, the dogs were fasted for 12 h and then received, for

three consecutive days and three times a day, the placenta of the pigs and the CNS of the piglets of the respective groups to which they were bound, as the sole source of feed.

During the 20 days following inoculation, the entire daily volume of feces from each dog was collected. This was sent to the Laboratory of Parasitology and Parasite Diseases of the CAV/UDESC and analyzed using the modified Sheather technique (Sheather, 1923).

On the 25th day after inoculation, blood was collected from the dogs to obtain serum for the detection of IgG (IFAT) against *N. caninum*.

2.9. Statistics

The experimental design was completely randomized, with the animals divided into four groups containing three replicates.

For the results obtained, i.e., litter size, weight, and sex of the piglets, placental weight, and piglet weight, the analysis of variance was performed using the Sisvar software and the means were compared using the Tukey's test ($\rho \geq 0.05$).

The Kappa Concordance Coefficient (confidence coefficient: 95%) was used to verify the agreement between the tests used in various tissues and fluids. Values below zero were considered insignificant agreement, values between 0 and 0.2 were weak, between 0.21 and 0.4 were reasonable, between 0.41 and 0.6 were moderate, between 0.61 and 0.8 were strong, and between 0.81 and 1 was almost perfect (Landis and Koch, 1977).

3. Results

3.1. Gilts

After inoculation of the Nc1 strain of *N. caninum*, all gilts of groups B and C seroconverted (IgG) on the fifth day while the females of group D seroconverted on the seventh day.

In all the inoculated groups, the highest IgG titers were seen between 14 and 28 DPI, reaching a maximum of 6400 and then decreasing after 28 DPI, then remaining constant during gestation.

As for IgM, all inoculated animals were found to be seroconverted on the fifth day after inoculation. The peak of antibody production in all gilts was between seven and 21 DPI, reaching a titer of 1600 in some animals. However, one of the gilts in group C presented with an IgM antibody titer of 1600 at 42 DPI, preceded by a decrease in antibody production (100) at 35 DPI.

From gilts in group B, blood samples were collected up to 130 DPI. Two females from this group were seronegative (IgM) at 55 DPI and one was seronegative at 70 DPI. However, at 100 DPI the three gilts again seroconverted, remaining positive until delivery, reaching IgM antibody titers of 1600.

When IgG and IgM antibodies were evaluated in milk, all nine inoculated gilts were IgG positive and eight were IgM positive. IgG titers in milk ranged from 400 to 409600 (Table 3). Group B gilts showed 800, 400, and 3200 titers, as well as those from group C, 12800, 1600 and 409600, and those from group D, 25600, 25600 and 51200. IgM titers in milk ranged from 100 to 800. The gilts of group B presented titers of 100, negative, 100, of group C, 200, 200 and 400, and those of group D, 400, 200 and 800.

In addition, the occurrence of IgG and IgM antibodies in the amniotic fluid was evaluated. Only five females showed a positive result for IgG; two females from group C and three from group D. The titers ranged from 100 to 400. The test for IgM in amniotic fluid was negative in all animals.

All females of group A remained negative throughout gestation and at delivery.

3.2. Piglets

No female had an abortion and only one in group A (control) expelled a stillborn piglet 12 h from labor onset.

Table 1
Number, size, estimated gestational age and PCR results of parasite DNA in the tissues of mummified fetuses from uninoculated and inoculated gilts. Inoculation was carried out with 2.9×10^7 tachyzoites of *N caninum* strain Nc1.

Group	Nº Female	Mummified fetuses					
		Nº fetus	Size (cm)	EGA (days)	PCR heart	PCR lung	PCR brain
A	14	1	8.5	47	–	–	–
	14	2	8.5	47	–	–	–
	14	3	8.5	47	–	–	–
	14	4	23.5	92	–	–	–
	16	5	4.5	34	–	–	–
	16	6	7.5	44	–	–	–
	16	7	11.5	56	–	–	–
	16	8	13.5	62	–	–	–
B	1	9	4.5	34	–	–	–
	1	10	11	54	–	–	–
	1	11	12	57	+	–	+
	3	12	28	105	–	–	–
C	8	13	9	48	–	–	+
	9	14	4.5	34	–	–	–
	9	15	9	48	–	–	–
	10	16	4.5	34	–	–	+
	10	17	9.5	50	+	+	+
	10	18	10.5	53	+	+	+
	10	19	15	66	–	+	+
	10	20	15.5	68	+	–	+
	10	21	18	75	–	+	+
D	11	22	15.5	68	–	–	–
	12	23	13	60	–	–	–
	12	24	13	60	–	–	+

Nº: number; EGA: estimation of gestational ag; -: PCR negative result; +: PCR positive result.

Nine of the 12 females expelled 24 mummified fetuses at the time of delivery, two females in group A (eight fetuses), two in group B (four), three in group C (nine) and two in group D (three). The estimated gestational age at which fetal deaths occurred and the PCR results using tissues from the mummified fetuses are shown in the Table 1.

Six piglets were born with fetal malformation. Three with defective epitheliogenesis of the anterior limbs; two descendants of a female in group D and one of a female in group A. A piglet, from a female in group B, was born with anal atresia. Two piglets, from groups B and C, were respectively born with partial duplication in a facial region and having two tongues and a bifid muzzle. These two piglets were euthanized just after birth.

Of the 126 piglets that were born alive, 20 died between within the first 24 h of birth. Several causes were noted which included crushing, lack of colostrum intake, umbilical hemorrhage, hypothermia caused by the cold temperature in the night of labor. Of the 20 animals, six were from group A, six were from group B, five from group C, and three from group D.

3.2.1. Size, weight, and gender

There was no statistically significant differences ($p > 0.05$) in litter number, size, weight, and sex, and the estimated placenta weight of each piglet (Table 2).

3.2.2. Serology

3.2.2.1. IgG. Only piglets from Group C and D were born with IgG antibodies against *N. caninum*. In total, 17 piglets were positive (16 positive serum and plasma and one positive in plasma) before the colostrum was ingested. Of these, two were from one female from group C and 15 from three females from group D (Table 3).

Serum and plasma antibody titers observed at time 0 (pre-colostral) ranged from 50 to 1600. The two animals in group C had titers of 1600, similar to the two animals in group D, one in serum and another in plasma.

Table 2
Number of littered (n), size (cm), weight (g) and sex of piglets, placenta weight (g) and the estimation of placenta weight (g) in inoculated and uninoculated pigs. Inoculation was done with 2.9×10^7 tachyzoites of *N. caninum* strain Nc1.

		Littered			Sex		Placenta	
	Nº Female	Piglets number	Middle-weight	AS (cm)	F	M	Weight	APWP
Group A	14	11	1265	30	5	6	3050	277.3
	15	12	1156	29	4	9	1750	145.8
	16	9	1107	28	1	8	2230	123
	Total (Mean)	32 (10.6)	1176	29	10 (3.4)	23 (7.7)	2343.3	182
Group B	1	13	1093	25	4	9	1910	147
	3	9	1130	30	4	5	2145	238.3
	4	13	1175	25	8	5	3660	281.5
	Total (Mean)	35 (11.6)	1133	26.7	16 (5.3)	19 (6.3)	2571.7	222.3
Group C	8	10	1321	27	6	4	2380	238
	9	10	1257	26	4	6	2095	209.5
	10	4	1239	26	0	4	1450	362.5
	Total (Mean)	24 (8)	1272.3	26.3	10 (3.3)	14 (4.7)	1975	270
Group D	11	10	1216	30	4	6	2145	214.5
	12	10	1258	30	4	6	2240	224
	13	15	1085	27	6	9	3085	205.7
	Total (Média)	35 (11.7)	1186.3	29	14 (4.7)	21 (7)	2490	214.7

AS: average size; F:Female; M: Male; APWP: Average placental weight per piglet.

Table 3
Piglets with anti-*N. caninum* IgG antibodies in serum and plasma at birth (pre-colostral) and serum 24 h after, descendants of uninoculated or inoculated sows with 2.9×10^7 tachyzoites of *N. caninum* strain Nc1. This inoculation titer correlated with the titer of IgG against *N. caninum* in breast milk at the time of delivery.

			Piglets 0 h			Piglets 24 h		
	Nº Female	Nº piglets	Serum	Plasma	% Positive Total	Serum	% Positive Total	Milk titration
Group A	14	11	0	0	0	0	0	0
	15	12	0	0	0	0	0	0
	16	9	0	0	0	0	0	0
	Total	32	0	0	0	0	0	
Group B	1	13	0	0	0	13	100	800
	3	9	0	0	0	6	85.71	400
	4	13	0	0	0	8	88.9	3200
	Total	35	0	0	0	27	93.1	
Group C	8	10	0	0	0	6	100	12800
	9	10	0	0	0	8	100	1600
	10	4	2	2	50	4	100	409600
	Total	24	2	2	8.33	18	100	
Group D	11	10	3	2	30	10	100	25600
	12	10	8	10	100	10	100	25600
	13	15	3	3	26.7	12	100	51200
	Total	35	14	15	48.57	32	100	

Nº: Number; %: percentage.

When the sera of piglets were analyzed 24 h after birth (after colostrum ingestion), all piglets in groups C and D and 93.1% of piglets in group B, had IgG antibodies against *N caninum strain* Nc1 (Table 3).

Immunoglobulin titers of class G piglets at 24 h ranged from 50 to 51200. The highest IgG titers were observed in piglets from groups D (up to 51200), followed by group C (up to 12800) and B (up to 400).

When the presence of IgG in the saliva of the piglets was investigated, 36 of them had antibodies at time 0; eight (22.85%) from group B, 10 (41.66%) from group C and 18 (51.42%) from group D. At 24 h, 54 animals were positive; 13 (44.82%) from group B, 10 (52.63%) from group C and 31 (96.9%) from group D.

Ten piglets had saliva titers higher than 25 (IFAT) within 24 h after birth; one piglet from female 4 (group B), two female piglets from female 10 (group C), and seven from group D (two with titers 25, three with title 50, and two with title 100). To justify this, it can be observed the of salivary titers of piglets with those of colostrum and amniotic fluid. We found that females from group D showed high titers of antibodies in the colostrum and amniotic fluid. In group B, female 4 had higher milk titers than the rest. In group C, animal 10 had higher titers in milk and amniotic fluid among all inoculated animals.

3.2.2.2. IgM. Only one piglet, belonging to group D, showed a positive result for IgM when the serum was analyzed at time 0. Already when the plasma was analyzed, two piglets from group D were positive.

At 24 h, 42 piglets had serum IgM antibodies, 16 (55.17%) from group B, 13 (68.42%) from group C, and 13 (41.93%) from group D.

The maximum titer of IgM antibodies detected in both plasma and serum piglets was 50.

When the presence of saliva IgM is evaluated, it can be observed that only four animals were positive at time 0, all of them coming from gilt 1 from group B. At 24 h, all piglets were already negative when saliva IgM was evaluated.

3.2.3. PCR for N. caninum detection

Of the nine inoculated gilts, three had positive PCR results for milk, one from each inoculated group. Furthermore, when the amniotic fluid

was used as a sample for *N. caninum* DNA detection, five gilts had positive PCR results: one gilt from group B, two from group C, and two from group D.

When placenta tissue was used as a sample for *N. caninum* DNA detection, five females positive PCR results; one from group B (4), one from group C (10), and three from group D.

Of the 24 mummified fetuses, nine (37.5%) had positive PCR results for *N. caninum*: one (11.1%) fetus from group B, seven (77.8%) from group C, and one (11.1%) from group D (Table 1). Of all the organs measured in the mummified fetuses, the brain showed the highest positivity for *N. caninum* (37.5%).

The analysis of the brain, heart, liver, diaphragm, lung, blood, and saliva in 0 and 24 h of offspring of female pigs inoculated with *N. caninum* showed that 93.61% (88/94) were PCR positive. All gilts inoculated gave rise to at least one positive piglet (Table 4)

Only two piglets out of the 94 inoculated gilts showed both negative serology and PCR, one piglet from group B (brown 4) and one from group C (brown 8).

Using PCR, saliva, at time 24 (survivors), gave the highest number of positive piglets (51/80) infected with the protozoan. This was followed by saliva at time 0 (47/94), heart (45/94), brain (42/94), blood (41/94), liver (35/94), lung (33/94), and diaphragm (30/94).

All piglets born with fetal malformation from females inoculated with the protozoan were positive in at least one of the evaluated organs using PCR.

3.2.4. Kappa coefficient of agreement

It was used to compare the serological results (IgM and IgG) and results from PCR obtained using samples from different tissues and fluids of the piglets.

There was almost perfect agreement (0.82, CI 0.67 - 0.97) when the serum and plasma results were analyzed at time 0 for anti-*N. caninum* IgG. When IgG was analyzed in the saliva and serum at the 24 h there was moderate agreement (0.48; CI 0.31-0.66). A reasonable concordance was observed when serum (IgG) and saliva (0.34, CI 0.14 - 0.55), and plasma (IgG) and saliva (IgG) (0.28, CI 0.07-0.49) were analyzed at time 0.

When comparing the results obtained from the different fluids, IgM results showed insignificant agreement (> 0) for all samples.

Table 5 shows the results obtained when comparing PCR results from tissues and fluids. The following tissues showed a strong agreement: diaphragm and lung (0.72, CI 0.58 - 0.86), and lung and liver (0.63; CI 0.48 - 0.79).

3.3. Bioassay in dogs

Three dogs who ingested placenta and CNS did not eliminate *N. caninum* oocysts and did not seroconvert throughout the experiment.

4. Discussion

All the inoculated gilts seroconverted (IgG and IgM), demonstrating that inoculation of the *N. caninum* Nc1 strain was effective. These results are consistent with those found by Dubey et al. (1996), who inoculated 2.5×10^6 *N. caninum* strain Nc1 tachyzoites in pigs and used the IFAT and ELISA techniques for further testing.

IgM antibody titers found in gilts inoculated during gestation suggest that *N. caninum* was reactivated in group B females (inoculated at days 30 or 45 of gestation), at approximately 100 DPI, corresponding to approximately 70 days of gestation.

IgM is associated with the acute phase of infection or after reinfection or reactivation. In cattle, the main form of vertical transmission of *N. caninum* is endogenous facilitated by the action of certain hormones and the immunosuppressed state during pregnancy. Under such conditions, the cyst of the protozoan releases bradyzoites that later transform in tachyzoites and then migrate to various organs of the body, including the placenta and subsequently the fetus. Depending on the stage of gestation at which this occurs, fetal death may or may not occur, depending on the development of the fetus' immune system (Mcallister, 2016). Based on this, we suggest that the transplacental transmission of *N. caninum* in B group females occurred approximately 70 days after gestation, due to the presence of IgM antibodies in gilts and the presence of protozoan DNA in the tissues of piglets.

In cattle, when endogenous transmission occurs, it is more commonly between the second and third gestational thirds, due to hormonal and immunological actions (Antonello et al., 2015). Therefore, we can affirm that the pathogenesis of the endogenous transmission of *N. caninum* in pigs is similar to that of cattle.

None of the females had an abortion although there was a large number of mummified fetuses. Currently, a rate of 1.0–3.4% of mummified pigs in Brazil, without an association with infectious agents, is only acceptable because of the increase in the number of fetuses per sow (Padilha et al., 2017).

Two gilts in the control group each had eight mummified fetuses. Gilt14 had four mummified piglets; three of them died near day 47 of pregnancy and could be caused by female stress during blood sampling that began on day 42 of pregnancy. Many non-infectious causes of fetal

Table 4
Detection of *Neospora caninum* in tissues and fluids of uninoculated vs inoculated piglets. Inoculation was done with 2.9×10^7 tachyzoites of *N. caninum* strain Nc1.

Group	Nº Female	nº piglets	Brain		Heart		Liver		Diaphragm		Lung		Blood		Saliva 0		Saliva 24		Positive	
			P	%	P	%	P	%	P	%	P	%	P	%	P	%	P	%	P	%
A	14	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	16	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	T	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	1	13	5	38.46	7	53.84	4	30.76	0	0	0	0	5	38.46	6	46.15	6	46.15	13	100
	3	9	1	11.11	0	0	1	11.11	1	11.11	1	11.11	1	11.11	5	55.55	6	66.66	8	88.9
	4	13	5	38.46	6	46.15	1	7.7	0	0	1	7.7	7	53.84	2	15.38	8	61.53	10	77
	T	35	11	31.42	13	37.14	6	17.14	1	2.85	2	5.71	13	37.14	13	37.14	20	57.14	31	88.57
C	8	10	1	10	3	30	3	30	0	0	0	0	5	50	1	10	1	10	8	80
	9	10	1	10	7	70	1	10	2	20	0	0	6	60	0	0	3	30	10	100
	10	4	4	100	2	50	3	75	4	100	4	100	2	50	2	50	2	50	4	100
	T	24	6	25	12	48	7	29.16	6	25	4	16.66	13	54.16	3	12.5	6	25	22	91.67
D	11	10	8	80	6	60	9	90	9	90	9	90	3	30	9	90	9	90	10	100
	12	10	9	90	7	70	5	90	6	60	9	90	6	60	9	90	8	80	10	100
	13	15	8	53.33	7	46.67	8	53.33	8	53.33	9	60	6	40	13	86.66	8	53.33	15	100
	T	35	25	71.43	20	57.14	22	62.85	23	65.71	27	77.14	15	42.85	31	88.57	25	71.42	35	100

Table 5
Kappa Coefficient of agrrement for PCR results obtained from tissues and fluids of piglets, descendants of uninoculated or inoculated sows with 2.9×10^7 tachyzoites of *N. caninum* strain Nc1.

	Brain	Heart	Liver	Diaphragm	Lung	Blood	Saliva 0	N° Total +
Brain	–	–	–	–	–	–	–	42
Heart	0.32 (0.14-0.48)	–	–	–	–	–	–	45
Liver	0.42 (0.25-0.59)	0.27 (0.09-0.45)	–	–	–	–	–	35
Diaphragm	0.50 (0.33-0.66)	0.38 (0.20-0.56)	0.56 (0.40-0.73)	–	–	–	–	30
Lung	0.60 (0.45-0.75)	0.34 (0.16-0.51)	0.64 (0.48-0.79)	0.73 (0.58-0.86)	–	–	–	33
Blood	0.23 (0.05-0.40)	0.37 (0.19-0.53)	0.10 (0.08-0.27)	0.12 (0.06-0.31)	0.20 (0.01-0.38)	–	–	41
Saliva 0	0.36 (0.18-0.52)	0.08 (0.03-0.25)	0.39 (0.21-0.56)	0.44 (0.26-0.60)	0.53 (0.37-0.68)	0.13 (0.04-0.30)	–	47
Saliva 24	0.34 (0.16-0.50)	0.33 (0.15-0.49)	0.31 (0.12-0.48)	0.38 (0.20-0.56)	0.40 (0.23-0.57)	0.18 (0.004-0.36)	0.30 (0.12-0.46)	51

+: positive.

death have been reported, including stress, extreme temperatures, toxic substances, and sunburn (Sobestiansky and Barcellos, 2007).

When the mummified fetuses were born by infected females, in group B, only one of such fetus was PCR-positive for *N. caninum*, and its death probably occurred at approximately 57 days of gestation, that is, at 87 DPI, which also coincides with the detection of IgM in the serum of the sows.

The gilt of group C had seven of the nine mummified fetuses positive for *N. caninum*. Also, all the mummified fetuses of gilt 10 were positive for the protozoan. By estimating the fetal age at which death occurred, we found that all but one of the positives died after inoculation. Only one fetus from the three mummies of group D had a positive result for *N. caninum* although the gestational age at which fetal death occurred did not coincide with the inoculation of the protozoa. However, it is worth noting that it is only an estimate since piglet size varies throughout pregnancy. Therefore, the weight of this particular piglet could have been smaller than that of other piglets of the same gilt had it not suffered fetal death.

Some mummified fetuses from the inoculated females had negative PCR results (7/16). This does not rule out the possibility that these fetuses may have been infected with *N. caninum*, since obtaining a false-negative result is a possibility given that degradation of DNA may occur after infection. In addition, this result may be due to the sensitivity of the test (Snak et al., 2018).

These results demonstrate that there is transplacental transmission of *N. caninum* in pigs, which may still be one of the causes of fetal mummification in this animal species. Nevertheless, fetal mummification caused by *N. caninum* in pigs tends to occur more frequently in infected females in the second third of gestation (about 45 days). These results are consistent with those obtained in bovine and sheep species, where fetal death is more common between the first and second gestational thirds (Mcallister, 2016; Camillo et al., 2010; Buxton et al., 1998).

However, in cattle, fetal death rarely occurs when the animal is infected in the final third of gestation (Camillo et al., 2010). Similar results were also observed in the present experiment when gilts were inoculated at 90 days gestation and 30 days before gestation. Conception (reactivation at 70 days of gestation) had a low fetal death rate but with the birth of positive piglets (IgG/IgM/PCR).

Corroborating this study, in which *N. caninum* infection was associated with reproductive problems in pigs, Kamga-Waladjo et al. (2009) analyzed 60 serum samples from wandering pigs in the Senegal region and found that 58.3% of the positive samples for *N. caninum* were associated ($p < 0.05$) with stillbirth, demonstrating that the protozoan can cause reproductive problems in pigs.

Furthermore, Jensen et al. (1998) inoculated six gilts with 2.5×10^6 tachyzoites of the *N. caninum* strain NC-SweB1 intramuscularly in order to verify the transplacental transmission of the parasite. However, these gilts were euthanized before delivery making it impossible to verify possible reproductive complications. All gilts seroconverted and, using immunohistochemistry, it was possible to detect the protozoan in three fetuses (3/64), which is indicative of transplacental transmission.

Therefore, the present study is the first study to comprehensively associate *N. caninum* infection with fetal death in swine.

When analyzed, there were no statistically significant differences in piglet weight and size and litter size and placental weight in the inoculated and uninoculated groups, suggesting that *N. caninum* did not interfere with these parameters

When we serologically tested (IgG) the live-born piglets at time 0 (pre-colostral), it was noticed that only the piglets in group D and two piglets coming from the nut 10 of group C, had antibodies against *N. caninum*. The swine placenta is an epitheliochorial type, where there is no maternal blood contact with the fetal blood (Sinkora and Butler, 2016); therefore, there is no passage of antibodies from the female o the piglet, and these antibodies were found to have been generated by the piglets during the infection. When IgM was evaluated in the serum and plasma of the piglets at birth, only three animals had a positive result. The development of fetal antibodies in pigs starts at approximately 45 and 58 days of gestation for IgM and IgG, respectively. However, although antibody production is very small (Sinkora and Butler, 2016) the low titer can still be observed in the serum.

When the serum was evaluated at 24 h, most offspring of females inoculated with *N. caninum* were positive for IgG (96.2%) and IgM (52.5%), demonstrating that females could pass the antibodies via colostrum.

When comparing the IgG antibody titers present in milk from seroconverted piglets, it can be seen that the females who had high antibody titers were from the groups that had the most positive piglets at 24 h (postcolostral). When comparing the milk antibody titers with the antibody titers of the piglets at the 24 -h time point, it is noted that the higher the milk antibody titer, the higher the antibody titers of the piglets as well.

The females of group B had lower antibody titers in milk, lower piglets seroconversion, and lower antibody titers after 24 h.

Also, when the saliva of the piglets was analyzed at birth and 24 h after birth, it was observed that the later the inoculation, the greater the number of positive piglets at both times. At the time of birth, the saliva of the piglets is basically amniotic fluid, and the higher the antibody titer in this, the greater the number of positive piglets at the time of birth. Also, the higher the antibody titers in the milk, the higher the number of positive piglets by saliva, 24 h after birth.

There was a higher number of positive piglets (IgG) for *N. caninum* in saliva when compared with serum and plasma at time 0. Protozoan

antibodies were already detected in the saliva of other animal species, such as cattle (Ooi et al., 2000); therefore, the saliva of the animals can be used as an alternative for the diagnosis of *N. caninum*.

PCR results showed that the nine females inoculated with *N. caninum* were able to transmit the protozoan to the piglets. Only six piglets had negative results in all organs and fluids; however, these negative results could be false negatives because four of the six PCR-negative piglets had antibodies (IgM and/or IgG) against the protozoan.

In addition to transplacental transmission in pigs, there is a chance for transmitting through milk and amniotic fluid, as DNA from the parasite was found in these fluids. *N. caninum* has already been detected in milk and amniotic fluid of other animal species such as cattle (Razmi and Barati, 2017; Ho et al., 1997); however, this is the first time its detection in swine is being reported.

The placenta is an important organ that should be evaluated when studying the causes of abortion. With regards to *N. caninum*, the placenta is one of the organs with the highest positivity rates when evaluated by PCR and isolation techniques (Costa et al., 2018; Sinnott et al., 2017; Mcallister, 2016). In this study, it was possible to detect the presence of the protozoan DNA in the placenta of five inoculated sows, corroborating the results seen in other species of animals (Snak et al., 2018; Costa et al., 2018).

When the organs and fluids of the piglets were analyzed through PCR, the one that showed the greatest positivity was saliva at time 24 h, followed by saliva at time 0 h. This is the first detection of *N. caninum* DNA in animal saliva. This fact can be justified by the presence of protozoal DNA in milk and amniotic fluid and may be present in the saliva of piglets.

A leukocyte layer can also be used to diagnose neosporosis in animals. In this study, satisfactory results were found using this type of sample, however, there is great difficulty in collecting blood from newborn piglets.

Among the evaluated organs, the ones with the highest PCR positivity rates were the heart, followed by the brain, liver, lung, and diaphragm (Table 4). These results corroborate those found in other animal species such as cattle, where the CNS is the main organ for detection of the protozoa, followed by the heart (Snak et al., 2018; Sinnott et al., 2017; Mcallister, 2016; Amaral et al., 2012).

The Kappa concordance coefficient analysis showed that although saliva (0 and 24 h) showed a higher number of positive piglets, the results did not completely corroborate that obtained using other organs; that is, they were not necessarily the same animals that have positive results in saliva 24 and brain, for example. Therefore, there is a need to evaluate two or more organs/fluids to obtain more accurate results. However, there was a strong concordance between lung and diaphragm and between lung and liver, demonstrating that these organs present similar results and, therefore, selecting only of these organs for an effective diagnosis will suffice.

Finally, the bioassay was performed in dogs to verify the viability of the bradyzoites and tachyzoites present in the placenta and the CNS of the piglets. None of the dogs seroconverted or eliminated protozoan oocysts, demonstrating that there was no transmission of *N. caninum* from fetal tissues to the definitive host. However, the isolation of *N. caninum* in dogs is difficult to perform. Several studies of experimental inoculation of the protozoan in dogs have not been successful, with dogs rarely eliminating oocysts or seroconverting (Cedillo et al., 2008). Although not yet completely clear, it is believed that several factors, such as tissue storage temperature containing the bradyzoites (2–8 °C for 14 days), amount of tachyzoites and bradyzoites in the animal tissue, and age of the dogs can interfere with the infection of dogs and, consequently, the elimination of the oocysts (Cedillo et al., 2008).

5. Conclusion

The results of the present study allow us to conclude that *N. caninum* infection in pigs can be transmitted via the placenta and can cause reproductive disorders, such as mummified fetuses, especially in the first and second gestational thirds. Due to reactivation of the infection, endogenous vertical transmission was observed in the sows inoculated in the final third of gestation.

Moreover, it can be observed that the main organs and fluids for the detection of *N. caninum* in congenitally infected piglets are saliva, brain, heart, and blood.

Declaration of Competing Interest

There is no conflict of interest.

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