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PROTOZOOLOGY - ORIGINAL PAPER

Experimental infection of tachyzoites of the NC1 strain of *Neosporacaninum* in female swine

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Abstract

Neospora caninum is a protozoan that can cause reproductive problems in several animal species. Although *N. caninum* infection has been reported in swine, the pathogenesis and clinical signs are not fully known in this species. The objective of this work was to evaluate the effect of experimental infection with tachyzoites of the *N. caninum* strain Nc1 in swine matrices at different stages of gestation. For that purpose, 12 gilts, seronegative for *N. caninum* and *T. gondii*, were selected and allocated into four groups of three animals each. Animals in group A were not inoculated (control) and animals in groups B, C, and D were inoculated intravenously with of 2.9×10^7 tachyzoites, 30 days before conception, and at 45 and 90 days of gestation, respectively. Temperature, heart rate, blood, saliva, and vaginal mucus samples from the animals were collected periodically until the time of delivery for the investigation of IgG and IgM antibodies against *N. caninum* using IFAT and PCR to detect the parasite DNA. All gilts sero-converted from 5 and 7 DPI (days postinoculation) to IgM and IgG, respectively. Two gilts showed hypothermia on the 5th and 7th DPI, and five inoculated animals had leukocytosis on the 7th DPI. It was possible to detect DNA of *N. caninum* in samples of saliva (33/84), vaginal mucus (17/84), and blood (2/84). Based on serology (IgM) and PCR, three animals in group B showed evidence of reappearance of the infection during pregnancy. It is concluded that *N. caninum* can cause clinical signs in infected swine females, in addition to indicating saliva as a suitable diagnostic biological material for the detection of *N. caninum* DNA in this animal species.

Keywords Neosporosis · Swine · Gilts · Inoculation

Introduction

Neospora caninum is a heteroxene protozoan of the Phylum Apicomplexa. It parasitizes several species of animals, while canines are the definitive hosts. After ingesting animal tissue containing bradyzoites, dogs excrete oocysts in their feces, contaminating the environment, food, and water that may be a source of infection to other animals (Cerqueira-Cezar et al. 2016). Herbivores are the main intermediate hosts, and among them, infection in the bovine species has been identified as the greatest importance, and consequently, the most studied due to the great losses in production caused by reproductive failures (Cerqueira-Cezar et al. 2016).

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However, infections in other intermediate hosts have also been reported, such as sheep, buffalo, and pigs. In pigs, experimental studies of *N. caninum* infection are rare. The first work involving this animal species was developed by Dubey et al. (1996) who inoculated two pigs with 2.5×10^6 tachyzoites (strain Nc1) in order to test the enzyme-linked immunosorbent assay (ELISA) and immunofluorescent antibody technique (IFAT) methods in which two animals sero-converted 2 weeks after inoculation. In a study by Jensen et al. (1998), six gilts were inoculated with 2.5×10^6 tachyzoites from the NC-SweB1 strain of *N. caninum* in order to verify the transplacental transmission of the parasite. The results showed that all gilts seroconverted and it was possible to detect the protozoan in two fetuses providing the first indication of transplacental transmission in pigs. More recently, Snak et al. (2019) demonstrated that experimental infection with the Nc1 strain of *N. caninum* can cause reproductive problems in pigs, including fetal death.

Natural infection with *N. caninum* in pigs has been reported throughout the world. The prevalence of *N. caninum* in swine

varies from 1.9 to 18.9%, depending on the region, type of production system, and diagnostic test employed. For example, in England, Helmick et al. (2002) identified 8.8% of 454 swine females with a history of abortion or infertile reproduction were sero-positive for *N. caninum* using ELISA. Bártová and Sedlák (2011), in the Czech Republic, analyzed 551 swine serum samples and found 3% of the animals presented antibodies against *N. caninum* using the ELISA technique. In Germany, 3.3% of the animals tested were sero-positive using the ELISA technique and 27.7% of the farms had at least one positive animal (Damriyasa et al. 2004). The first description of *N. caninum* in pigs was in China when Gui et al. (2019) analyzed 1500 serum samples using the ELISA technique of which 1.9% were positive. In Brazil, there are reports of infection by *N. caninum* in domestic swine in the State of Paraíba where two studies identified approximately 3% of pigs from the slaughterhouses were sero-positive for *N. caninum* using IFAT (Azevedo et al. 2010; Feitosa et al. 2014). However, a higher sero-prevalence has been identified in the States of Santa Catarina and Mato Grosso in commercial (18.9%; 94/498) (da Silva et al. 2020) and backyard pigs (13.49%; 144/1070) (Minetto et al. 2019) respectively.

Brazil is one of the main pork producers for human consumption in the world (Jank et al. 2020). However, to date, there are no studies verifying the changes that *N. caninum* can cause in pregnant and newly infected female swine. This lack of knowledge is a limiting factor in the development of disease prevention programs in Brazil and worldwide. Therefore, the objective of this work was to verify the effect of experimental infection with Nc1 strain of *N. caninum* in different types of pregnancy of swine matrices, evaluating immunological, hematological, and clinical responses of infection, in addition to assessing which diagnostic samples (saliva, vaginal mucus, blood) are best suited for detecting *N. caninum* DNA.

Material and methods

Ethics committee

The present study was submitted to the CAV/UDESC Ethics Committee on Animal Use (CEUA), being approved under protocol number 7997170717.

Gilt selection

Twelve healthy, 10-month-old gilts were selected, weighing approximately 130 kg, from the commercial strain Agroceres Pic Camborough, and were determined to be seronegative for *Toxoplasma gondii* and *N. caninum* using IFAT 2 days prior to inoculation. The animals were kept in individual pens in the swine sector at the Center for Agricultural Sciences (CAV), State University of Santa Catarina (UDESC), where they received routine food and handling. Estrus was detected by daily direct contact with a healthy male, and all twelve gilts were fertilized through intrauterine artificial insemination. The pregnancy was confirmed with the use of transabdominal ultrasound, which was performed at 45 days and at 90 days of pregnancy, to assess the maintenance of pregnancy and fetal viability.

Inoculation

For inoculation, 2.9×10^7 tachyzoites of the Nc1 strain of *N. caninum* (Dubey et al. 1988) per animal were obtained through cell culture with VERO ATCC CCL-81 cells, enriched daily with RPMI medium and fetal bovine serum (10%), as described by Williams et al. (1997). The inoculum was diluted with sterile PBS pH 7.2 solution (about 2 mL). The experimental design was completely randomized, with the animals divided into four groups with three animals each. Group A animals were kept as uninoculated controls. The animals in groups B, C, and D were inoculated, intravenously, 30 days before conception and at 45 and 90 days of gestation, respectively.

Clinical exams

In the postinsemination period until the 110th day of gestation, the gilts were kept in individual pens and observed three times a day, in order to detect possible abortion or clinical changes. Clinical examinations of gilts (temperature and respiratory frequency) were performed on the same days as blood samples described below (Table 1). These data were tabulated and analyzed to check for possible changes resulting from the acute phase of infection in the animals or reappearance during pregnancy.

Sampling

Whole blood, serum, plasma, saliva, and vaginal mucus samples from the animals were collected periodically from two days before inoculation until delivery (Table 1). Blood samples were collected from the vena cava using 18G gauge needle into prepared tubes with EDTA and dry tube. For the collection of saliva and vaginal mucus, sterile swabs were used, which were placed in conical tubes containing 200 μl of PBS, kept at rest for one hour and then centrifuged at 1700*g* for 3 min, and the samples thus obtained were stored (− 20 °C) until processed.

Complete blood counts on all whole blood samples collected from gilts was performed, with the aid of the hematological device SDH-3 VET® (company, city, country). Confirmation of the leukocyte differential was determined by reading a blood smear. In addition, measurements of glutamic-oxaloacetic transaminase (AST) and gamma-glutamy

Table 1 Corresponding days of gestation based on the inoculation days of *N. caninum* in swine females

DPI	Corresponding days of gestation			
	Group A	Group B	Group C	Group D
− 2	43	− 32	43	43
2	47	− 28	47	47
5	50	− 25	50	50
7	52	− 23	52	52
14	59	− 16	59	59
21	66	− 9	66	66
28	73	− 2	73	73
35	80	5	80	80
42	87	12	87	87
55	100	25	100	100
70	UD	40	UD	UD
85	UD	55	UD	UD
100	UD	70	UD	UD
115	UD	85	UD	UD
130	UD	100	UD	UD

DPI Days postinoculation

ND Not done

ltransferase (GGT) levels in serum were performed sing the automatic analyzer LABMAX PLENNO® (Labtest, Lagoa Santa, Brazil). The reference values used for GGT and AST were 10 to 60 IU/L and 32 to 84 IU/L respectively.

Serum, plasma, vaginal mucus, and saliva samples were used to determine the presence of *N. caninum*–specific IgG and IgM antibodies by means of the indirect immunofluorescence test (IFAT) as described below. In addition, a buffy coat was prepared from 0.2 ml of the whole blood samples and together with the saliva and vaginal mucus prepared for use in polymerase chain reaction (PCR) for the detection of parasite DNA.

Indirect immunofluorescence reaction for IgG and IgM antibodies against *N. caninum*

IFAT was performed to search for IgG and IgM antibodies against *N. caninum* in all samples of serum, plasma, saliva and vaginal mucus using a 1:50 dilution as a cutoff point (Azevedo et al. 2010). The slides used for IFAT were adsorbed with tachyzoites of the Nc1 strain of *N. caninum*. For that purpose, 20–30 tachyzoites for field at × 400 magnification, from cell culture as described above were used. The tachyzoites were prepared by washing three times with PBS pH 7.2 and centrifugation at 1500 rpm for 10 min to remove cell debris and residual culture medium before being deposited on slides marked with Teflon adhesive (12 wells). The slides were dried at room temperature, fixed with absolute methanol, and stored at − 20 °C until needed.

The serum and plasma samples were prepared by diluting with PBS in ten two-fold dilutions starting at 1:50. The saliva and vaginal mucus samples were initially evaluated by IFAT (IgG and IgM) without dilution. Any positive samples were then diluted with PBS in ten two fold dilutions starting a 1:25. Twelve microliters of each sample dilution was added to each immunofluorescence slide well and incubated for 40 min at 37 °C. Afterwards, three washes were performed with PBS pH 7.2 for 10 min. The slide was dried in an oven for 3–5 min at 37 °C. Then 12 μL of the rabbit antipig IgG FITC (1:150; Sigma®, St. Louis, USA) or goat antipig IgM FITC (1:100; Bethyl Laboratories®, Montgomery, USA) diluted with PBS was added to each well and incubated for 40 min at 37 °C. Three washes were performed with PBS as described above, and the slide was dried in an oven at 37 °C as previously described. Afterward, two drops of buffered glycerin were added, and the slide was covered with a coverslip. The slide was read as described by Paré et al. (1995) in an epifluorescence microscope at 400 times magnification.

Polymerase chain reaction

The PCR technique was used to detect *N. caninum* DNA in the buffy coat, saliva, and vaginal mucus of the inoculated gilts. DNA extraction from buffy coat, saliva, and vaginal mucus from gilts was performed as described by Cavalcante (2010). For PCR, a 337 base pair region of the *N. caninum* Nc5 gene of was selected as the target sequence for DNA amplification using the primers Np21/Np6 (5'-CCCAGTGC GTCCAATCCTGTA-3 ')/(5'-CTCGCCAGTCAACC TACGTCTTCT-3') (Muller et al. 1996).

The reaction was carried out with a final volume of 50 μL, containing × 10 buffer, 200 μM dNTP, 1.5 mM $MgCl_2$, 20 μM of each primer, 1.25 U Taq (AmpliTaq Gold DNA Polymerase, Thermo Fischer Scientific, Waltham, USA), with initial denaturation at 95 °C for 5 min, 40 cycles at 94 °C for 1 min/63 °C for 1 min/74 °C for 3.5 min and final extension at 74 °C for 10 min. The amplified samples were submitted to electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized under UV light. The positive control consisted of DNA extracted from tachyzoites of the Nc1 strain of *N. caninum*, and as a negative control, autoclaved ultrapure water was used.

Statistics

To verify the agreement between the tests used in different tissues and fluids, the Kappa Agreement Coefficient (confidence coefficient: 95%) was used, while values below zero were considered insignificant agreement, values between 0 and 0.2 were weak, reasonable between 0, 21, and 0.4,

moderate between 0.41 and 0.6, strong between 0.61 and 0.8, and almost perfect agreement between 0.81 and 1 (Landis and Koch 1977).

Results

Respiratory rate and temperature

Regarding temperature, all three animals in group B showed hyperthermia at the beginning (2nd DPI) and lower body temperature at the end of the experiment (100, 115, 130 DPI). Two animals, one from group C and the other from group D, had temperatures below 36 °C, on the 7th (35.3 °C) and 5th (35.8 °C) DPI, respectively, in addition to showing signs of apathy. Regarding respiratory rate (RR), only the three animals in group B, on the -2nd DPI had a lower RR compared to the rest of the days and animals.

Complete blood count, leukogram, and biochemical analysis

Five of the nine inoculated animals (group B $n = 3$, group C $n = 1$, group D $n = 1$) showed leukocytosis on the 7th DPI (LT: 25650; 23510; 23950; 26130; 30140), two in group B had neutrocytosis (N: 10516; 11017) and three (group B $n = 2$, group D $n = 1$) with lymphocytosis (L: 13594.5; 13635.8; 22906.4). In addition, three animals (group B $n = 1$, group C $n = 1$, group D $n = 1$) had higher amounts of band cells (classified as immature neutrophils).

When the serum levels of AST and GGT were evaluated, the three animals in group B showed high levels on the 21th DPI (AST: 148; 112; 198) and, in two of these animals, these levels remained until 28 DPI (AST: 141; 86), returning to normal after this period. The animals showed no changes in GGT levels.

Other than what is specifically noted above, the blood counts and biochemistry of the gilts remained normal during the experiment.

Serology for *Neospora caninum*

Serum

When the presence of IgM was analyzed in the serum of gilts after inoculation, all animals in groups B, C, and D sero-converted on the fifth day postinoculation. The highest antibody titer (1:800) was on the 14th DPI for all animals, with the IgM titers decreasing thereafter. Of note, the group B animals, which were negative for IgM between 55 and 85 DPI displayed positive IgM titers (ranging from 1:200 to 1:800) on the 100th DPI which were maintained at an equal or higher titer when tested on 115 DPI before dropping on 130 DPI suggesting a possible reexposure or reactivation of *N. caninum* infection (Fig. 1).

All gilts in groups B and C and animal in group D were positive for IgG antibodies (> 1:50) against the protozoan on the fifth day postinoculation. The remaining two group D females tested positive for IgG (1:200) on the seventh day postinoculation. The highest IgG titers in all inoculated groups were found after 14 DPI, and in most animals in group B and C, these values decreased after 28 DPI, remaining constant and positive during pregnancy (Fig. 1).

Plasma

When analyzing IgM in plasma, only four animals tested positive in the fifth DPI, one from group B (1:50), one from group C (1:100), and two from group D (1:50, 1:100). However, after the seventh DPI, all animals showed IgM antibodies against the protozoan ranging from 1:100 to 1:800. Higher antibody titers (1:800) were observed between 14 and 21 DPI, decreasing after this period. As in serum, the three animals in group B were negative on the 85th DPI and IgM antibody production returned on the 115th DPI (Fig. 1).

When the presence of IgG against *N. caninum* in the plasma of gilts was evaluated, it was found that only two animals sero-converted (1:50) on the fifth DPI, one from group B and the other from group D. As with serum, the highest IgG titers in all inoculated groups were found between 14 and 28 DPI. Most animals did not show high titers, compared to serum, remaining constant throughout pregnancy (Fig. 1)

Saliva and vaginal mucus

IgG antibodies against *N. caninum* as determined by IFAT were detected in the vaginal mucus without dilution. Three animals, one from each inoculated group, showed a positive result (1:25) from 14 DPI. Of the inoculated animals, only three were negative in all samples, one from group C and two from group D (Table 2).

The presence of IgG antibodies against *N. caninum* in saliva was observed in the three sows in groups B and C. In group B, one animal was positive on the 70th DPI, in group C, one animal was positive on the 28th DPI and another on the 42th DPI. Group D did not show positive animals.

When IgM was analyzed, the three animals in group B showed antibodies *N. caninum* in the vaginal mucus after 14 DPI, and only one animal in groups C showed a positive result for IgM, on the 28th DPI. No group D animals presented IgM against *N. caninum* in the vaginal mucus.

When analyzing saliva for IgM, only one animal from group C was positive at 14 DPI, and was also positive on the 21th and 35th DPI.

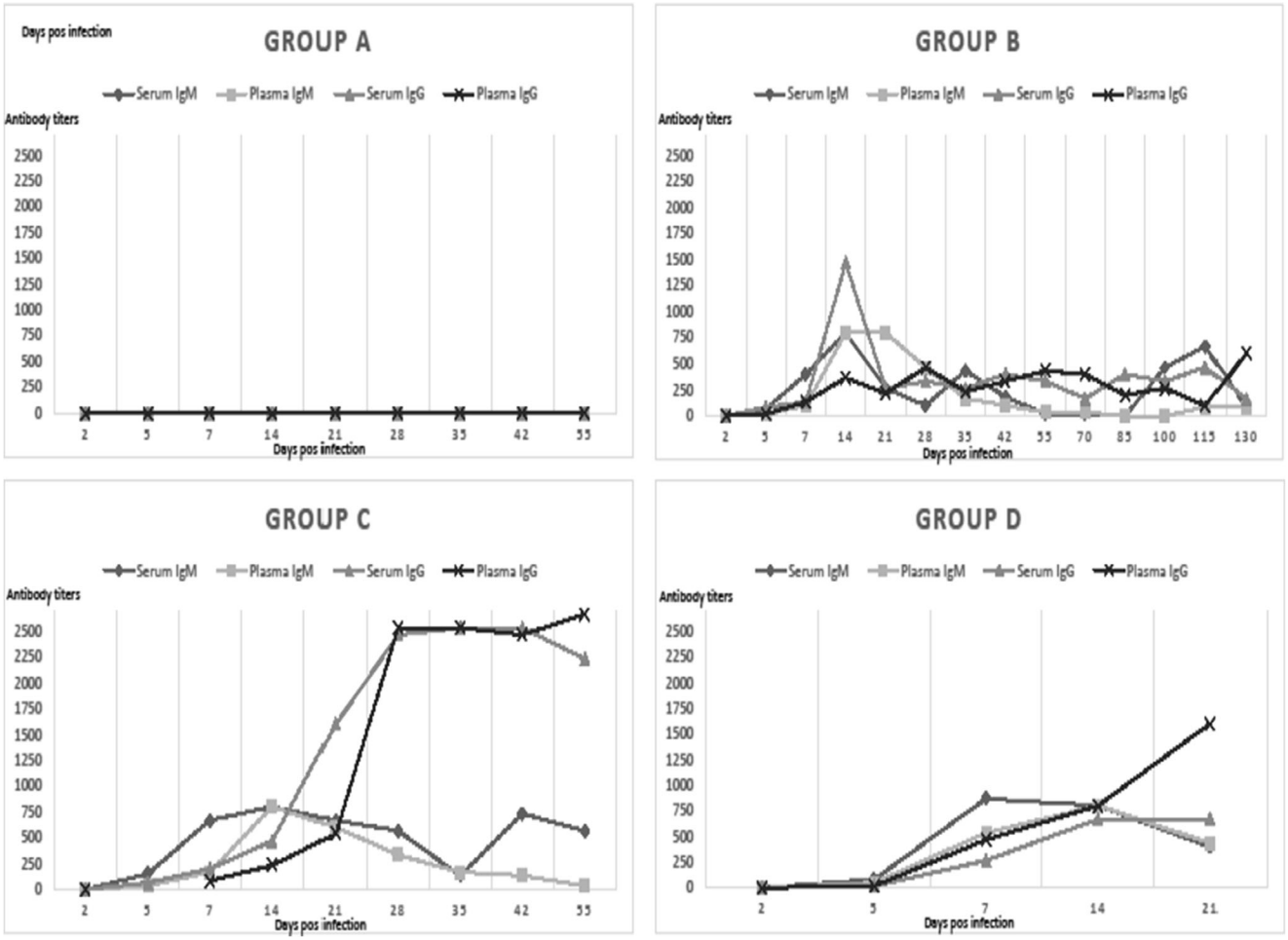

Fig. 1 Mean titers of IgG and IgM antibodies in serum and plasma from gilts experimentally inoculated with 2.9×10^7 tachyzoites of the Nc1 strain, from day 2 to day 130 postinoculation (cutoff 1:50)

Table 2 Results of IgG (IFAT) research against *N. caninum* in the vaginal mucus of inoculated gilt, or not, with 2.9×10^7 tachyzoites of the Nc1 strain

Day	Group A animals			Group B animals			Group C animals			Group D animals		
	14	15	16	1	3	4	8	9	10	11	12	13
-2	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	+	-	-	-	-	+	-	+	-
21	-	-	-	+	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	+	UN	UN	UN
35	-	-	-	+	-	-	-	-	+	UN	UN	UN
42	-	-	-	+	+	-	+	-	+	UN	UN	UN
55	-	-	-	+	+	-	+	-	-	UN	UN	UN
70	UN	UN	UN	-	-	-	UN	UN	UN	UN	UN	UN
85	UN	UN	UN	-	+	+	UN	UN	UN	UN	UN	UN
100	UN	UN	UN	-	-	-	UN	UN	UN	UN	UN	UN
115	UN	UN	UN	-	-	-	UN	UN	UN	UN	UN	UN
130	UN	UN	UN	-	-	-	UN	UN	UN	UN	UN	UN

[-] Negative; [+] Positive; *UN* unperformed

Polymerase chain reaction

Only two inoculated animals, one in group B and one in group C were positive for *N. caninum* DNA in blood by PCR on the 5th and 7th DPI, respectively.

When the gilts' saliva was analyzed by PCR, all nine inoculated animals returned at least one positive PCR from saliva samples. Two animals from group B and one animal in group D were positive on the 2nd DPI, with 8/9 animals returning a positive PCR result from a saliva sample by the 5th DPI (Table 3).

The results of the PCR for *N. caninum* of vaginal mucus demonstrated that it was possible to detect DNA in 6/9 inoculated animals, with DNA detected in one animal from group D on the 2nd DPI, three animals in group B, and one animal in group D on the 5th DPI. During the sampling period only 3/9 inoculated animals, two in group C and one in group D, did not return a positive sample (Table 3).

All nine inoculated animals showed a positive PCR result in serum and 6/9 in vaginal mucus at some point in the experimental period (Table 3). In addition, animals in group B, inoculated 30 days before conception, showed positive results in vaginal mucus and/or saliva up to 35 DPI. In the samples collected on the 42th, 55th, and 70th DPI, all animals were negative, returning to positive results on the 85th and 100th DPI, results that are in line with those found when analyzing the serum and/or plasma IgM titers.

Cohen's Kappa coefficient

Both, the results of the research of IgG and IgM in serum, saliva, vaginal mucus, and plasma, as well as the results of PCR of blood, saliva, and vaginal mucus, were analyzed using the Kappa coefficient.

When the results of IgG were evaluated, almost perfect agreement was obtained between serum and plasma (0.83 (CI: 0.70–0.96)) and weak agreement between serum and saliva (0.013 (CI: − 0.35–0.38)), serum and vaginal mucus (0.07 (CI: − 0.25–0.40)), plasma and saliva (0.01 (CI: − 0.34–0.38)), plasma and vaginal mucus (0.08 (− 0.23–0.40)), and vaginal saliva and mucus (0.18 (CI: − 0.14–0.51)).

The results of the Kappa coefficient for IgM showed strong agreement between serum and plasma (0.63 (CI: 0.43–0.69)), weak agreement between serum and saliva (0.01 (CI: − 0.34–0.37)), serum and vaginal mucus (0.04 (CI: − 0.29–0.39)), plasma and saliva (0.02 (CI: − 0.33–0.38)), plasma and vaginal mucus (0.05 (CI: − 0.28–0.39)), and negligible agreement between vaginal mucus and saliva (− 0.04 (CI: − 0.2–0.12)).

Analyzing Kappa coefficient for the results of the PCR, there is insignificant agreement between all collected samples: blood and saliva (− 0.02 (CI: − 0.32–0.37)), blood and vaginal mucus (− 0.03 (CI: − 0.35–0.28)), and vaginal saliva and mucus (− 0.06 (CI: − 0.28–0.16).

Table 3 Result of *Neospora caninum* PCR in saliva and vaginal mucus of gilts experimentally inoculated with 2.9×10^7 tachyzoites of the Nc1 strain, from day 2 to day 130 postinoculation

	Group A						Group B						Group C						Group D					
Animals	14		15		16		1		3		4		8		9		10		11		12		13	
DPI	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M
− 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-
5	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	-	+	-	+	-	+	-	+	+
7	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	+	-	+	-	+	-
14	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	+	-
21	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-
28	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	-	UN	UN	UN	UN	UN	UN
35	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	UN	UN	UN	UN	UN	UN
42	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	UN	UN	UN	UN	UN	UN
55	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	UN	UN	UN	UN	UN	UN
70	UN	UN	UN	UN	UN	UN	-	-	-	-	-	-	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN
85	UN	UN	UN	UN	UN	UN	-	+	-	-	-	+	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN
100	UN	UN	UN	UN	UN	UN	+	-	-	-	+	-	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN
115	UN	UN	UN	UN	UN	UN	+	-	-	-	+	-	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN
130	UN	UN	UN	UN	UN	UN	+	-	+	-	+	-	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN	UN

[+] Positive; [-] Negative; *UN* unperformed; *S* saliva; *M* vaginal mucus, *DPI* days postinoculation

Discussion

This is the first study carried out to evaluate the clinical signs of neosporosis in swine, in addition to laboratory methods to diagnose the presence of *N. caninum*.

Throughout the gestational period and postinoculation, clinical examinations were performed on the inoculated animals. Two animals, one from group C and the other from group D, presented hypothermia and apathy shortly after inoculation (5 and 7 DPI). Hypothermia is generally not associated with infection by *N. caninum* in animals; however, hypothermia has already been reported in a newborn lamb born from a ewe inoculated with *N. caninum* (Pivatto 2017). In addition, hypothermia has been reported in kittens infected with the related protozoan *T. gondii* via transplacental or transmammary pathways (Galvão et al. 2014) and in a cat with skin lesions and breathing difficulties associated with *T. gondii* infection (Anfray et al. 2005). In this study, when the respiratory rate was observed, there was no change in the monitored periods, demonstrating that *N. caninum* does not affect this parameter in the clinical analysis of animals.

In addition, leukocytosis due to lymphocytosis and/or neutrophilia was observed in five of the nine inoculated gilts on the 7th DPI, which may be associated with infection by *N. caninum*. Studies involving *N. caninum* that report leukocytosis are rare. Pivatto (2017) inoculated tachyzoites of the Nc-1 strain of *N. caninum* in pregnant sheep and observed leukocytosis by eosinophilia on the 14th and 21th DPI. Valeriano et al. (2015) also reported leukocytosis and lymphocytosis in a dog with neurological signs due to infection with *N. caninum*. Serrano et al. (2011) observed leukopenia in bovine females chronically infected with *N. caninum*.

Three inoculated animals presented larger numbers of band cells on the 7th DPI. The band cells were identified as immature neutrophils which are usually found in cases of acute infections where there is a greater demand for leukocytes. Therefore, the presence of band cells can be associated with the inoculation of *N. caninum* tachyzoites.

When the liver enzymes AST and GGT were evaluated, the three animals in group B showed slightly high levels of AST (twice as the reference value) after inoculation. This enzyme is related to liver damage and has been associated with *N. caninum* infection in dogs with skin lesions (Murakami et al. 2017) and with *T. gondii* infection in dogs due to liver necrosis (Dubey and Lappin 2006).

The results of serology showed that all gilts sero-converted from 5 DPI. Class M immunoglobulins are associated with the acute phase of the infection, whereas IgG are found in greater quantities in the chronic phase of the infection, remaining present for long periods. In this study in swine, it can be seen that the detection of IgM occurs from the fifth DPI, with the titers increasing until the 14th DPI and then the production begins to decrease, becoming undetectable, in the serum and plasma, from days 55–70 postinfection, respectively. Immunoglobulins G, on the other hand, begin to be detected from the fifth day after infection, showing greater production peaks between the 14th and 28th postinoculation, remaining constant after this period. These results, as well as those of other authors (Cadore et al. 2010), demonstrate that for the detection of *N. caninum* infection in herd animals, in this case of swine, the search for IgG is more efficient because it stays longer in the bloodstream of animals.

Mineo (2007) evaluated the immune response, through IFAT, of a calf and eight dogs inoculated with *N. caninum*. In the calf, it was possible to detect IgM and IgG from day 7 postinoculation, with peak IgM production on day 35 postinoculation, decaying after this period. In the inoculated dogs, it was possible to detect IgM against *N. caninum* from day 35 postinoculation and IgG from day 5 postinoculation, maintaining low titers during the experiment. Evaluating these results, it is possible to affirm that the immune response of pigs to infections of *N. caninum* appears to develop earlier when compared to dogs and cattle.

Saliva and vaginal mucus did not show satisfactory results for the search for IgG and/or IgM antibodies in pigs. When saliva was evaluated, only three animals tested positive for IgG and one for IgM, during the entire postinoculation follow-up. When the vaginal mucus was evaluated, only three animals were negative for IgG and three animals were positive for IgM throughout the experiment. Although vaginal mucus showed a greater number of positive animals, they did not remain positive in all samples. In cattle, Ooi et al. (2000) analyzed serum, saliva, and vaginal mucus samples from 613 cattle using the IFAT (IgG) technique, finding a greater number of animals positive for *N. caninum* in the serum, followed by vaginal mucus and saliva. These results are in agreement with those found in this study in swine.

In addition, postinoculation parasitemia was evaluated through PCR of buffy coat, saliva, and vaginal mucus. Only two animals had a positive result when the buffy coat was analyzed, six animals when the vaginal mucus was analyzed, and all animals were positive when the saliva was analyzed, showing positive results from 2 DPI. These results suggest saliva as an alternative for collecting samples in the field to search for *N. caninum* DNA, with results superior to those found when using buffy coat or vaginal mucus. It is important to note that saliva samples are rarely used as a means of diagnosing *N. caninum*; however in the case of *T. gondii,* there are several studies that report the presence of parasite DNA in animal and human saliva (Bresciani et al. 2008; Vitor and Pinto 1991; Terragna et al. 1984). With this study, we can conclude that, like *T. gondii*, *N. caninum* can also be found in the saliva of infected animals.

Group B animals were followed up to 130 DPI, a longer period compared to the other inoculated groups. These

animals were inoculated 30 days before conception, being in chronic infection during the gestational period. Animals in this group, after a period (55–85 DPI) without showing IgM antibodies in serum and plasma (Fig. 1), returned to present IgM antibodies on the 100th DPI. When analyzing the results of the PCR, it is clear that it was not possible to detect DNA of the parasite, both in saliva, in vaginal mucus and in the blood, from 42 to 70 DPI, with *N. caninum* appearing again on the 85th DPI (Table 3). Relating the result to those found in the IFAT to search for IgM antibodies, it can be said that the infection reappeared acutely in this period (second gestational third), due to the presence of antibodies and parasitemia. These results are similar to what occurs in other animal species such as cattle and sheep where there is endogenous transplacental transmission (Mcallister 2016; Jolley et al. 1999).

Comparing (kappa test) the techniques performed with all samples collected from pigs to search for antibodies against and DNA of *N. caninum*, it is possible to use both, serum and plasma, to perform serology, to the search for IgG and IgM, with no change in the results. These data have already been reported in the literature for other species of animals and protozoa, such as *T. gondii*, using the IFAT technique (Navarro et al. 1997). As for the PCR technique, there was no agreement between the samples collected (saliva, vaginal mucus, and blood), but the one with the highest number of positive animals throughout the experiment was saliva, showing that, among the samples collected, it was best suited to detect *N. caninum* in swine herds. It should also be noted that saliva is easier to collect compared to blood and may be an alternative for the diagnosis of neosporosis.

Conclusion

Neospora caninum infects swine and can cause clinical signs such as hypothermia and leukocytosis in the acute stage of infection. Infection by *N. caninum* can acutely reappear in chronically infected swine during pregnancy. For serological diagnosis, both serum and plasma can be used as samples, with similar results. For the search for DNA by PCR, it is recommended to collect saliva, as it has a larger number of positive animals compared to blood and vaginal mucus.

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Data availability Not applicable

Compliance with ethical standards

Ethics approval The present study was submitted to the CAV/UDESC Ethics Committee on Animal Use (CEUA), being approved under protocol number 7997170717.

Consent to participate Not applicable

Conflict of interest The authors declare no conflict of interest.

Consent for publication Not applicable

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