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Experimental infection with *Neospora caninum* in pregnant bitches

Infecção experimental com *Neospora caninum* em cadelas prenhes

Guacyara Tenorio Cavalcante[1]; Rodrigo Martins Soares[2]; Sandra Mayumi Nishi[3]; Stéfano Carlo Filippo Hagen[4]; Camila Infantoni Vannucchi[5]; Paulo Cesar Maiorka[6]; Anaiá Sevá Paixão[2]; Solange Maria Gennari[2]*

[1]Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo – USP, São Paulo, SP, Brasil

[2]Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo – USP, São Paulo, SP, Brasil

[3]Escola de Medicina Veterinária, Universidade Federal da Bahia – UFBA, Salvador, BA, Brasil

[4]Departamento de Cirurgia, Universidade de São Paulo – USP, São Paulo, SP, Brasil

[5]Departamento de Reprodução, Universidade de São Paulo – USP, São Paulo, SP, Brasil

[6]Departamento de Patologia, Universidade de São Paulo – USP, São Paulo, SP, Brasil

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Abstract

In this study, transplacental transmission of *Neospora caninum* in bitches at different stages of pregnancy was evaluated. Three bitches were inoculated in the 3rd week and three in the 6th week of gestation with 10^8 tachyzoites of *N. caninum* (Nc-1 strain). All the infected bitches and at least one of their offspring presented anti-*N. caninum* antibodies according to the indirect fluorescent antibody test (IFAT ≥ 400). The pups and their mothers were sacrificed and tissues from the central nervous system (CNS), popliteal lymph nodes, skeletal muscle, brain, lungs, heart and liver were analyzed for the presence of *N. caninum* using the nested polymerase chain reaction (nested PCR), restriction fragment length polymorphism (RFLP) and immunohistochemistry (IHC). The parasite was found in the pups in lymph node, CNS, heart and liver tissues using nested PCR. There was no difference in perinatal mortality between the offspring from bitches infected in the 3rd week of gestation (60%) and in the 6th week (53.8%).

Keywords: *Neospora caninum*, dogs, transplacental transmission, experimental infection.

Resumo

Neste estudo a transmissão transplacentária de *Neospora caninum* foi avaliada em fêmeas em diferentes estágios de gestação. Três cadelas foram inoculadas na 3ª semana e três na 6ª semana de gestação com 10^8 taquizoítos de *N. caninum* (cepa Nc-1). Todas as cadelas infectadas, e pelo menos um de seus filhotes, apresentaram anticorpos anti-*N. caninum* por imunofluorescência indireta (RIFI ≥ 400). Os filhotes e suas mães foram sacrificados e tecidos de sistema nervoso central (SNC), linfonodo poplíteo, músculo esquelético, cérebro, pulmões, coração e fígado foram analisados para a presença de *N. caninum* pela reação em cadeia da polimerase (*nested* PCR), polimorfismo de comprimento de fragmentos de restrição (RFLP) e imunoistoquímica (IHQ). O parasita foi encontrado em filhotes em linfonodo, SNC, coração e fígado pela *nested* PCR. Mortalidade perinatal não apresentou diferença entre os filhotes das cadelas infectadas na 3ª semana (60%) ou na 6ª semana de gestação (53,8%).

Palavras-chave: *Neospora caninum*, cães, transmissão transplacentária, infecção experimental.

Introduction

Neospora caninum is a protozoan parasite of domestic and wild animals. Coyotes and dogs are the definitive hosts of the parasite. Neonatal mortality and abortions are major problems in cattle (DUBEY et al., 2007). The parasite can be transmitted transplacentally, during pregnancy from an infected dam to her fetus, or postnatally through ingestion of drinking water or food contaminated with sporulated oocysts or through ingestion of tissues infected with tachyzoites or tissue cysts (McALLISTER et al., 1998; LINDSAY et al., 1999; GONDIM et al., 2004; DUBEY et al., 2007).

It is not completely understood how dogs are infected with *N. caninum* in nature. In experimentally infected dogs, transplacental

*Corresponding author: Solange Maria Gennari
Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo – USP, CEP 05508-270, São Paulo, SP, Brasil
e-mail: sgennari@usp.br

transmission has been demonstrated (DUBEY; LINDSAY, 1989; DUBEY, 1992; COLE et al., 1995; DUBEY et al., 2007). In most cases of neonatal neosporosis, clinical signs are not apparent until 5 to 7 weeks after birth, thus suggesting that *N. caninum* is transmitted from the dam to the neonates postnatally through ingestion of milk or at the terminal stages of gestation (DUBEY; LINDSAY, 1996; DUBEY et al., 2007).

In nature, bitches that delivered pups congenitally infected with *N. caninum* remained clinically normal (DUBEY et al., 2007), and infected bitches can transmit the parasite to their fetuses in successive generations (DUBEY, 1992; BARBER; TREES, 1998; LINDSAY; DUBEY, 2000; CROOKSHANKS et al., 2007). Barber and Trees (1998) showed that the frequency of vertical transmission in naturally infected dogs is low, such that approximately 3% of pups are born infected from seropositive bitches. It is believed that postnatal infection must occur in order to maintain the infection by *N. caninum* in dogs, because vertical transmission alone does not sustain the infection (DUBEY; LINDSAY, 1996; DUBEY, 1992).

Because of the great scarcity of reports showing transplacental transmission of *N. caninum* in dogs, we present the results from a study that aimed to determine occurrences of transplacental transmission in bitches at different stages of pregnancy.

Materials and Methods

1. Dogs

Seven mixed-breed adult bitches (dogs 1, 2, 3, 4, 5, 6 and 7) that were seronegative for *N. caninum* (IFAT ≤ 50) and *Toxoplasma gondii* (IFAT ≤ 16) were selected. All the animals had been vaccinated against canine distemper virus, parvovirus, adenovirus type 2, parainfluenza, hepatitis, coronavirus and leptospirosis (Duramune® Max5CvK- Fort Dodge, Brazil), dewormed and tested for *Brucella abortus* using a blood PCR assay (KEID et al., 2007), prior to infection with tachyzoites of *N. caninum*. Two adult mixed-breed male dogs were used for mating with the bitches.

The animal management and veterinary procedures were in accordance with the standards of the Animal Use Ethics Committee of the Biomedical Science Institute and the School of Veterinary Medicine of the University of São Paulo (protocol number 1020/2007).

2. Experimental infection with N. caninum

The six bitches were inoculated with 10^8 tachyzoites of *N. caninum* (strain NC-1) subcutaneously: three bitches (dogs 3, 6 and 7) were inoculated in the 3rd week of gestation (GI) and three bitches (dogs 2, 4 and 5) in the 6th week of gestation (GII), while one bitch (dog 1) was kept as a control without infection (GIII). The animals were housed individually in stalls, fed commercial dry food and received water *ad libitum*.

3. Detection of pregnancy and evaluation of fetuses

All the bitches underwent ultrasound examinations before pregnancy to evaluate the conditions of the uterus, ovary and lymph nodes, and to confirm the absence of reproductive problems. For the males, sperm concentration, motility, membrane integrity and acrosome were evaluated by means of a spermiogram.

The pregnancy was confirmed using ultrasonography around day 21 after mating. Thereafter, for each of the bitches, two more follow-up ultrasound pregnancy tests were performed: on days 50 and 55 of gestation.

During the gestational follow-up ultrasound examinations, the morphology of the embryo (fetus) was determined. In this regard, the fetal development was evaluated by using measurements of length, biparietal distance, intercostal space, abdominal diameter, stomach diameter, lung differentiation and liver appearance. To assess fetal viability, the echogenicity, volume of fetal fluid, appearance of the fetal membranes, definition and pulsation of the umbilical cord, fetal movements, frequency of fetal heartbeat and placental morphology were evaluated. The number of fetuses was also evaluated. The pups that were born were clinically evaluated for their physical and neurological condition until the time of sacrifice.

4. Necropsies on the dogs

The mothers were sacrificed along with their litters in order to detect *N. caninum*. CNS, muscle (thigh), popliteal lymph node, liver, lung and heart tissue were collected to perform immunohistochemical and molecular techniques.

For the sacrifice, the animals were anesthetized using xylazine (Kensol®, König, Brazil) and ketamine (Vetaset®, Fort Dodge, Brazil) and then received an intracardiac injection of iodide and embutramide mebezone (T-61®, Intervet, Brazil).

5. Detection of antibodies (IFAT)

Serum samples from the dogs were tested for anti-*N. caninum* and anti-*T. gondii* antibodies using the indirect fluorescent antibody test (IFAT) as described by Dubey et al. (1988) using anti-dog antibodies (Immunology Consultants Laboratory and Sigma, USA), and intact tachyzoites of *N. caninum* (strain NC-1) and *T. gondii* (RH strain) as the antigens. The cutoff was set at 1:50 (McALLISTER et al., 1998) for *N. caninum* and 1:16 (CAMARGO et al., 1977) for *T. gondii*. Serum samples were collected when the animals arrived at the kennel: three days before infection and immediately after sacrificing or natural death. Blood samples from the pups were collected at the time of sacrifice.

6. Nested PCR

DNA for PCR was extracted from CNS, muscle (thigh), popliteal lymph node, liver, lung and heart tissue from each bitch and its respective litter. Each tissue sample was homogenized with TE (10 mM of Tris-HCl and 1 mM of EDTA, pH 8.0) in the

proportions of one part of tissue to four parts of TE (weight/volume), followed by vortex homogenization.

The material was suspended in 500 µl of lysis buffer (10 mM of Tris-HCL, pH 8.0; 25 mM of EDTA, pH 8.0; 100 mM of NaCl; and 1% SDS). Proteinase K was added to a concentration of 10 µg/mL. The suspension was incubated at 37 °C. After overnight digestion, the DNA was extracted using a mixture of phenol-chloroform, isoamyl-alcohol (25:24:1) and ethanol precipitate, as described by Sambrook et al. (1989).

The tissue DNA was tested by means of nested PCR, based on primers directed towards the 18S and 5.8Sr RNA coding genes, which are common to all Toxoplasmatinae (SOARES et al., 2011). Amplicons were digested using the restriction enzymes Taq I and Rsa I in order to differentiate between genetic sequences derived from *Neospora* spp., *T. gondii*, *Hammondia heydorni* and *H. hammondi*.

7. Immunohistochemical analysis

Tissue fragments from the dams and their offspring (CNS, lymph nodes, skeletal muscle, lungs, heart and liver) were preserved in 10% formalin and prepared for histopathology and immunohistochemical analysis. When it was possible to collect the offspring that died before the scheduled date for necropsy, samples from these animals were also set aside for analysis. *N. caninum* detection was performed by means of an immunohistochemical test using an anti-*N. caninum* primary antibody (VMRD, Pullman, USA). The tissue samples were initially incubated in 3% hydrogen peroxide solution for 20 minutes to block endogenous peroxidase. Antigen retrieval was performed using 0.1% trypsin for 10 minutes at 37 °C followed by heat (microwave on full power for 2 minutes) with the slides immersed in citrate buffer, pH 6.0 (1 L of distilled water, 2.1 g of C6H8O7, adjusted to pH 6.0 with 0.5% NaOH). Nonspecific labeling was reduced by applying 5% reduced-fat milk for 15 minutes. The primary antibody was applied for 1 hour at room temperature followed by 20 minutes with linking solution (LSAB kit, Universal, K0690, Dako Corporation, Carpinteria, CA, USA) and 25 minutes with streptavidin-peroxidase, using phosphate-buffered saline washings between steps. Labeling was performed using the chromogen 3.5-diaminobenzidine tetra-hydrochloride as the substrate (DAB, Dako K3468, USA) for 10 minutes. Hematoxylin, applied for 1 minute, was used as a counterstain.

Results

Perinatal mortality of 60% (6/10) of the offspring from the bitches infected in the 3rd week of gestation was observed, and the deaths occurred up to 48 hours after birth. The offspring from bitches infected in the 6th week of gestation died from 5 to 10 days after birth and presented a mortality rate of 53.8% (7/13) as shown in Table 1. During pregnancy, both the bitches and the fetuses remained healthy, and *N. caninum* was not detected in any of the bitch tissues examined using immunohistochemical or molecular techniques.

The pups did not develop neurological or clinical signs compatible with neosporosis. Not all the pups that died were evaluated serologically, since in some cases it was not possible to collect blood because of the interval between the animal's death and collection of the fetus.

The *Neospora caninum* antibody titers for the infected and control bitches and their respective offspring are presented in Table 2. All the infected dogs (GI and GII) seroconverted to anti-*N. caninum* antibodies (IFAT ≥ 50). Among the adults, the lowest titer (3,200) was found in dog 3, which was infected in the 3rd week of gestation. The highest titer (12,800) was observed in bitches infected in the 3rd week of pregnancy (dog 6) and the 6th week (dogs 2 and 5). The antibody titers were higher in pups from mothers infected in the 6th week of gestation. The titers among the pups ranged from 400 to 12,800. Among the 13 pups from which it was possible to obtain serum, two showed no evidence of antibodies against the parasite, although both the mothers and the siblings of these seronegative dogs were IFAT positive. The control dog (dog 1) and its offspring remained negative for the presence of anti-*N. caninum* antibodies throughout the experimental period.

Among the 10 pups examined by means of nested PCR, from mothers inoculated in the 3rd week of gestation (GI), 20% (two pups) were positive. The agent was found in the heart and liver of one pup from mother 3 and in the heart of one pup from mother 6. Among the 13 pups examined by means of nested PCR, from mothers inoculated in the 6th week of gestation (GII), 7.7% (one pup from mother 2) were positive, and the agent was found in the CNS and in lymph nodes, as presented in Table 3.

None of the tissue samples from the bitches and pups examined were immunohistochemically positive.

Discussion

Few studies have been conducted on the serological profile of pregnant bitches that were experimentally infected with *N. caninum*, or on their offspring. Little is known with regard to whether, in dogs, *N. caninum* may act similarly to what is observed among cattle, i.e. showing that fetuses exposed in early gestation are more likely to be aborted and fetuses exposed later are more likely to survive (BARBER et al., 1997; BARBER; TREES, 1998).

In this study, perinatal mortality occurred to 60% of the offspring from the bitches infected in the 3rd week of gestation,

Table 1. Reproductive observations among pregnant bitches infected with 10^8 tachyzoites of *N. caninum* (subcutaneously) in the 3rd and 6th weeks of gestation, and the non-infected control.

Group	Week of infection	Bitch number	Total of fetuses	Total of perinatal death
I	3rd	3	5	3
I	3rd	6	2	1
I	3rd	7	3	2
II	6th	2	5	3
II	6th	4	6	4
II	6th	5	2	0
III	control	1	2	0
Total			25	13

Table 2. Titers of anti-*N. caninum* antibodies in dogs infected in the 3rd week (GI) and 6th week (GII) of gestation and in the non-infected control (GIII) and their respective offspring according to IFAT (≥50) on day 35 postpartum.

Group	Bitch Nº	Titer (IFAT)						
		Bitch	Offspring					
			1	2	3	4	5	6
I	3	3,200	3,200	1,600	*	*	*	
I	6	12,800	800	*				
I	7	6,400	800	*	*			
II	2	12,800	12,800	400	3,200	800	*	
II	4	6,400	N	*	6,400	12,800	*	*
II	5	12,800	400	N				
III	1	N	N	N				

*Not done; N: negative.

Table 3. Detection of *Neospora caninum* by means of nested PCR on offspring of bitches infected during the 3rd (GI) and 6th (GII) weeks of gestation.

Tissues	Group	Identification of positive offspring (mother number)
Heart	I	1 (6)
	I	1 (3)
Liver	I	1 (3)
CNS	II	1 (2)
Lymph nodes	II	1 (2)

and the deaths occurred up to 48 hours after birth. On the other hand, the offspring from the bitches infected in the 6th week of gestation died later and presented a mortality rate of 53.8%. Dubey and Lindsay (1989) infected a bitch beagle with 1.5 x 10^6 tachyzoites of *N. caninum* from the same strain used in this study, in the 5th week of gestation, subcutaneously, and observed that two pups died (25%), two days after birth. All the infected bitches and at least one of the offspring from each of them seroconverted to anti-*N. caninum* antibodies (IFAT ≥ 50), thus confirming the occurrences of infection. The range of antibody titers among the pups of the present study was similar to what was observed by Barber and Trees (1998), with values ranging from 50 to 12,800, and higher than what was reported by Dubey and Lindsay (1989) among the offspring from bitches infected in the 5th week of gestation. These differences may have been due to the doses of inoculum administered and the individual response from each animal against the infection. The antibody titers were higher among the pups from bitches infected in the 6th week of gestation, probably because the challenge was implemented later.

Viable offspring did not show any neurological symptoms after birth, and neither did their mothers. This finding is in agreement with other observations, since clinical signs caused by neosporosis are rarely present in dogs (DUBEY; LINDSAY, 1996, DUBEY et al., 2007). However, in a study by Cole et al. (1995), a bitch inoculated with 5 × 10^6 tachyzoites of *N. caninum* (NC-1 strain) in the 3rd week of gestation had two pups that presented proprioceptive deficit, increased muscle tone and spasticity in both pelvic limbs before the 4th week after birth. In the same study, one of the bitches inoculated died.

The role of *N. caninum* in relation to abortion, stillbirth, reabsorption or development of pyometra in dogs is still little known. Barber and Trees (1998) estimated that the loss of pups from infected mothers during the peripartum period would range from 15 to 30%, but studies on experimental infection have observed values between 4.6 and 83% (DUBEY; LINDSAY, 1989, 1990; COLE et al., 1995; BARBER et al., 1997).

Although the mothers seroconverted to anti-*N. caninum* antibodies, the parasite was not detected in any tissues from these bitches, by means of PCR or immunohistochemistry, and these results are in accordance with what was found by Dubey and Lindsay (1989).

In the present study, it was not possible to collect blood from the pups before ingestion of colostrum, and thus it was not possible to determine whether the antibodies observed were from within the animals or from the colostrum. However, in some of these seropositive pups, *N. caninum* was found by means of other diagnostic techniques, thus confirming the transplacental infection. It was not possible to detect the agent by means of molecular or histopathological methods in any of the serologically negative dogs.

The presence of *N. caninum* in some pups was confirmed by means of nested PCR, and the parasite was detected in samples from lymph node tissue, CNS, heart and liver, thus indicating that the multiplication of this parasite occurred in different organs after transplacental infection. In the present study, the stage of pregnancy seemed to have little importance.

The lack of influence from the bitches' gestational period regarding *N. caninum* infection may be related to these animals' shorter duration of gestation. Among cows (approximately 9 months), it is known that *N. caninum* infection may be influenced by gestational period.

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