Incidence and transplacental transmission of *Neospora caninum* in primiparous females from *Bos indicus* slaughtered in Presidente Prudente, São Paulo, Brazil

Incidência e transmissão transplacentária de *Neospora caninum* em fêmeas primíparas da raça *Bos indicus* abatidos em Presidente Prudente, São Paulo, Brasil

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Abstract

To produce an epidemiological map of neosporosis in Brazil and identify the types of transmission of this disease, the present study evaluated the occurrence of *Neospora caninum* in Nelore cattle (*Bos indicus*) in Presidente Prudent, west region of Sao Paulo state; its vertical transmission; and the early stage in which fetuses are infected. To achieve this, serum samples from 518 slaughtered pregnant heifers and their fetuses were tested by ELISA technique and fetal brain tissues subjected to PCR. One hundred and three heifers (19.88%) had antibodies to *N. caninum*, as well as 38 (36.8%) of fetuses from 4 months of gestation. The conventional PCR failed to detect *N. caninum* DNA. These findings show that neosporosis occurs in the area studied and that it may be transmitted the transplacental route, althought *N. caninum* had not detected in brain tissue from non-aborted fetuses. The use of nested PCR it would be applied to increase the sensitivy of test.
Key words: Epidemiology, cattle, *Neospora caninum*.

Resumo

Para produzir um mapa epidemiológico da neosporose no Brasil e identificar os tipos de transmissão dessa doença, o presente estudo avaliou a ocorrência de *Neospora caninum* em fêmea Nelore (Bos Indicus) em Presidente Prudente, região oeste do Estado de São Paulo e o risco de infecção fetal nos estágios iniciais da gestação. Para a realização deste estudo, amostras de soro de 518 novilhas prenhas abatidas e seus fetos foram testadas pela técnica de ELISA e para avaliação de transmissão vertical, tecido cerebral fetal foi submetido à reação da polimerase em cadeia (PCR). Dessas novilhas, 103 (19,88%) tinham anticorpos para *N. caninum* dos quais 38 (36,8%) estavam no 4 mês de gestação. Esses achados mostram que a Neosporose ocorre na área estudada e que pode ser transmitido pela via placentária, embora o *N. caninum* não tenha sido detectado em tecido cerebral de fetos não abortado. O uso de nested PCR poderia ser aplicado como forma de aumentar a sensibilidade do teste.
Palavras-chave: Epidemiologia, gado, *Neospora caninum*

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Introduction

The levels of antibodies to *Neospora caninum* in Brazilian cattle are similar or higher than those of other countries (GONDIM; SARTOR, 1997). These levels are impressive since some consequences of neosporosis, such as cattle abortion, lead to substantial losses. In California, 42.5% of cattle abortions were attributed to *N. caninum* (ANDERSON et al., 1991), and this disease caused economic losses of 35 million dollars in dairy cattle production (LINDSAY, 1998). Estimations of neosporosis losses in Brazil have not been calculated, but based on the occurrences reported; the expenses caused by this infection probably exceed those of other abortive diseases such as brucellosis and leptospirosis.

For neosporosis diagnosis, the infecting agent in the infected tissues can be detected by polymerase chain reaction (PCR) and immunohistochemistry technique. Brain tissue is commonly used in PCR because cyst formation is common in this structure. In epidemiological surveys the detection of antibodies to the parasite is achieved by ELISA technique or indirect immunofluorescence (IFAT) (THURMOND; HIETALA, 1999; ELLIS et al., 1999). Even though these procedures are well known, a great variation in the infection rate of neosporosis is found among cattle herds (THILSTED; DUBEY, 1989; RAGOSO, 2003). This variation is related to the location and density of cattle rearing, as well as the breed and management procedures. Sampling procedures may also affect the results since higher neosporosis incidence is found in cattle from herds with higher abortion rate (PITUCO et al., 1998) compared to cattle sampled randomly (THILSTED; DUBEY, 1989). Finally, differences among results are found when different techniques are used to analyze a same sample (THILSTED; DUBEY, 1989). Despite these difficulties, successive epidemiological surveys are needed in Brazil since different breeds of both *Bos indicus* and *Bos Taurus* are distributed throughout the country (CORBELLINI et al., 2002). In addition, differences in cattle management in the various areas of the country lead to differences in neosporosis transmission.

N. caninum protozoa are maintained in nature by vertical or horizontal transmission. The horizontal transmission occurs almost exclusively by the ingestion of water and/or food contaminated by oocysts expelled in the feces of infected dogs (BARTELS et al., 1999; WOUDA et al., 1999; DIJKSTRA et al., 2001) or other wild carnivores such as coyote *Canis latrans* (GONDIM et al., 2004). The vertical transmission disseminates and maintains this pathogen in the herds for successive generations (BJÖRKMAN et al., 1996). The transplacental transmission of *N. caninum* in cattle is a type of vertical transmission that appears to be more common in the last third of gestation. However, the few studies available on this issue are not consistent enough to support this fact. Based on these premises, this study aimed to: 1) evaluate the infection occurrence in primiparous females and their respective fetuses; 2) determine the earlier phase of gestation in which the infection occurs; and 3) test whether the PCR technique applied to fetal tissues is suitable for neosporosis diagnosis since this procedure has been performed only after calf birth. This study was carried out in Presidente Prudente, in the west of Sao Paulo state, because most of the beef cattle in the state are reared in this region.

Material and Methods

The 518 pregnant Nelore heifers (*Bos indicus*) from the Presidente Prudente region, (west Sao Paulo state) were slaughtered in a local commercial processing facility. The experiment was conducted in this same municipality, and the experimental procedures were approved by the Chamber of Ethics in Animal Experimentation, School of Veterinary Medicine and Zootechny of the UNESP campus at Botucatu (Protocol 023/2002-CEEA).

During the slaughtering, 10-mL of blood was collected from the heifers by cardiac punction. Just after heifers were sacrificed, 10 mL of blood was collected from the dead fetuses by cardiac punction; fetal brain tissue was also harvested. Blood samples

were collected in tubes without any anticoagulant, and the tubes kept in coolers with recyclable ice. During necropsy fetuses were weighed and the length of their spinal cord from the occipital-atlantal junction to the sacral base was measured for age determination (SARTOR et al., 2003).

The serum diagnosis for Neosporosis of dams (N= 518) and fetuses (N=518) was realized at Instituto Biologico using a commercial ELISA kit (IDEXX® – Herdcheck) according to instructions by manufacturer. The serum of dams was diluted 1:100 according of instructions of kit and fetus 1:10 to detection low levels of specific antibodies.

For detection of *Neospora caninum* in fetus, brain tissues were analyzed by PCR. Two grams of each brain were prepared in 8 ml in stomacher for 30 seconds and clarified with 20% chlorophorm. For DNA extraction was used Trizol (Invitrogen™) according to instructions by manufacturer. Primers pairs Np6-Np21 of pNC-5 gene of *Neospora caninum* (Np6 - 5'CTC GCC AGT CAA CCT AGG TCT TGT3' and Np21 – 5'CCC AGT GCG TCC AAT CCT GTA AC3') were selected for PCR which amplify 337 bp (MÜLLER et al., 1996). The sensitivity of PCR was 100 tachyzoites/ml. PCR amplification as target DNA with the same PCR mixture with 40 cycles at 95ºC for 5 minutes, 94ºC for 1 minute, anneling at 60ºC for 1 minute, and extension at 72ºC for 1 minute, 72ºC for 10 minutes and 4ºC. Amplicons were resolved a 2% agarose gel with ethidium bromide and photographed under UV light. Positive controls (NC-1 strain of *N. caninum*) and negative controls (bovine brain) were included in each PCR run.

A chi-square test was used to assess the occurrence of *N. caninum* in the heifers and in the fetuses from the infected heifers.

Results

Out of the 518 heifers sampled, 103 (19.88%) had antibodies to *N. caninum,* and only 38 fetuses from these infected heifers were also infected (Table 1). Infected fetuses were found from the fourth month of gestation onward (Table 2). The PCR not revealed any positive fetuses.

Table 1. Tested serum with *ELISA to *Neospora caninum* in primiparous cows (1:100) and its respectives fetus (1:10), obtained in slaughterhouses of west São Paulo State.

	Mother	Fetus
Positives	103 (19,9)	38 (7,3)
Negatives	415 (80,1)	480 (92,7)
Total	518 (100)	518 (100)

*ELISA-IDEXX®-Herdcheck $\chi^2 = 34,69$
Observation: The afection vertical transmission appeared in 36,8% (38 of 103) in tested fetus.

Table 2. Pregnancy period and infection average in fetus of primiparous cows infected with *Neospora caninum*. Fetal serum tested to ELISA (1:10) and maternal (1:100)

Gestational month	Infected fetuses		Non-infected fetuses	
	Cases	(%)	Cases	(%)
3			7	6.80
4	4	3.9	23	22.33
5	6	5.8	14	13.60
6	9	8.74	13	12.62
7	9	8.74	03	2.91
8	9	8.74	02	1.94
9	1	0.97	03	2.91
TOTAL	38	36,89	65	63,11

*ELISA-IDEXX®-Herdcheck $\chi^2 = 34,69$
Observation: vertical transmission was present after four or more months of pregnancy.

Discussion

The prevalence of antibodies to *N. caninum* was within the range found in other studies conducted in Brazil and other countries, which used similar sampling conditions and technique (ROBERTS, 1984; MORALES et al., 2001; GUIMARÃES JUNIOR, 2003; GARCIA FILHO, 2004). As showed Table1, there was a significant incidence, because at least 36,8% of fetuses presented antibodies indicating transplacental trasmission.

The titer 10 was chosen to analyze fetal blood to obtain a higher antibody concentration in the serum. In fact, the immunological system is incomplete in fetuses, and thus a lower level of antibodies is expected. Osawa et al. (1998) also suggest that dilutions of fetal blood should be lower than those recommended by blood testing manufacturers. However, an ideal dilution rate still has not been established. Cross reaction with other Apicomplex a was not evaluated.

The rate of infected fetuses (Table 2) has a low error probability, i.e., false negative results are unlikely. In fact, the blood dilution used for antibody tittering was lower than that used by Wouda et al. (1999), who consider blood dilution of 1:25 as specific for tittering antibodies to *N. caninum*.

The results show *N. caninum* prevalence in fetuses from 3 to 9 months of gestation. The transplacental transmission occurred in 36.8% of the fetuses obtained from the infected heifers, evidencing this as a potential route for neosporosis transmission. A higher infection rate could still be found if pregnancy was not interrupted in the 63.2% non-infected fetuses whose mothers were infected. In fact, these fetuses would be under transplacental-infection risk in the remaining gestation. The earlier transplacental transmission of *N. caninum* was detected in fetuses from 4 months of gestation onward. From this period onward, it was not possible to identify the infection period.

The use of PCR for *N. caninum* diagnosis was evaluated because it provides rapid diagnostic results with high sensitivity and specificity. For instance, Pitel et al. (2001) studied 104 aborted heifer fetuses and detected 22 neosporosis cases using PCR. In contrast to that study, the PCR analyses of brain tissues from the fetuses studied herein did not indicate any case of neosporosis infection. The discrepancy between these studies is possibly due to the conditions of the fetuses. Because Pitel et al. (2001) used naturally aborted fetuses, their infection should be more severe than that in the fetuses used in the present study, and neosporosis was diagnosed by PCR because the parasite count was higher. In fact, PCR was not sensitive enough to detect neosporosis in other studies (ELLIS, 1998) probably because very few protozoa were present in the tissues of the infected animals (DUBEY et al., 1988; THURMOND; HIETALA, 1999).

A second amplification could be performed on the type of tissue used. According to De Paula (2003), the threshold for *N. caninum* detection by the conventional technique is 100 Tak/mL for suspension of frozen cattle brain; however, a second amplification allows detection of 1 Tak/mL. Ho et al. (1997) found that *N. caninum* cannot be detected in all the tissue sections, and De Marez et al. (1999) found that *N. caninum* DNA is not detected in all the repetitions from a same sample.

It is possible that the method proposed by Ellis (1998) and Holmdahl, Mattson (1996), who found a high specificity and sensitivity of nested PCR for *N. caninum* diagnosis, should have been applied in this study. According to these authors, the internal transcription spacer of ribosomal DNA is ideal for PCR use because it has a high copy number and keeps the sequence of bases of the species well described for *N. caninum*.

In conclusion, neosporosis is expressive in the Presidente Prudente region, and vertical transmission is an important mode of *N. caninum* propagation. PCR is a valuable confirmatory tool to diagnose *N. caninum* abortion in cattle since the sensitivity of test has been adjusted. In addition, the choice of an appropriate diagnosis method for neosporosis must be made cautiously since the conventional PCR is not adequate to diagnose *N. caninum* in brain tissues of non-aborted fetuses. However, *N. caninum* antibodies can be detected by the ELISA technique.

References

ANDERSON, M. L.; BLANCHARD, P. C.; BARR, B. C.; DUBEY, J. P.; HOFFMAN, R. L.; CONRAD, P. A. *Neospora*-like protozoa infection as a major cause of abortion in California dairy cattle. *Journal of the American Veteterinary Medical Association,* Schaumburg, v. 198, n. 2, p. 241-244, 1991.

BARTELS, C. J. M.; WOUDA, W.; SCHUKKEN, Y. H. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). *Theriogenology,* New York, v. 52, n. 2, p. 247-257, 1999.

BJÖRKMAN, C.; JOHANSSON, O.; STELUD, S.; HOMDAHL, O. J. M.; UGGLA, A. *Neospora* species infection in a herd of dairy cattle. *Journal of the American Veterinary Medical Association,* Schaumburg, v. 208, n. 9, p. 1441-1444, 1996.

CORBELLINI, L. G.; DRIEMEIER, D.; CRUZ, C. F. E.; GONDIM, L. F. P.; WALD, V. Neosporosis as a cause of abortion in dairy cattle in Rio Grande do Sul, southern Brazil. *Veterinary Parasitology***,** Amsterdam, v. 103, n. 3, p. 195-202, 2002.

DE MAREZ, T.; LIDDEL, S.; DUBEY, J. P.; JENKINS, M. C.; GASBARRE, L. Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses. *International Journal for Parasitology,* Elmsford, v. 29, n. 10, p. 1647-1657, 1999.

DE PAULA, V. S. O. *Avaliação de uma reação em cadeia pela polimerase para detecção de Neospora caninum em fetos bovinos*. 2003. Dissertação. (Mestrado em Epidemiologia Experimental Aplicada às Zoonoses) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo.

DIJKSTRA, T. H.; BARKEMA, H. W.; EYSKER, M.; WOUDA, W. Evidence of post-natal transmission of *Neospora caninum* in Dutch dairy herds. *International Journal for Parasitology,* Elmsford, v. 31, n. 2, p. 209-215, 2001.

DUBEY, J. P.; HATTEL, A. L.; LINDSAY, D. S.; TOPPER, M. J. Neonatal *Neospora caninum* infection in dogs: Isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medical Association,* Schaumburg, v. 193, n. 10, p. 1259-1263, 1988.

ELLIS, J. T. Polymerase chain reaction approaches for the detection of *Neospora caninum* and *Toxoplasma gondii*. *International Journal for Parasitology,* Elmsford, v. 28, n. 7, p. 1053-1060, 1998.

ELLIS, J. T.; McMILLAN, D.; RYCE, C.; PAYNE, S.; ATKINSON, R.; HARPER, P. A. W. Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA. *International Journal for Parasitology,* Elmsford, v. 29, p. 1589-1596, 1999.

GARCIA FILHO, A. *Ocorrência de anticorpos anti-Neospora caninum em rebanhos de bovinos leiteiros e de corte da região de Presidente Prudente, SP*. 2004. Dissertação. (Mestrado em Medicina Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Julio de Mesquita Filho", Botucatu.

GONDIM, L. F. P.; McCALLISTER, M. M.; PITT, W. C.; ZEMLICKE, D. E. Coyotes(Canis latrans) are definitive hosts of *Neospora caninum*. *International Journal for Parasitology,* Elmsford, v. 34, p. 159-161, 2004.

GONDIM, L. F. P.; SARTOR, I. F. Detecção de anticorpos contra *Neospora caninum* em vacas leiteiras numa propriedade com histórico de aborto. In: SEMINÁRIO BRASILEIRO DE PARASITOLOGIA VETERINÁRIA, 10., 1997, Itapema. *Anais...* Itapema: Colégio Brasileiro de Parasitologia Veterinária, 1997. p. 346.

GUIMARÃES JUNIOR, J. S. *Neospora caninum em bovinos de exploração leiteira: soroprevalência, fatores de risco e comparação de técnicas sorológicas*. 2003. Tese (Doutorado em Epidemiologia Experimental e Aplicada às Zoonoses) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo.

HO, M. S. Y.; BARR, B. C.; ROWE, J. D. Detection of *Neospora* sp. from infected bovine tissues by PCR and hybridization. *Journal of Parasitology,* Lancaster, v. 83, p. 508-511, 1997.

HOLMDAHL, O. J. M.; MATTSON, J. G. Rapid and sensitive identification of *Neospora caninum* by in vitro amplification of the internal transcribed spacer. *Parasitology*, Cambridge, v. 112, n. 2, p. 177-182, 1996.

LINDSAY, D. S. Question: What is the economic impact of the disease? *The Compendium on Continuing Education for the Praticing Veterinarian,* Princeton, v. 20, n. 1, p. 13, 1998.

MORALES, E.; TRIGO, F. J.; IBARRA, F.; PUENTE, E.; SANTACRUZ, M. Seroprevalence study of bovine neosporosis in Mexico. *Journal of Veterinary Diagnostic Investigation***,** Columbia, v. 13, n. 5, p. 413-415, 2001.

MÜLLER, N.; ZIMMERMANN, V.; HENDRICH, B.; GOTTSTEIN, B. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *Journal Clinical Microbiology,* Washington, v. 34, n. 11, p. 2850-2852, 1996.

OSAWA, T.; WASTLING, J.; MALEY, S.; BUXTON, D.; INNES, E. A. A multiple antigen ELISA to detect *Neospora* specific antibodies in bovine sera, bovine fetal fluids, ovine and caprine sera. *Veterinary Parasitology,* Amsterdam, v. 79, n. 1, p. 19-34, 1998.

PITEL, P. H.; PRONOST, S.; CHATAGNON, G.; TAINTURIER, D.; FORTIER, G.; BALLET, J. J. Neosporosis in bovine dairy herds from the west of France: detection of *Neospora caninum* DNA fetuses, seroepidemiology of *N. caninum* in cattle and dogs. *Veterinary Parasitology,* Amsterdam, v. 102, n. 4, p. 269-277, 2001.

PITUCO, E. M.; SOARES, J. A. G.; OKUDA, L. H.; STEFANO, E. Ocorrência de neosporose bovina em rebanhos com histórico de abortamento no Brasil. *Arquivos Instituto Biológico,* São Paulo, v. 65, n. 1, p. 70, 1998.

RAGOSO, A. M. A. Ocorrência de anticorpos anti-*Neospora caninum* em soros bovinos procedentes de seis estados brasileiros. 2003. Dissertação. (Mestrado em Epidemiologia Experimental e Aplicada às Zoonoses) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo.

ROBERTS, S. J. *Obstetricia veterinaria y patologia de la Reproducion*. Buenos Aires: Hemisfério Sur, 1984.

SARTOR, I. F.; HASEGAWA, M. Y.; CANAVESSI, A. M. O.; PINCKNEY, R. D. Ocorrência de anticorpos de *Neospora caninum* em vacas leiteiras avaliados pelos métodos ELISA e RIFI no município de Avaré, SP. *Semina*, Londrina, v. 24, n. 1, p. 3-10, 2003.

THILSTED, J. P.; DUBEY, J. P. Neosporosis-Like abortions in a herd of dairy cattle. *Journal of Veterinary Diagnostic Investigation,* Columbia, v. 1, n. 3, p. 205-209, 1989.

THURMOND, M. C.; HIETALA, S. K. *Neospora caninum* infection and abortion in cattle. In: SMITH, H. (Ed.). *Current Veterinary Therapy 4*: food animal practice. Philadelphia: Saunders, 1999. p. 425-431.

WOUDA, W.; DIJKSTRA, T.; KRAMER, A. M. H.; MANEN, C.; BRINKHOF, J. M. A. Seroepidemiological evidence for a relationship between *Neospora caninum* infection in dogs and cats. *International Journal for Parasitology,* Elmsford, v. 29, n. 10, p. 1677-1682, 1999.