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Bovine Abortion Associated with *Neospora caninum*: Diagnosis and Epidemiological Aspects of a Dairy Cattle Herd in the Northeast Region of São Paulo State, Brazil

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Abstract

The objective of this study was to report the presence of *Neospora caninum*-associated abortion in bovines at a farm in the northeast region of São Paulo State. In January 2010, it was sent to the Department of Pathology, UNESP-Jaboticabal, a bovine fetus with an estimated age of seven months, which was natural of a dairy farm with 300 animals and an average daily production of 3,000 liters of milk, nearly 20 liters per cow. The animals were vaccinated against rabies, foot and mouth disease, carbuncle, brucellosis, leptospirosis, bovine herpes virus type I and bovine viral diarrhea virus. The herd consisted of purebred Holstein animals, Jersey, and mostly by crossbred animals 7-8 (gir x holstein). During necropsy, samples of the serosanguineous liquid present at the thoracic cavity and the heart of the fetus were collected for the detection of anti-*Neospora caninum* antibodies through Indirect Immunofluorescence Assay (IFA). Fragments of brain, cerebellum, tongue, liver, heart and kidneys were collected for the execution of histopathology (HP), immunohistochemistry (IHC) and Polymerase Chain Reaction. In order that IFA could be performed, the owner was requested blood samples without anticoagulants of the mother and other cows in the farm, with or without a history of abortion. At necropsy, it was verified a severe autolysis of the fetus. The serology of the fetus was 1:25, while the serology of the mother was 1:3,200. At HP, it was observed discrete multifocal non-suppurative encephalomyelitis characterized by gliosis and mononuclear inflammatory infiltration associated with cellular debris. DNA amplification of *N. caninum* was positive in fragments of brain, tongue, cerebellum, heart and kidneys. At IHC, it has been observed immunoreactivity to a cyst located in the tongue. The owner reported that his herd showed endemic episodes of abortion, while 27.69% (18/65) of the 65 animals sampled were seropositive. Although it has not been a significant difference ($p>0.05$), a higher seropositivity was observed in animals with a history of abortion (10/26) 38.46%, in comparison with animals without previous abortion (8/39) 20.51%. These findings show that the abortion under study was provoked by the protozoan *N. caninum*, while this is the first report concerning cattle in the northeast region of São Paulo State.

Key Words: Neosporosis, cattle, abortion

Introduction

Neospora caninum is an obligate intracellular protozoan considered as one of the most important causes of bovine abortion worldwide (16). Fetuses die in utero, between the third and the eighth month of pregnancy (3), most frequently between fifth and the sixth month (5); in addition, fetuses are usually expelled showing moderate state of autolysis.

Abortion may occur due to a primary infection during pregnancy, or due to the recrudescence of the parasite resulting from the predominance of Th2 response during pregnancy; despite the Th2 response is important for fetal development (8), it cannot

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effectively control the infection, hence resulting in increased parasite load in the mother (8; 13).

Although abortion is regarded as the greatest loss, the overhead such as help from professionals, establishment of diagnosis, invigoration of animals that aborted, replacement of culled animals, eventual losses in milk production and value of the wasted fetus should be considered (4, 11).

Once vertical infection is effective in cattle (2), definitive diagnosis requires a panel with a set of information such as sero-epidemiological investigation of the farm, serology of mother and aborted fetus, the exact period of pregnancy when abortion has occurred, identification of compatible microscopic wounds in the tissues, as well as PCR and markings at the positive immunohistochemistry (7).

The objective of this study was to describe the first *Neospora caninum*-associated abortion in dairy cattle at a dairy farm in the northeastern region of São Paulo State, by associating laboratory tests to epidemiological aspects.

Material and Methods

In January 2010, it has been sent to the Department of Pathology, UNESP-Jaboticabal, a bovine fetus with an estimated age of seven months, which was natural of a dairy farm pertaining to the city of Taiúva (South Latitude 21 º 08 'west longitude and 48 º 27') with 300 animals and an average daily production of 3,000 liters of milk, nearly 20 liters per cow. The farm had a semi-intensive production system with provision of feed concentrate during the dry period. The animals were vaccinated against rabies, foot and mouth disease, carbuncle, brucellosis, leptospirosis, bovine herpes virus type I (IBR) and bovine viral diarrhea virus (BVDV), while the herb consists of purebred Holstein , Jersey, and mostly by crossbred animals 7-8 (gir x holstein); as for the reproduction mechanism, both natural mating and artificial insemination were used. The mother of the fetus was a crossbreed 7-8 (gir x holstein) aged 9 years, with a history of five parturitions and two episodes of abortion. During the necropsy, samples of serosanguineous liquid present in the thoracic cavity and the heart of the fetus were collected for the detection of anti-*N. caninum* antibodies by IFA (18), using a cutoff point of 1:25.

Fragments of brain, cerebellum, tongue, liver, heart and kidneys were kept in 10% buffered formalin for 24 hours, and after fixation, transferred to 70% ethanol to conduct Histopathology (HP) and Immunohistochemistry (IHC) (6). At IHC, polyclonal serum from a bovine naturally infected to *N. caninum* and negative to *Toxoplasma gondii* - in a dilution of 1:1000 - has been used as primary antibody.

In order to perform the polymerase chain reaction (PCR), fragments of the organs described above were collected and kept at -70ºC. DNA was extracted from 25 mg of each tissue by means of the Dneasy kit (Qiagen™) according to the recommendations of the manufacturer while the PCR was conducted using the Np6 (5'-CAG TCA ACC TAC GTC TTC T-3') and Np21 (5'-GTG CGT CCA ATC CTG TAA C-3') primers, which amplified has approximately 328 base pairs (19). Amplification reactions were performed with final volume of 25 µL: 5 µL of each DNA sample, 0.5 µM of each oligonucleotide primer, 1mM of MgCl2, 200 mM of a compound of dNTP and Taq polymerase (Invitrogen 1U/reação), and ultrapure distilled water to complete the volume. The amplifications were processed in a PTC-200 thermocycler (MJ Research) with 40 cycles programmed for denaturation (94°C/1 minute), annealing (50°C/1 minute) and extension (74°C/3.5 minutes).

After the amplification reactions, the PCR products were submitted to horizontal electrophoresis in 1.0% agarose gel containing 0.5 µL/mL of ethidium bromide in TEB running buffer (40 mM Tris-acetate, 2 mM EDTA pH 8.0). 10 µL of each sample plus 2 µL of sample buffer (glycerol 40% and 0.02% bromophenol blue) were applied in the gel channels. The electrophoresis was performed at 100V, 350 mAp, for 110 minutes, while the PCR products were visualized and photographed under ultraviolet light transilluminator and photographed by a coupled image analyzer.

Blood samples without anticoagulant were collected from the mother and 64 other cows of the farm, which had no history of abortion, for the execution of IFA in serum of these animals, using a cutoff point of 1:200. Results were distributed in a 2x2 contingency table and the chi-square test with Yates correction factor was performed (14).

Results

At necropsy, it was verified severe fetal autolysis, while microscopy showed discrete multifocal non-suppurative encephalomyelitis characterized by gliosis and mononuclear inflammatory infiltration associated with cellular debris (Figure 1A-B). The remaining tissues were autolysed. Serology of the fetus was 1:25, while the serology of the mother was 1:3200. The DNA amplification of *N. caninum* was positive in fragments of brain, tongue (Figure 1C), cerebellum, heart and kidneys. At IHC, it has been verified the presence of a 12 µm cyst located on the tongue (Figure 1D). The owner reported that his herd had endemic episodes of abortion. Serology showed that 65 sera of the sampled cows, 27.69% (18/65) were seropositive. Although it has not been a significant difference ($p>0.05$), a higher seropositivity was observed in animals with a history of abortion, 38.46% (10/26), in comparison with animals without previous abortion 20.51% (8/39).

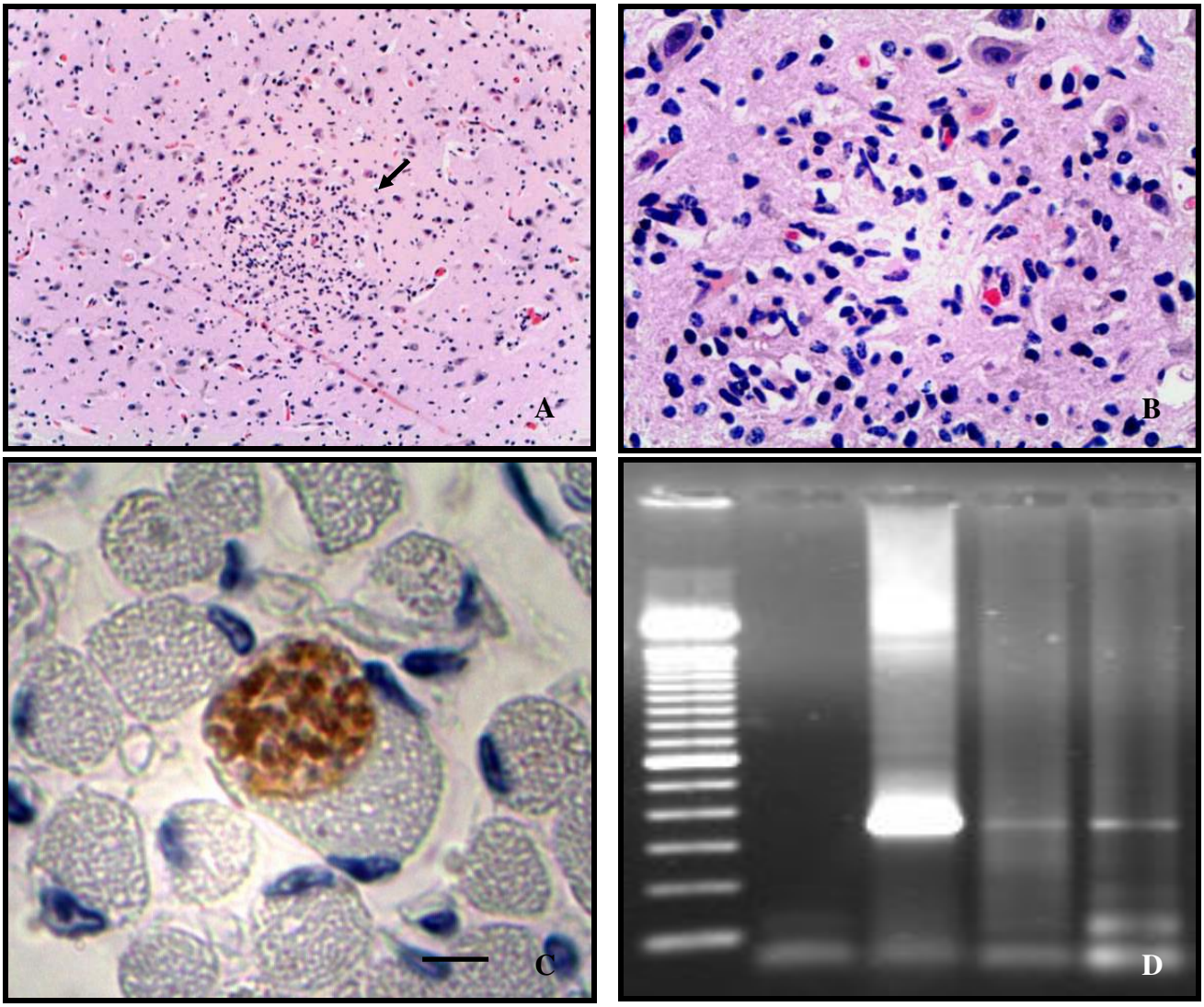


Figure 1. *Neospora caninum*-associated bovine abortion at dairy farms in the northeastern region of São Paulo State. A - Non-suppurative encephalomyelitis characterized by gliosis and mononuclear inflammatory infiltration associated with cellular debris (⟵), x100, H&E; B - Higher magnification in the region indicated in the previous figure (400x, H&E); C - Cyst on the tongue (1000x) marked by IHC (Polyclonal antibody+LSAB+DAB) (bar=5µm); D - Photography of electrophoresis on agarose gel 1.0% stained with Ethidium Bromide. The amplimers shown in the photo concern the PCR for *N. caninum*, according to the partial sequence of the 5S rRNA gene. Channel M: molecular marker on a scale of 100 base pairs (Invitrogen®); Channel 1: negative control; Channel 2: positive control; Channel 3 and 4: positive samples of brain and tongue of the aborted fetus for *N. caninum* at the PCR Np6/Np21.

Discussion

The presence of positive serology of the fetus (20), high titration of the mother at the time of abortion (1), and properties with the highest number of abortions in seropositive cows vaccinated against other infectious agents that cause abortions (15) are only indications that the cause of abortion has been associated with *N. caninum*, once in most cases, seropositive cows give birth to calves clinically normal but chronically infected (5). The presence of lesions consistent with infection and identification of the protozoa on the tissues of the fetus are therefore additional elements needed to support this verification.

Thus, the area of necrosis with mononuclear cell infiltrations observed in the brain, the dissemination of *N. caninum* on tissues, detected at PCR, the cyst observed on the tongue marked by IHC, associated with the epidemiological characteristics, allow us to affirm that neosporosis was cause of abortion.

Once the parasite has been detected in the skeletal musculature (12), our observations on positive PCR and IHC on the tongue - also described in a calf experimentally infected (9) - suggest the inclusion of

the organ in the identification of the parasite on tissues of the suspected animals. The absence of IHC staining in the brain and other organs may have occurred due to the low sensitivity of the technique (17), although the most likely is that it has resulted from the autolysis process (10).

The confirmation of *N. caninum*-associated abortions requires special attention of farmers in regard to their herds; in this, the determination of serum-positive animals and the monitoring of reproductive and zootechnical indexes of these animals, the use of seronegative recipients in the transfer of embryos, the use of seronegative bull semen and the prevention that dogs feed on placental rests or fetal materials, as well as have contact with food and water available for the cattle are important measures to be taken.

Conclusions

The abortion under study was provoked by the protozoan *N. caninum*, while this is the first report concerning cattle in the northeast region of São Paulo State.

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