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Short communication

Increased incidence of DNA amplification in follicular than in uterine and blood samples indicates possible tropism of *Neospora caninum* to the ovarian follicle

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ABSTRACT

This study evaluated the presence of *Neospora caninum* in ovarian follicle aspirates and uterine flushes obtained from *N. caninum* seropositive dairy cows. Ninety-two cows that aborted within the previous 90 days were sampled to determine the presence of antibodies against *N. caninum*. Thirteen seropositive cows were chosen for collection of blood leukocytes, uterine flushes (UF; $n = 12$) and follicular aspirates (OPU; $n = 13$). Samples were centrifuged and the cellular sediment from the follicular fluid, uterine flushes and blood leukocytes were used for DNA extraction and PCR. Follicular aspirates had the highest frequency of DNA amplification for *N. caninum* ($p < 0.05$, 92.3%; 12/13). Whereas uterine (4/12) and blood leukocyte (5/13) samples had similar ($p > 0.05$) rate of positive results. Nonetheless, there was no agreement between blood leukocytes and follicular samples taken from the same animal (Cohen Kappa = −0.16). Similarly, blood leukocytes and uterine results had moderate agreement between them (Cohen Kappa = 0.47). This study indicates that *N. caninum* is present in the ovarian follicle and uterus of seropositive cows, suggesting a possible risk of neosporosis transmission between females during oocyte and embryo collection and transfer. Hence, precautions should be taken to minimize the risk of *N. caninum* transmission. Furthermore, the high incidence of positive results in follicle samples, that exceeded that of their paired blood leukocytes, suggests a possible tropism of *N. caninum* for the ovarian follicle.

1. Introduction

Neospora caninum is an intracellular protozoa, first observed causing encephalomyelitis in dogs (Bjerkas et al., 1984). Before 1988, the infection was misdiagnosed as *Toxoplasma gondii*, until Dubey et al. (1988) showed that a new antigenically and ultra structurally different pathogen was responsible for abortions in cattle.

In the last decade, neosporosis rose as a major cause of abortion and reproductive disorders in dairy cows worldwide (Anderson et al., 2000; Dubey, 2003; McAllister et al., 1998). In California, USA, 42.5% of abortions were attributed to *N. caninum* (Anderson et al., 1991), elevating production costs from conception failure, increase in unproductive days, a higher need for replacement heifers and greater veterinary costs (Barr et al., 1997; Dubey et al., 2002). In Mexico, *N. caninum* in cattle is present across the country, with prevalences as high as 72% (Morales et al., 2001; Meléndez et al., 2005).

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Prevention of neosporosis needs to focus in both horizontal and vertical transmission. Control of the definitive host is paramount as, after ingestion of infective tissue, dogs and coyotes shed unsporulated *N. caninum* oocysts (Dijkstra et al., 2002; McAllister et al., 1998) contaminating water, food and pastures. Vaccination against *N. caninum* has shown limited success, mainly reducing abortions but not preventing foetal or placental infections (Innes et al., 2001; Reichel and Ellis, 2009). Further, crossbred pregnancies have a reduced risk of abortion when compared to dairy cows (López-Gatius et al., 2005; Almería et al., 2009), indicating a genetic susceptibility to the disease. Vertical transmission is considered the dominant route of infection in cattle, as 95% of calves born to infected cows are positive to the disease (Paré et al., 1996), and it is an important way to spread neosporosis (Schares et al., 1998; Hall et al., 2005). Thus, persistently infected cattle could transmit the pathogen to further generations without involvement of a definitive host.

Selective culling of infected cows is another alternative for the control of *N. caninum* transmission. Nonetheless, it results impractical due to the high cost of culling productive cattle. Baillargeon et al. (2001) showed that embryos recovered from *N. caninum* seropositive cows and treated with trypsin before being transferred into a seronegative recipient cows, produced calves free of *N. caninum* infection. Further, Campero et al. (2003) tested embryo transfer (ET) for the control of *N. caninum* and found that progeny obtained by ET from seropositive donor cows remained seronegative to *N. caninum*. Modern technologies in ET use transvaginal ovum pick-up (OPU) to increase the number of oocytes yield for *in vitro* maturation and fertilization (IVM/IVF). The spread of neosporosis through semen was shown experimentally and embryo development was significantly reduced in infected heifers (Serrano et al., 2006). However, there are no studies evaluating the presence of *N. caninum* in follicle aspirates and whether oocytes from infected cattle could be a source of contamination for *in vitro* produced embryos. The objective of this study was to evaluate the presence of *N. caninum* in follicle aspirates and uterine flushes obtained from *N. caninum* seropositive dairy cows.

2. Materials and methods

2.1. Animals and samples

The study was conducted in Tizayuca dairy complex, where 25,000 cows in 126 dairies are located within 120 ha, where *N. caninum* is widely spread with 72% of herds having at least one cow seropositive for the disease (Morales et al., 1997, 2001). Seven dairies that had never been vaccinated against *N. caninum* were selected for this study.

Ninety-two dairy cows between 2 and 5 years old, with a history of abortion in the previous 90 days, were sampled to determine the presence of antibodies against *N. caninum* using an indirect ELISA kit (Civtest Bovis Neospora, Laboratories HIPRA, S.A, Spain). Results were expressed as IRPC ratio, based on a positive and a negative control.

Thirteen seropositive cows with a relative IRPC ratio above 6 (range 15–104) were chosen for collection of uterine flushes (UF; $n = 12$) and follicular aspirates (OPU; $n = 13$).

2.2. Collection of follicular and uterine fluids

Feces were removed from the rectum and each cow received epidural analgesia (5–10 mL of 2% lignocaine) and 5 mg acepromazine (i.v.) (Goodhand et al., 1999). Follicular fluid was collected by OPU as described by Fouladi-Nashta et al. (2009), using a Sonovet 600 ultrasound and a 6.5 MHz trans-vaginal probe. A single lumen needle (18G; 60 cm long) was used for puncturing and aspirating the follicle content into a 50 mL sterile centrifuge tube.

To perform uterine flushing, a n°16 Foley catheter was passed through the cervix and the tip placed in the uterine body, caudal to its bifurcation and flushed four times with 1 L of *Hartmann* solution.

Fluid samples from uterine flushes and follicular aspirates were centrifuged at 8000 rpm for 1 h and the pellet was stored at −20 °C for DNA evaluation.

2.3. Leukocytes

To rule out false positive results due to blood contamination of follicular aspirates and uterine flush samples, blood was collected from the caudal vein. After centrifugation, plasma was separated and leukocytes were recovered with a Pasteur pipette. Aliquots were stored at −20 °C for antibody detection and DNA identification through PCR.

2.4. DNA extraction

Leukocytes from blood and sediments from follicular and uterine fluids were collected by centrifugation at 12,000 RCF for 15 min. The cell pellet was mixed with DNAzol reagent (Invitrogen) and allowed to stand for 5 min (Palaniappan et al., 2005). The lysate was transferred to Eppendorf tubes; the genomic DNA was precipitated with 2 volumes of 95% ethanol and washed again with cold 75% ethanol. Pellets were dried and resuspended in water. Concentration of DNA was determined by spectrophotometry at 280 nm.

2.5. PCR primers and amplification

PCR was performed as previously described (Baszler et al., 1999; Sánchez et al., 2009) with *N. caninum*-specific primers Np7 (5′-GGGTGAACCGAGGGAGTTG-3′) and Np4 (5′-CCTCCCAATGCGAACGAAA-3′), which amplify a 275bp fragment. Each sample was ran in duplicate by PCR. *N. caninum* vaccine (Bovilis Neo Gard, Intervet) was used as a positive control and no DNA was added in the negative control. The PCR amplification products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and viewed under UV light.

2.6. Statistical analysis

Agreement between the results of paired leukocyte and OPU samples and of uterine flushes and leukocyte