Artificial insemination of cows with semen in vitro contaminated with *Neospora caninum* tachyzoites failed to induce neosporosis

Nuno Canada, Carla Sofia Meireles, Paulo Ferreira, José Manuel Correia da Costa, António Rocha

Abstract

Neosporosis is a major cause of abortion in cattle all over the world. Congenital transmission as well as horizontal transmission by ingestion of oocysts has been described. The detection of *Neospora caninum* DNA in bull semen warrants the investigation of possible transmission through the use of contaminated semen. In this experiment four cows were artificially inseminated with frozen-thawed semen contaminated in vitro with viable *N. caninum* tachyzoites (group A) and four control cows were inseminated with tachyzoites-free frozen-thawed semen, from the same bull (group B). Serum samples were collected 15 days before the artificial insemination (AI) and at days 10, 14, 21, 28, 45, 60 and 75 post-insemination. All sera samples were tested for neosporosis by direct agglutination test (DAT). Three of the cows from group A had negative DAT titers (<1:20) in all of the samples, while the fourth cow from this group had a low titer of antibodies (1:80) at day 10, and became negative at day 45, suggesting a stimulation of the immune system by the tachyzoites placed in uterus, rather than the induction of an infection. All of the cows from group B had negative DAT titers (<1:20) in all of the samples. These results suggest that transmission of neosporosis by artificial insemination with frozen-thawed semen is an unlikely event.

Keywords: Neosporosis; Transmission; Artificial Insemination; Cattle

1. Introduction

*Neospora caninum* is a major pathogen of cattle and dogs, and neosporosis is widespread in five continents (Dubey, 2003). In cattle, neosporosis is a
major cause of abortion and may be also responsible by decrease of milk yield, increase involuntary culling and consequent decrease of genetic progress (Thurmond and Hietala, 1996; Thurmond et al., 1997).

The disease has both a vertical and a horizontal transmission (Dubey, 2003). In the bovine, congenital transmission is very efficient and is responsible by the perpetuation of the disease in the herd (Pare et al., 1996; Hernandez et al., 2002; Piergili Fioretti et al., 2003). Nevertheless, some authors consider that vertical transmission alone is not sufficient to sustain the infection in cattle herds (French et al., 1999). Horizontal transmission seems to be responsible for the spread of the disease during epidemic abortion outbreaks (Thornton et al., 1994; McAllister et al., 1996; Thurmond et al., 1997). Dogs, the definitive hosts most likely to infect cattle, shed a low number of oocysts (McAllister et al., 1998; Lindsay et al., 1999; Dijkstra et al., 2001), and it seems to be necessary a high number of oocysts to induce abortion in the cow (Trees et al., 2002; Gondim et al., 2004). Therefore, the search of other routes of transmission is warranted. Recently, the presence of *N. caninum* DNA was reported in semen from naturally infected bulls (Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004; Ferre et al., 2005) and the possibility of venereal transmission in bovine neosporosis has been suggested (Ferre et al., 2005). Transmission of *N. caninum* through contaminated semen would have important repercussions on cattle semen trade. Moreover, transmission by artificial insemination (AI) could explain the worldwide distribution of the disease, as well as its higher incidence in dairy when compared to beef cattle, as AI is the predominant breeding method of the former.

The objective of the present work was to assess the infection status of cows after insemination with frozen-thawed semen, contaminated in vitro with *N. caninum* tachyzoites.

### 2. Materials and methods

#### 2.1. Animals and artificial insemination

Eight Holstein-Friesian cows kept at the teaching and research farm of the University of Porto, Portugal, were used for this study. Cows were between 3 and 6 years old, were brucellosis-free and had been vaccinated against IBR, BVD and leptospirosis with Triangle 9® (Fort Dodge, USA). Right at the beginning of the experiment, two experienced clinicians palpated the cows and no genital pathologies were noticed. The cows were barn-housed and were fed corn silage, straw and commercial concentrates.

Cows were randomly assigned to group A (inseminated with in vitro contaminated semen) or to a group B (control group—inseminated with non-contaminated semen). Estruses were synchronized in all cows, by placing intravaginal devices containing 1.55 g of progesterone and 10 mg of oestradiol benzoate (PRID, Ceva, Libourne, France) for 12 days. All cows were artificially inseminated twice, at 48 and 72 h after the removal of the intravaginal devices. Frozen-thawed semen in 0.25 ml straws, from a single bull and from the same batch, was used for all the inseminations. Pellets containing $6.5 \times 10^7$ and $1.8 \times 10^7$ *N. caninum* tachyzoites were utilized to contaminate the thawed semen in the first and second days of insemination, respectively. For that, and in each day of insemination, the pellet of *N. caninum* tachyzoites was resuspended in 1 ml of tissue culture medium (TCM) 199 with Earls salts buffered with 25 mM HEPES, supplemented with 10% heat-inactivated FCS, 3 mg of bovine Follicle Stimulating Hormone, 6 mg of bovine luteinizing hormone, 100 units of penicillin and 100 ug streptomycin sulfate/ml, and gently mixed with the contents of four 0.25 ml straws thawed in a water bath at 37 °C during 7 s. The mixture of semen and parasites was than aspirated into eight sterile 0.25 ml straws. Immediately after that, the straws were loaded into AI pistolets. All cows were inseminated trans-cervically either with 1 (group B) or 2 (group A) straws, in each insemination day. The average number of *N. caninum* tachyzoites inseminated into the cows of group A was $1.63 \times 10^7$ for the first and $4.5 \times 10^6$ for the second inseminations, respectively. At thawing, the mean progressive motility of spermatozoa before and after the addition of the parasites was estimated to be 55% and 50%, respectively, using a phase-contrast microscope (Zeiss, Axiostar 1122-100). Semen to inseminate group B was thawed as described above, but no assessment of spermatozoa motility was done. All cows were inseminated within 15 min after thawing of the semen straws. The same operator performed all inseminations.
2.2. Culture of *N. caninum*

*N. caninum* tachyzoites (NC-1 strain) were cultured and serially passaged in Vero cells maintained at 37 °C in minimum essential medium (MEM) supplemented with 10% FCS, Earl’s salt, L-Glutamine, penicillin (100 UI/ml) and streptomycin (100 µg/ml) (all from Sigma, St. Louis, USA) in a humified atmosphere of 5% CO2 as previously described (Canada et al., 2002a, 2004a). To obtain *N. caninum* tachyzoites, the supernatants of the cultures were collected when approximately 50% of the cells were destroyed and then purified and immediately used for the experiment.

2.3. Pathogenicity and parasitological viability tests

To assess viability and pathogenicity of the parasites after the contamination of semen, five doses of 1 × 10^7 tachyzoites mixed with 0.25 ml of semen were inoculated onto two flasks with confluent monolayers of Vero cells as previously described (Canada et al., 2002a, 2004a) and in three immunodepressed Swiss Webster mice as described by Canada et al. (2002b).

For positive control, five doses of 1 × 10^7 tachyzoites from the same batch were inoculated onto two flasks with confluent monolayers of Vero cells and in three immunodepressed Swiss Webster mice. For negative control five doses of 0.25 ml of semen were inoculated onto two flasks with confluent monolayers of Vero cells and in three immunodepressed Swiss Webster mice. The number of sperm cells per straw was 22 × 10^6, as assessed by hemocytometer counts.

2.4. Sera sampling

Blood samples were collected by tail venipuncture. Serum samples were collected 15 days before the AI (day 0) and at the days 10, 14, 21, 28, 45, 60 and 75 post-insemination, to evaluate the kinetic of the specific antibodies anti-*N. caninum* produced by the cows. Blood samples were left to clot, centrifuged and the serum separated and frozen at −20 °C until used. All sera samples were tested for neosporosis by direct agglutination test.

2.5. Direct agglutination test (DAT) for detection of specific antibodies anti-*N. caninum*

A DAT as described by Romand et al. (1998) using intact formalin-treated tachyzoites of *N. caninum* NC-1 strain was used to detect specific antibodies anti-*N. caninum* in serum samples. The titer used as cut-off value was 1:40 as determined in a previous study (Canada et al., 2004b) where this DAT titer had the highest Youden’s *J*-statistic, which maximized the sensitivity (100%) and specificity (90.4%) of the test.

2.6. Pregnancy diagnosis

Pregnancy diagnosis was performed 33 days after the last AI by ultrasonography, using a Aloka SSD-500, with a 5.0 MHz linear-array probe. Confirmation of the diagnosis was performed at day 60 post-AI by rectal palpation.

3. Results

Cytopatic effect and several free tachyzoites were observed in groups where tachyzoites and tachyzoites mixed with semen were inoculated onto two flasks with confluent monolayers of Vero cells. Cytopatic effect was not observed in the groups where only semen was inoculated onto flasks with Vero cells.

*N. caninum* tachyzoites were seen in the peritoneal exudates of the immunodepressed Swiss Webster mice 4 days after the inoculation in all of the mice infected with tachyzoites and tachyzoites mixed with semen. Isolated tachyzoites were successively utilized to reinfect several groups of mice as previously described, for a period of approximately 2 months.

Results of the direct agglutination test performed with sera from cows from groups A and B indicate that three of the cows from group A and all cows from the group B were negative for neosporosis in all samples. One of the cows from group A (cow no. 2) presented a low titer of specific antibodies anti-*N. caninum* (1:80) at day 10, which decreased (1:40) by day 21, becoming negative at day 45 post-AI.

Only one cow in group B (cow no. 7) was found pregnant, by visualization of an embryo with heart beat at day 33 and maintenance of pregnancy was
confirmed at day 60 by rectal palpation of the embryo and positive slip test.

4. Discussion

Several studies have shown that endogenous infection of the dam can result in congenital infection with foetopathy (Thurmond et al., 1997; Wouda et al., 1998; Guy et al., 2001). Exogenous infection in pregnancy is also presumed to potentially cause foetopathy. As yet, this presumption is based on experimental challenges with tachyzoites (Dubey et al., 1992; Barr et al., 1994; Williams et al., 2000) and epidemiological evidence suggesting a point source of exposure to infection (McAllister et al., 1996, 2000). To the authors’ knowledge, there is only one published study reporting an experimental oocyst infection of pregnant cows with oral administration of oocysts that produced foetopathy or abortion (Gondim et al., 2004). In that study, 19 pregnant cows were administered 1500–115 000 oocysts through esophageal tube, and only one out of nineteen aborted (Gondim et al., 2004). Considering that dogs excrete few \( N. \) *caninum* oocysts (McAllister et al., 1998; Lindsay et al., 1999; Dijkstra et al., 2001) and that a high number of oocysts (>1500) seem to be necessary to induce abortion in cattle, other sources of infection may exist. Semen as a source of infection could explain some epidemiological aspects of the infection. The maximum parasite load observed in positive semen samples from naturally infected bulls was 10 parasites/ml of semen, equivalent to an ejaculate containing approximately 100 parasites (Ferre et al., 2005). In the present work, approximately \( 1 \times 10^7 \) tachyzoites were placed in the uterus in each AI. It should be noted that no differences were found between the viability and pathogenicity of the tachyzoites resuspended in either TCM medium or mixed with frozen/thawed semen, which indicated that the content of the semen straw did not interfere with the viability or the pathogenicity of the parasites. In this experiment we used \( N. \) *caninum* tachyzoites, because the parasite DNA found in semen is more likely to be from tachyzoites rather than bradyzoites (the tissue cyst form). \( N. \) *caninum* tachyzoites are found in many cell types and tissues, while cysts containing bradyzoites have only been reported in the nervous system and in skeletal muscle of cattle (Barr et al., 1991; Wouda et al., 1997; Peters et al., 2001; Thompson et al., 2001). Evidence of the presence of \( N. \) *caninum* tachyzoites in bull semen was based in PCR positive semen samples and it is not possible to evaluate the viability of the parasites by PCR (Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004; Ferre et al., 2005). Furthermore, attempts at isolation of \( N. \) *caninum* in PCR positive bull semen samples by bioassay in Balb/c \( nu/nu \) mice were unsuccessful (Ferre et al., 2005). In our study, massive amounts of inseminated viable tachyzoites failed to induce the infection, with one of the cows from group A showing a positive low titer of antibodies (1:80) at day 10 after AI, but at day 45 became seronegative (1:20). This suggests that there was an immune response to the tachyzoites placed in uterus but not an infection, because in an acute infection, it should be expected a sustained increase of specific antibodies anti-\( N. \) *caninum* (Andrianarivo, 2001; Williams et al., 2003), and not a positive low titer that decreases in a short period of time, as seen here. Furthermore, this low transient serologic response in animals that do not develop an infection has already been described (Hietala and Thurmond, 1999). Our results are in sharp contrast with data from a recent report (Serrano et al., 2006). The differences between serological tests, as well as multiple versions of test protocols in use, make it difficult to compare test results from different studies (Frossling et al., 2003). Furthermore, the cut-off for serodiagnosis in cattle by ELISA tests are not padronized and a wide range of cut-offs are used in different ELISA test from different laboratories (von Blumroder et al., 2004), which may contribute for discrepant results between laboratories (Atkinson et al., 2000; von Blumroder et al., 2004). For that reason we used a DAT for detection of specific antibodies anti-\( N. \) *caninum* (Romand et al., 1998) with a 1:40 cut-off that maximize the sensitivity (100%) of this test (Canada et al., 2004b). This cut-off obtained by statistical analysis was validated by immunoblot (Canada et al., 2004b). Serrano et al. (2006) detected DNA from \( N. \) *caninum* in blood and tissues from heifers previously inseminated with semen contaminated with \( N. \) *caninum* tachyzoites. However, results presented by van Maanen et al. (2004) showed that occasional false-positive results may occur and cross-contamination at several levels is a danger in routine diagnostic use of PCR. In our opinion, parasitological identification and/or isolation remains the unequivocal evidence of infection.
Similarly to our results, the venereal transmission of a close-related coccidian, *Toxoplasma gondii*, could not be demonstrated despite its detection in semen of experimentally infected rams (Blewett et al., 1982; Teale et al., 1982) and goats (Dubey and Sharma, 1980).

The only pregnancy seen in this study was obtained with insemination of *N. caninum*-free semen. The low (25%) conception rate in this control group could be attributed, among others, to the use of a fixed-time insemination of progesterone estrus-synchronized cows, without additional utilization of PGF2α and/or induction of ovulation with GnRH to avoid asynchronous ovulations in relation to the timed AI. Presence of parasitic forms in the inseminated semen could have contributed to the absence of conceptions in the treated group, by a deleterious effect on the semen itself or through interference in the fertilization process, among others. However, the experimental design of this study was not intended to assess the effect of the parasite on the fertilizing ability of semen and no assumptions can be made on that respect.

Even considering that in vitro contamination of semen may not fully mimic what happen in vivo, the present results do not support the hypothesis that bovine neosporosis could be transmitted by insemination with *Neospora*-contaminated frozen-thawed semen.

**Acknowledgements**

We gratefully acknowledge the support of laboratory work done by Soraia Pinto as well as the revision of this manuscript by Dr. James Harrison (CIBIO, University of Porto).

**References**


Dubey, J.P., Sharma, S.P., 1980. Prolonged excretion of *Toxoplasma gondii* by experimentally infected rams (Blewett et al., 1982; Teale et al., 1982) and goats (Dubey and Sharma, 1980).}

N. Canada et al. / Veterinary Parasitology 139 (2006) 109–114

113


